Identification of FHL1 as a therapeutic target for Duchenne muscular dystrophy

Journal: Human Molecular Genetics

Manuscript ID: HMG-2013-D-00489.R2

Manuscript Type: 2 General Article - UK Office

Date Submitted by the Author: n/a

Complete List of Authors: D'Arcy, Colleen; Monash University, Biochemistry and Molecular Biology
Feeney, Sandra; Monash University, Biochemistry and Molecular Biology
McLean, Catriona; Alfred Hospital, Anatomical Pathology
Gehrig, Stefan; University of Melbourne, Physiology
Lynch, Gordon; University of Melbourne, Physiology
Smith, Jaclyn; Monash University, Biochemistry and Molecular Biology
Cowling, Belinda; Monash University, Biochemistry and Molecular Biology; Institut de Génétique et de Biologie Moléculaire etCellulaire, Department of Translational Medicine and Neurogenetics
Mitchell, Christina; Monash University, Faculty of Medicine, Biochemistry & Molecular Biology
McGrath, Meagan; Monash University, Biochemistry and Molecular Biology

Key Words: FHL1, Duchenne muscular dystrophy, utrophin, NFATc1, therapy
Identification of FHL1 as a therapeutic target for Duchenne muscular dystrophy
Colleen E. D’Arcy¹,⁴, Sandra J. Feeney¹, Catriona A. McLean², Stefan M. Gehrig³, Gordon S. Lynch³, Jaclyn E. Smith¹, Belinda S. Cowling¹,⁵, Christina A. Mitchell¹*# and Meagan J. McGrath¹##.

¹Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia.
²Department of Anatomical Pathology, Alfred Hospital, Prahan, Victoria 3181, Australia.
³Basic and Clinical Myology Laboratory, Department of Physiology, The University of Melbourne, Victoria 3010, Australia.

Present Affiliations:
⁴Alfred Hospital, Prahan, Victoria 3181, Australia.
⁵Department of Translational Medicine and Neurogenetics, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, 67404, France.

# Denotes equal senior authors
* Corresponding author

Professor Christina A. Mitchell
Department of Biochemistry and Molecular Biology,
Monash University,
Clayton, Victoria 3800
Australia.
christina.mitchell@monash.edu
Phone 61-3-9905 4318
Fax 61-3-9902 9500
ABSTRACT

Utrophin is a potential therapeutic target for the fatal muscle disease, Duchenne muscular dystrophy (DMD). In adult skeletal muscle utrophin is restricted to the neuromuscular and myotendinous junctions and can compensate for dystrophin loss in mdx mice, a mouse model of DMD, but requires sarcolemmal localization. NFATc1-mediated transcription regulates utrophin expression and the LIM protein, FHL1 which promotes muscle hypertrophy, is a transcriptional activator of NFATc1. By generating mdx/FHL1-transgenic mice, we demonstrate that FHL1 potentiates NFATc1-activation of utrophin to ameliorate the dystrophic pathology. Transgenic FHL1 expression increased sarcolemmal membrane stability, reduced muscle degeneration, decreased inflammation and conferred protection from contraction-induced injury in mdx mice. Significantly, FHL1 expression also reduced progressive muscle degeneration and fibrosis in the diaphragm of aged mdx mice. FHL1 enhanced NFATc1 activation of the utrophin promoter and increased sarcolemmal expression of utrophin in muscles of mdx mice, directing the assembly of a substitute utrophin-glycoprotein complex, and revealing a novel FHL1-NFATc1-utrophin signaling axis that can functionally compensate for dystrophin.
INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common, severe, childhood muscular dystrophy affecting males with an incidence of 1 in 3,500. This X-linked recessive disorder is characterized by progressive proximal muscle weakness, which leads to loss of ambulation and premature death due to cardiopulmonary failure (1). There is no cure for DMD and current treatments including corticosteroids provide only limited efficacy and can cause significant side effects (2). DMD is caused by dystrophin (DMD) gene mutations, resulting in the absence of dystrophin protein from the sarcolemma of muscle fibers (3). Dystrophin functions as a scaffold for the assembly of the dystrophin glycoprotein complex (DGC), a series of integral and peripheral membrane proteins located at the sarcolemma which connect the intracellular actin cytoskeleton to the extracellular basal lamina (4). The DGC stabilizes the sarcolemma, and provides structural integrity to muscle fibers during muscle contractions. In DMD, the absence of dystrophin initiates a pathological cascade of primary and secondary events including contraction-induced instability of the sarcolemma, Ca\(^{2+}\) influx, protease activation and a cytotoxic immune response, which collectively culminate in severe ongoing muscle degeneration and necrosis (5). Although skeletal muscle has the innate ability to regenerate, this is insufficient to compensate for the chronic muscle degeneration in DMD.

Utrophin is a homolog protein of dystrophin that has potential therapeutic promise for DMD (6). Utrophin shares an 80% amino acid sequence homology with dystrophin, binds many proteins within the DGC and can form a substitute utrophin-glycoprotein complex (UGC) at the sarcolemma (7). Increased utrophin, via transgenic expression, in the dystrophin-deficient mdx mouse model of DMD (8, 9) and the canine CXMD model (10) can compensate for dystrophin, ameliorating the dystrophic pathology and improving muscle function. In mature muscle, utrophin expression is restricted to the neuromuscular and myotendinous junctions (11). However, in developing and regenerating muscle, utrophin is located at the sarcolemma. To compensate for dystrophin in DMD, incorporation of utrophin at the sarcolemma and into the UGC of mature fibers is required to prevent sarcolemmal damage. Emerging research is focusing on elucidating the precise molecular
pathways that increase endogenous utrophin protein expression at the sarcolemma, with the aim of identifying pharmacologically-targetable pathways for a utrophin-based treatment for DMD.

The calcineurin/NFATc1 pathway is a key activator of utrophin expression in skeletal muscle. Calcineurin is a Ca\(^{2+}\)/calmodulin-activated serine/threonine protein phosphatase which dephosphorylates the cytosolic family of transcription factors, nuclear factor of activated T cells (NFAT), to induce their nuclear localization and activation of gene transcription (12). The utrophin gene (UTRN) promoter contains an NFATc1-binding site and is activated by the calcineurin/NFATc1 pathway (13). Calcineurin activation in mdx mice attenuates the dystrophic pathology (14, 15) and protects mds muscles from contraction-induced injury (16). In contrast, inhibition of Ca\(^{2+}\)/calmodulin signaling in mdx mice reduces utrophin expression and exacerbates the dystrophic phenotype (17). The slow oxidative myofiber program is also regulated by the calcineurin/NFATc1 pathway (18, 19). Oxidative muscle fibers express more utrophin (13, 20) and show reduced damage in DMD (21) and mdx mice (22), compared to fast glycolytic fibers. Therefore the therapeutic potential of the calcineurin/NFAT pathway to DMD lies in its ability to activate the slow myogenic program, coupled with increased utrophin expression. The identification of drug targets which potentiate calcineurin/NFATc1-mediated utrophin expression may be useful for treating DMD. However, to achieve this aim it is critical to identify the complement of transcriptional co-activators of NFATc1, which stimulate utrophin expression downstream of calcineurin.

Four and a half LIM domain protein 1 (FHL1) is essential for healthy muscle, with the recent identification of FHL1 mutations as causative of several human muscle diseases including Reducing body myopathy, Scapuloperoneal myopathy, X-linked myopathy with postural muscle atrophy (XMPMA), Emery-Dreifuss muscular dystrophy (reviewed in (23)) and hypertrophic cardiomyopathy (24). Over 30 different FHL1 mutations have been reported in multiple studies, highlighting the significant current interest in the role of FHL1 in human muscle disease. Loss of FHL1 function in mice results in skeletal muscle myopathy accompanied by myofibril disorganization and impaired muscle oxidative capacity (25). Our previous study identified FHL1
as a novel activator of the calcineurin/NFATc1 pathway in skeletal muscle (26). FHL1 directly
binds and coactivates NFATc1-mediated transcription, downstream of calcineurin activation. We
generated a skeletal muscle-specific FHL1 transgenic mouse, which develops skeletal muscle
hypertrophy associated with increased calcineurin/NFATc1 signaling. Consistent with a role for
FHL1 in activating the calcineurin/NFATc1 pathway \textit{in vivo}, muscles from FHL1-transgenic mice
also exhibit a shift from fast glycolytic to slow oxidative fiber types. As FHL1 regulates muscle
mass, via activation of NFATc1 and promotes the slow oxidative myofiber program, we
hypothesized that FHL1 may be a potential therapeutic target for DMD by regulating the expression
of utrophin. In this study we test this hypothesis by investigating whether increased FHL1
expression is sufficient to ameliorate the dystrophic phenotype of dystrophin-deficient \textit{mdx} mice.
This study identifies a novel FHL1/NFAT/utrophin signaling axis which may act as a candidate
therapeutic target for DMD.
RESULTS

Generation of mdx mice with increased muscle-specific expression of FHL1.

To test the hypothesis that FHL1 promotes utrophin protein expression and has potential as a therapeutic target for DMD, we crossed FHL1-transgenic mice with the dystrophin-null mdx mice. The mdx mouse carries a point mutation in exon 23 of the murine dystrophin gene, resulting in a premature stop codon and the absence of dystrophin protein expression (27). FHL1-transgenic mice were generated and described previously whereby the human skeletal muscle α-actin (HSA) promoter drives skeletal-muscle specific expression of the HA-tagged FHL1 transgene (26). Immunoblot analysis of the gastrocnemius muscle confirmed the absence of dystrophin protein expression in both mdx and mdx/FHL1-Tg mice, which was present in wild type muscle (Fig 1A). Expression of the transgenic HA-FHL1 protein in various muscles from mdx/FHL1-Tg mice, but not mdx or wild type littermates, was detected by immunoblotting lysates with a HA-specific antibody (Fig 1B). Immunoblotting with a FHL1 antibody followed by densitometry analysis indicated that total FHL1 protein expression was moderately increased (~2-4 fold) in the muscles of mdx/FHL1-Tg relative to mdx mice (Fig 1B and 1C). Moreover, in mdx/FHL1-Tg mice, FHL1 expression was preferentially increased in skeletal muscles with a high proportion of fast glycolytic IIb fibers such as the gastrocnemius and the tibialis anterior (TA), compared to the soleus with a high slow oxidative fiber content (28), consistent with the preferential activation of the HSA promoter in type IIb fibers (8, 29).

FHL1 protects against muscle degeneration in dystrophic mdx mice.

The onset and progression of pathology in skeletal muscles of mdx mice is well defined (reviewed in (30)). In hind limb muscles an acute onset of myofiber necrosis at ~ 3-4 weeks of age, reduces to chronic low level damage by 8 weeks and persists throughout life. In addition, fast-muscle fibers in both DMD (21) and mdx mice (22) are generally more susceptible to damage. Therefore, the gastrocnemius and TA muscles of mdx versus mdx/FHL1-Tg littermates aged 4 and 16 weeks were
selected primarily for further analysis in addition to the diaphragm at 16 weeks. Analysis of total body mass (Fig 1D) and individual muscle mass (Fig 1E-F) did not reveal a significant difference between mdx and mdx/FHL1-Tg mice. Histological examination of H & E stained transverse muscle sections revealed a significant improvement in the pathology of TA, gastrocnemius and diaphragm muscles from mdx/FHL1-Tg mice compared to mdx littermates (Fig 2A, B high magnification images). In muscles from healthy wild type mice, myofibers were closely packed and of uniform size with peripherally located nuclei. Muscles of mdx mice exhibited characteristic dystrophic pathology, including variation in myofiber size, large foci of myofiber degeneration and necrosis (Fig 2A and B arrows), regenerating muscle fibers revealed by centralized myonuclei and pronounced immune cell infiltration. In contrast, muscles from mdx/FHL1-Tg mice had less variation of myofiber size, with only a few scattered areas of myofiber degeneration (Fig 2A and B arrows) and mononucleated cell infiltrates were reduced. Measurement of the mean myofiber diameter indicated a small but significant (p<0.001) increase in the TA, gastrocnemius and diaphragm muscles of mdx/FHL1-Tg relative to mdx muscles (Fig 2C-E), consistent with the known role of FHL1 in promoting skeletal muscle hypertrophy (26).

To further assess the benefit of FHL1 expression in mdx mice, the percentage of degenerating (necrotic) muscle fibers was quantified. Necrotic fibers were identified in H & E stained transverse muscle sections, by the presence fragmented sarcoplasm, basophilic and/or hypercontracted muscle fibers as performed previously (31, 32). Quantification of the proportion of necrotic myofibers revealed a significant 3-4 fold reduction in muscles of mdx/FHL1-Tg compared with mdx mice (Fig 2F-H). The reduced muscle degeneration in mdx/FHL1-Tg mice was consistently observed in all muscles examined at 4- weeks and was sustained in adult mice at 16-weeks of age. To further assess muscle degeneration serum creatine kinase was measured, a clinical indicator of muscle damage in DMD (1) and mdx mice (33). At 16-weeks of age serum creatine kinase was decreased by 2-fold in mdx/FHL1-Tg mice relative to mdx littermates (Fig 2I). The decreased number of degenerating myofibers in mdx/FHL1-Tg muscle, coupled with reduced serum creatine kinase indicates that FHL1 reduces muscle damage in mdx mice.
Sarcolemmal integrity is improved by FHL1 expression in mdx mice.

The absence of dystrophin in DMD leads to a reduction in the localization of DGC proteins at the sarcolemma, and as a consequence reduced sarcolemmal stability (5). Unrepaired micro-lesions in the sarcolemma facilitate the entry and accumulation of proteins that are normally restricted to blood, including IgM, into degenerating myofibers (15). Abundant IgM-stained myofibers were present in the TA, gastrocnemius and diaphragm of mdx mice indicating significant sarcolemmal membrane fragility (Fig 3A-D). In contrast, only a few IgM positive myofibers were observed in mdx/FHL1-Tg mice, reduced 2-4 fold relative to muscles of mdx mice, an improvement that was sustained in older mice at 16 weeks of age. Therefore, expression of FHL1 improves the stability and integrity of the sarcolemma in dystrophin-deficient muscle fibers.

FHL1 reduces inflammation in dystrophin-null mdx muscle.

A secondary and highly significant consequence of chronic muscle degeneration in DMD and mdx mice is the activation of an inflammatory response, mediated largely by the infiltration of macrophages into degenerating muscle, which significantly exacerbates muscle damage (34, 35). The extent of macrophage infiltration into the muscles of mdx versus mdx/FHL1-Tg mice was examined using a Mac-1 specific antibody (15). Only scant, transiting intravascular macrophages were observed in muscles of healthy wild type mice, which did not invade myofibers (not shown). Large numbers of Mac-1 positive macrophages infiltrated muscles of mdx mice (Fig 4A-D). In contrast, a significant reduction in macrophages was observed in mdx/FHL1-Tg muscles, to almost half that observed in mdx mice. This suggests that FHL1 is also beneficial in reducing secondary inflammation in dystrophic muscles, an effect which likely stems from the protective effect of FHL1 in reducing sarcolemmal damage.

Muscle regeneration in mdx versus mdx/FHL1-Tg mice.

Muscle degeneration in DMD and mdx mice triggers recurrent bouts of myofiber regeneration, which can be identified by the presence of centrally rather than peripherally located myonuclei (30).
Interestingly, despite evidence of reduced degeneration in muscles of mdx/FHL1-Tg mice, the proportion of myofibers with central nuclei was not significantly different from muscles of mdx mice (Fig 5A, D-F). In both mdx and mdx/FHL1-Tg mice almost half (~40%) of all myofibers exhibited central nuclei; i.e. evidence of substantial muscle regeneration. Similarly, in longitudinal TA muscle sections the average number of nuclei per muscle fiber was not significantly different in mdx/FHL1-Tg mice relative to mdx mice (Supplementary Fig 1A-B). Muscle sections were further immunoreacted for developmental myosin heavy chain (dMHC) which is expressed in regenerating muscle fibers (14). No significant difference was observed in the proportion of dMHC-stained regenerating myofibers in mdx versus mdx/FHL1-Tg mice (Fig 5B, G-I). Immunoblotting for dMHC in lysates prepared from TA muscle, followed by densitometry analysis also confirmed no difference dMHC protein expression in mdx versus mdx/FHL1-Tg TA mice (Supplementary Figure 2A-B).

Muscle stem (satellite) cells facilitate muscle regeneration and can be identified by immunostaining for Pax-7 (36). There was no significant difference in the number of Pax-7 stained satellite cells in muscles of mdx compared with mdx/FHL1-Tg mice, except for an increase in the TA muscles of 4 week old mdx/FHL1-Tg mice (Fig 5C, J-L), which is consistent with our previous study which showed increased numbers of Pax-7 stained satellite cells in the muscle of FHL1-Tg mice (26). However qRT-PCR analysis revealed expression of other developmental genes associated with regeneration (MyoD and myogenin) did not differ significantly between mdx/FHL1-Tg and mdx TA muscle (Supplementary Fig 2C-D).

**FHL1 reduces dystrophic changes in the diaphragm of older mdx mice.**

The diaphragm of mdx mice exhibits a more severe pathology than that in limb muscles, with the progressive replacement of muscle fibers with connective tissue (30). This more closely resembles the severe pathology in DMD where loss of diaphragm structure and function leads to respiratory failure (1). Foci of necrotic myofibers are present in the diaphragm of mdx mice aged > 30 days, and progressively worsens thereafter (30) Histological analysis of H & E stained diaphragm
sections revealed that FHL1 expression reduced the dystrophic pathology in diaphragm muscles of older mdx mice aged 9 months (Fig 6A). Fibrosis was examined using collagen immunostaining and revealed similar deposition in young wild type, mdx, and mdx/FHL1-Tg mice at 16-weeks of age (Fig 6B). However by 9 months of age, marked fibrosis was observed in the mdx diaphragm (Fig 6B, arrows) covering ~half the total muscle area (Fig 6C). Significantly, expression of FHL1 reduced fibrosis in the mdx diaphragm by 2-fold. Collectively, these results reveal FHL1 protects against the progressive muscle degeneration observed in the diaphragm of mdx mice.

**FHL1 increases sarcolemmal expression of utrophin by coactivation of NFATc1-mediated transcription.**

To determine the molecular mechanism by which FHL1 reduces the dystrophic pathology in muscles of mdx mice, we examined utrophin expression. Western blot followed by densitometry analysis indicated utrophin protein expression was increased by ~6-fold and ~2-fold in the TA and diaphragm muscles respectively of mdx/FHL1-Tg mice, compared to mdx mice (Fig 7A-B). Quantitative RT-PCR analysis using primers specific for the utrophin A isoform, which is expressed in slow oxidative myofibers and regulated by the calcineurin/NFATc1 pathway (13), revealed increased levels of utrophin A mRNA (1.5-2 fold) in muscles of mdx/FHL1-Tg relative to mdx mice (Fig 7C). Utrophin is expressed in myoblasts (37) and C2C12 myoblasts over- or under-expressing FHL1 were used to further demonstrate a direct role for FHL1 in regulating utrophin expression. Transient overexpression of HA-tagged FHL1 in C2C12 myoblasts resulted in a significant increase in utrophin protein (Fig 7D) and mRNA (Fig 7E), relative to vector control transfected or untransfected myoblasts. Furthermore, the FHL1-mediated increase in utrophin mRNA was reduced by myoblast treatment with the cell-permeable NFAT inhibitory peptide VIVIT (38) and also by the calcineurin inhibitor cyclosporine A (CSA) (Fig 7E). In contrast, in myoblasts with shRNA-mediated knockdown of FHL1, utrophin protein (Fig 7F) and mRNA (Fig 7G) were significantly reduced indicating FHL1 is essential for utrophin expression. We have previously reported FHL1 binds NFATc1 (26) and here show FHL1 regulation of utrophin is
NFAT-dependent. To determine whether FHL1 regulates activation of the utrophin gene directly via transcriptional coactivation of NFATc1, luciferase assays were performed using a utrophin A promoter-LacZ reporter construct (13). Myoblasts were co-transfected with the utrophin A promoter-reporter together with Myc-NFATc1 +/- HA-FHL1 (or Myc-vector and HA-vector controls) as indicated (Fig 7H). When expressed individually NFATc1 and FHL1 exhibited only low level activation of the utrophin A promoter, which increased significantly following NFATc1 and FHL1 co-expression. Therefore FHL1 functions synergistically with NFATc1 to activate the utrophin A promoter. In addition to activating the utrophin promoter, the calcineurin/NFATc1 pathway also regulates utrophin expression by increasing utrophin mRNA stability (39). Given that FHL1 promotes increased utrophin mRNA both in vivo in muscles of mdx mice (Fig 7C) and also in C2C12 myoblasts (Fig 7E), we determined if FHL1 promoted increased utrophin mRNA stability. The half-life of utrophin mRNA was examined by qRT-PCR in myoblasts over-expressing HA-FHL1 or HA-vector, following a timecourse of actinomycin D treatment, which inhibits transcription (40) (Fig 7I). Over expression of FHL1 enhanced utrophin mRNA stability, relative to vector transfected myoblasts. Collectively these results reveal FHL1 is both necessary and sufficient to promote utrophin protein expression via a mechanism which involves direct activation of NFAT-dependent transcription, and also enhanced utrophin mRNA stability.

**FHL1 promotes utrophin localization at the sarcolemma and formation of the UGC.**

The contrasting localization patterns for dystrophin and utrophin in muscle fibers pose a challenge for utrophin-based DMD treatments. Regenerating myofibers exhibit sarcolemmal localization of utrophin. In healthy adult muscle fibers dystrophin is present along the entire length of the sarcolemma, but utrophin is confined to the neuromuscular (NMJ) and myotendinous (MTJ) junctions (11). Given differences in the localization of utrophin in regenerating (sarolemma) versus mature (NMJ and MTJ) muscle fibers it is preferential to assess utrophin upregulation and sarcolemmal localization in mature muscle fibers in mdx mice for two main reasons; First, dystrophin localizes to the sarcolemma in mature muscle fibers, therefore utrophin must be
redirected to the sarcolemma from the NMJ and MTJ in mature fibers, its required site of action for replacement of dystrophin function. Second, analysis of utrophin localization in all muscle fibers would include the large number of regenerating muscle fibers that are present in the mdx mouse and express sarcolemmal utrophin independent of therapeutic intervention (11). Therefore, examining mature muscle fibers permits investigation of utrophin regulation independent from any differences in muscle regeneration that may occur in mdx versus mdx/FHL1-Tg mice, which would indirectly influence the proportion of muscle fibers with sarcolemmal utrophin. To this end, we determined a direct role for FHL1 in regulating utrophin sarcolemmal localization by examining only the mature muscle fibers as has been detailed previously (16, 41). For this analysis transverse muscle sections from wildtype, mdx and mdx/FHL1-Tg mice were co-stained for utrophin and TO-PRO-3 iodide to detect nuclei. Quantification of the proportion of fibers exhibiting sarcolemmal localization of utrophin was limited to adult muscle fibers (distinguished by peripherally located nuclei) and regenerating muscle fibers (with central nuclei) were excluded. Co-staining of muscle sections with a vinculin antibody (42), or α–bungarotoxin (43) was also used to detect the sarcolemma and NMJ respectively. Adult myofibers from wild type and mdx mice exhibited utrophin localization restricted to the synaptic compartment of the NMJ, where it co-localized with α–bungarotoxin, as reported (Fig 8A, closed arrows) (11, 43). In contrast adult myofibers from mdx/FHL1-Tg mice exhibited strong sarcolemmal utrophin staining which co-localized with vinculin (Fig 8A, open arrows) and, increased 3-4 fold above that in muscles of mdx mice (Fig 8B). Further co-staining of muscle sections with a utrophin antibody together with a dMHC-specific antibody which recognizes regenerating muscle fibers, confirms that utrophin localizes to the sarcolemma of mature muscle fibers (dMHC-negative) in mdx/FHL1-Tg mice, but not mdx mice (Fig 8C, arrows). In support of a direct role for FHL1 in promoting utrophin expression and sarcolemmal recruitment, this sarcolemmal utrophin staining correlated with expression of transgenic HA-tagged FHL1 (detected by HA antibody co-immunostaining) in transverse muscle fibers from mdx/FHL1-tg mice (Fig 8D-E). In ~85% of mature muscle fibers a correlation between sarcolemmal utrophin and HA-FHL1 immunostaining was observed in mdx/FHL1-tg muscle (Fig 8E). Furthermore, transgenic HA-FHL1
(using HA antibody immunostaining) also correctly localized to the sarcolemma in transverse muscle sections, consistent with the reported localization of endogenous FHL1 (44) (Fig 8D). Critically, the sarcolemmal localization of utrophin in mdx/FHL1-Tg muscle also correlated with nuclear localization of NFATc1 (Fig 8F-G). This suggests that FHL1 promotes expression of utrophin in dystrophin-deficient muscles in vivo, by enhancing its transcriptional activation by NFATc1.

We next assessed whether FHL1-mediated localization of utrophin to the sarcolemma in mdx mice was sufficient to restore components of the DGC. In the TA muscle dystrophin immunoreactivity was detected at the sarcolemma only in wild type, but not in mdx or mdx/FHL1-Tg mice (Fig 9A). Critically, immunofluorescence analysis demonstrated that all components of the DGC examined (α-dystroglycan, β-dystroglycan, β-sarcoglycan, γ-sarcoglycan and syntrophin) were reduced or absent from the sarcolemma of muscle fibers in mdx mice, but were restored in mdx/FHL1-Tg mice. In mdx/FHL1-Tg mice co-staining of TA muscle sections revealed a positive correlation between mature muscle fibers (peripheral nuclei) which exhibited strong sarcolemmal staining of utrophin and high level recruitment of the DGC proteins α- and β- dystroglycan and syntrophin to the sarcolemma (Fig 9B, asterix). In contrast in mdx/FHL1-Tg muscle fibers with low level utrophin sarcolemmal staining were associated with only weak co-staining of α- and β-dystroglycan and syntrophin (Fig 9B, hash symbol). The sarcolemmal recruitment of utrophin was also observed in the diaphragm of mdx/FHL1-Tg mice, and not wildtype or mdx diaphragm, and coincided with the sarcolemmal localization of β-dystroglycan (Fig 9C). Collectively, this indicates that FHL1-mediated expression of utrophin at the sarcolemma is sufficient to direct formation of a compensatory utrophin-glycoprotein complex (UGC) in dystrophin-deficient muscle.

FHL1 protects mdx muscle from contraction-induced injury.

Loss of the DGC in dystrophin-deficient muscle and the resulting instability of the sarcolemma makes myofibers more susceptible to contraction-induced muscle injury. In muscles of mdx mice
this is observed as a decrease in maximum force producing capacity after lengthening (eccentric) muscle contractions (ECC) and assessment of this force deficit is used to assess the efficacy of potential therapies (16, 45-47). The magnitude of the force deficit during the cumulative contraction-induced injury protocol was assessed in diaphragm muscle strips in vitro (Fig 10A) and the TA muscle in situ (Fig 10B) in wild type, mdx and mdx/FHL1-Tg mice. The TA muscles of mdx mice exhibited significantly greater force deficits with contraction-induced injury, relative to wildtype mice (p<0.05; Fig 10B). In contrast, TA muscle from mdx/FHL1-Tg mice exhibited an attenuated force deficit compared with mdx mice, albeit at lower level than wild type mice, indicating expression of FHL1 offers protection from contraction-induced injury in dystrophin-deficient muscles (*p<0.05). For the TA, contractile injury susceptibility for both the mdx and mdx/FHL1-Tg mice merges at higher levels of stretch (40-50% of Lo), this is likely because there is almost complete damage to the contractile filaments at these magnitudes of strain. After these stretches very little contractile force can be produced which, blurs any potential difference. It is at the more physiological strain magnitudes where the benefit (of an improved force deficit) is realized in the mdx/FHL1-tg TA muscle. No difference in susceptibility to contraction-induced injury was observed in the diaphragm of mdx mice compared to mdx/FHL1-Tg mice (Fig 10A). The lack of effect of FHL1 transgenic expression on susceptibility to contractile damage in the diaphragm muscle strips of mdx mice may be attributed to the reduced damage that this muscle sustains compared with the TA muscle, using this injury protocol. As can be seen in Figure 10A, the diaphragm showed only ~30% loss of force after the full contraction injury protocol, whereas the TA muscle (Fig 10B) exhibited greater than 80% loss of force-producing capacity. Therefore, a much greater opportunity for intervention in the TA muscle compared with the diaphragm may occur, accounting for the differences in susceptibility to contractile damage. Furthermore, Western blot analysis revealed that utrophin protein expression was increased ~6-fold in the TA of mdx/FHL1-Tg mice compared with only ~2-fold in the diaphragm, relative to mdx mice (Fig 7A-B). It is possible therefore, that the relative altered susceptibility to contraction-induced injury in the TA and diaphragm of mdx/FHL1-Tg mice is also related to differences in the expression of
utrophin. However, despite these differences in utrophin expression, immunofluorescence analysis demonstrated that in both the TA (Fig 8A) and diaphragm (Fig 9C) of mdx/FHL1-Tg mice utrophin localized to the sarcolemma of mature muscle fibers, the site of assembly of other DGC proteins (Fig 9A and C). Most importantly specific (normalized) force was higher in the diaphragm muscle strips from mdx/FHL1-Tg mice (*p<0.05; Fig 10C), but not the TA (Fig 10D), indicating that FHL1 imparts a significant functional improvement to muscles of mdx mice.
DISCUSSION

The findings reported here reveal transgenic muscle-specific expression of FHL1 significantly improves several pathological parameters in the pre-clinical mdx mouse model of DMD, by increasing the expression and localization of utrophin at the sarcolemma. FHL1 expression increased sarcolemmal stability, decreased muscle degeneration and inflammation and protected against contraction-induced muscle damage in mdx mice. Notably, FHL1 expression reduced progressive muscle degeneration and fibrosis in the diaphragm of older mdx mice; the muscle being the most clinically relevant for DMD (30). FHL1 enhanced NFATc1-dependent utrophin expression both in vitro and in vivo in mdx mice. Taken together these results reveal FHL1 regulation of utrophin as a potential therapeutic target for DMD.

FHL1 expression fulfills the criteria for a possible utrophin-based DMD therapy (6), since it increases endogenous utrophin protein expression in dystrophin-null muscle fibers, directly at the sarcolemma. Utrophin A mRNA was increased ~2 fold in mdx/FHL1-Tg muscles, and although this level of utrophin induction is moderate, numerous previous studies have revealed this is sufficient to mitigate the dystrophic pathology in mdx mice (43, 48, 49). Expression of activated calcineurin has been shown previously to increase utrophin expression by 2-fold and reduce muscle degeneration in mdx mice (15). FHL1 also increased utrophin protein expression in mdx muscle (~6-fold in TA, ~2-fold in diaphragm), accompanied by a concomitant reduction in the degeneration of individual myofibers and a significant decrease in serum creatine kinase, indicative of an overall improvement in the dystrophic pathology (33). Critically, FHL1 expression also significantly attenuated the progression of muscle degeneration in the diaphragm of older mdx mice, resulting in decreased fibrosis. Therefore the increased utrophin expression induced by FHL1 exceeds the therapeutic threshold required for the pre-clinical mdx model of DMD.

As shown here FHL1 is a novel activator of the extra synaptic sarcolemmal localization of utrophin, its required site of action to compensate for dystrophin. The select expression of utrophin at the NMJ of adult myofibers is regulated by local transcription in synaptic nuclei following activation by nerve-derived factors such as heregulin (50). Less is known about the signaling
pathways which regulate the extra synaptic localization of utrophin. Utophin protein expression is regulated by both transcriptional (13, 50, 51) and post-transcriptional events (20, 41, 52). The calcineurin/NFATc1 pathway regulates both the synaptic (53) and extrasynaptic (15) expression and localization of utrophin. In the present study FHL1 and NFATc1 co-operated synergistically to activate the human utrophin promoter in vitro. The calcineurin/NFATc1 pathway also enhances utrophin mRNA stability (39) and here expression of FHL1 which activates NFATc1 (26) also resulted in increased utrophin mRNA in skeletal muscle in vivo and enhanced utrophin mRNA stability in myoblasts in vitro. In C2C12 myoblasts FHL1 knockdown also reduced utrophin expression, revealing a direct causal role for FHL1 in regulating utrophin levels.

In our previous study FHL1-transgenic mice exhibited features consistent with activation of the calcineurin/NFATc1 pathway in skeletal muscle in vivo, including, increased nuclear localization of NFATc1, expression of the NFAT-target protein GATA2, and a shift to a slow muscle phenotype (26). In mdx/FHL1-Tg mice the increased sarcolemmal localization of utrophin was observed in mature muscle fibers which exhibited expression of the HA-FHL1 transgene and also enhanced nuclear NFATc1 localization. Moreover, both utrophin (13, 20) and FHL1 (54) are expressed preferentially in oxidative muscle fibers which exhibit greater resistance to damage in DMD (21) and mdx mice (22), relative to glycolytic muscle fibers. We therefore propose a model whereby FHL1 promotes the extrasynaptic expression of utrophin and reduces muscle degeneration in mdx mice through activation of the calcineurin/NFATc1 pathway (Fig 10E).

In mdx/FHL1-Tg mice we observed both increased utrophin expression and muscle fiber hypertrophy, both of which are consistent with a role for FHL1 in activating the calcineurin/NFAT pathway in vivo (15, 16). The observation that myofiber diameter is increased in mdx mice with FHL1 expression, is consistent with our previously reported role for FHL1 in promoting skeletal muscle hypertrophy (26). Therefore FHL1 could impart several benefits to mdx mice by increasing both muscle fiber size (although this did not result in an overall increase in muscle mass) and by increasing expression of the dystrophin homolog, utrophin. Extensive regeneration is also maintained in muscles of mdx/FHL1-Tg mice, despite considerable evidence indicating a significant
reduction in muscle damage. The presence of regenerating muscle fibers in mdx/FHL1-Tg mice is consistent with the increased numbers of muscle fibers with centralized nuclei and satellite cells that are observed in FHL1-Tg mice, in the absence of any muscle damage (26). Some previous studies have reported that utrophin is increased in regenerating muscle fibers in the mdx mouse (37, 55), while a more recent study has shown increased utrophin expression in the mdx mouse does not occur in response to muscle regeneration (56). However, the proportion of regenerating muscle fibers was not significantly different between mdx and mdx/FHL1-Tg mice and the expression of developmental genes associated with regeneration (MyoD, myogenin and dMHC) did not significantly differ between mdx/FHL1-Tg and mdx muscle. Moreover, in mdx/FHL1-Tg muscle we observed increased sarcolemmal expression of utrophin in muscle fibers which did not express dMHC. Collectively this indicates that the observed increase in utrophin expression in mdx/FHL1-Tg mice is not a consequence of increased numbers of regenerating muscle fibers, but rather direct transcriptional activation by FHL1.

FHL1-mediated expression of utrophin at the sarcolemma was sufficient to facilitate formation of a substitute UGC complex including the critical myofiber stabilizing connection to the basal lamina via α-dystroglycan (4). We examined the localization of a complement of dystrophin-associated proteins, including intracellular (syntrophin), transmembrane (β- and γ-sarcoglycans, β-dystroglycan) and extracellular (α-dystroglycan) components (4). All of these were enriched at the sarcolemma with utrophin only in muscles of mdx mice expressing increased FHL1. However, expression of FHL1 in mdx mice resulted in only 7-9% of adult myofibers in the TA exhibiting sarcolemmal utrophin; a 3-5 fold increase above that observed in mdx mice. Our data revealed that between 40-60% of muscle fibers in the mdx and mdx/FHL1-Tg mice were undergoing regeneration, as indicated by the presence of a central nuclei, and these regenerating muscle fibers were excluded from our analysis of utrophin sarcolemmal localization. Therefore only approximately half of the total muscle fibers (mature) were examined for the presence of utrophin at the sarcolemma in our study. Furthermore, only a proportion of these mature fibers would express
the HA-FHL1 transgene which is driven by the human skeletal α-actin (HSA) promoter (26), that is active preferentially in type 2B muscle fibers (8, 29). Type 2B fibers represent ~40-50% of all muscle fibers in the TA of C57BL6 mice, which were used in this study (28). Therefore, when considered collectively, it is feasible that sarcolemmal utrophin staining was observed in only 7-9% of mature muscle fibers in mdx/FHL1-Tg mice relative to mdx mice. Despite this modest increase of utrophin positive myofibers, muscles from mdx/FHL1-Tg mice exhibited a significant enhancement of sarcolemmal stability as indicated by reduced myofiber penetration by serum IgM, coupled with decreased susceptibility to contraction-mediated damage in the TA. Therefore the limited sarcolemmal expression of utrophin induced by FHL1 was sufficient to direct the formation of a functional UGC and impart a structural benefit to dystrophin-deficient muscle, suggesting that even low level enhancement of utrophin localization to the sarcolemma may provide benefit. To further improve efficacy, activation of the calcineurin/NFATc1/FHL1 pathway which regulates utrophin at a transcriptional/translational level could be used in synergy with other therapies to promote increased utrophin protein stability and enhanced recruitment at the sarcolemma (41, 57).

This study identifies FHL1 as a potent therapeutic target for ameliorating muscle degeneration in the pre-clinical mdx mouse model of DMD. Studies in animal models, such as we report here for FHL1, have provided compelling evidence that upregulation of utrophin is a viable therapeutic strategy to be evaluated in DMD patients. Critically, only a modest increase in FHL1 protein expression (~ 2-6 fold, in this study) imparted significant structural and functional improvements to dystrophin-null muscles, through its ability to modulate utrophin expression. At present a pharmacological utrophin-based therapy is yet to be established as a routine treatment for DMD patients. The degree of utrophin upregulation that would be therapeutically relevant in the human condition is still largely unknown. Encouragingly, evidence has shown a positive correlation between small differences in utrophin expression in DMD patients and age of wheelchair dependence (58). Current knowledge of pathways which regulate endogenous FHL1 expression in skeletal muscle is limited and will be examined in future studies. Therapies aimed at pharmacological targeting of the NFATc1/FHL1/Utrophin pathway identified here, may offer
promise as a potential treatment for DMD. Numerous recent studies identifying \textit{FHL1} mutations as causative of several human muscle diseases (\textit{reviewed in} (23)) have indicated a requirement for FHL1 expression for healthy muscle. This current study expands significantly upon current understanding of the role of FHL1 in muscle disease, identifying FHL1 also as a therapeutic target for DMD.
MATERIALS AND METHODS

Generation of mdx/FHL1-Tg mice

*Mdx* mice (C57BL/10ScSn-DmD<sup>mdx</sup>/ARC) were purchased from the Animal Resources Centre, Western Australia. We previously generated the skeletal muscle-specific FHL1 transgenic mice (FHL1-Tg) (26). Female heterozygous *mdx*/FHL1-Tg mice (FHL1-Tg/dystrophin<sup>+/−</sup>) mice were crossed with non-transgenic *mdx* male mice (non-Tg/dystrophin<sup>+/−</sup>) to generate the three genotypes of male mice that were used in this study; “wild type” (non-Tg; dystrophin<sup>+/+</sup>), “mdx” (non-Tg; dystrophin<sup>−/−</sup>) and “mdx/FHL1-Tg” (FHL1-Tg/dystrophin<sup>−/−</sup>), such that littermates were used for all analyses. *Mdx* allele transmission was determined by a two-step process. Firstly, PCR amplification of the region encompassing the mutation in exon 23 of the murine *dystrophin* gene from genomic DNA, followed secondly by sequencing of the PCR product to confirm the presence of the C to T mutation, as previously described (27). Genomic identification of FHL1-Tg mice was performed as previously described (26). All mice colonies were maintained at the Monash Animal Research Platform, Monash University, Australia, with a 12-hour day/night cycle with access to food and water *ad libitum*. Experiments were performed in accordance with the Monash University Animal Ethics Committee, Australia (approval number SOBSB/B/2007/46) and the Anatomy and Cell Biology, Neuroscience, Pathology, Pharmacology and Physiology Animal Ethics Committee, University of Melbourne, Australia (approval number 1112223) and under the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Serum Creatine Kinase

Blood was collected immediately post-mortem from the right ventricle using a 25 gauge needle. Serum creatine kinase levels were measured by Gribbles Veterinary Pathology Service, Clayton, Australia. At least n ≥ 16 mice were analyzed per genotype and data represent the mean ± SEM. Results from *mdx* and *mdx*/FHL1-Tg mice were expressed relative to serum creatine kinase measurements obtained from wild type mice, which was arbitrarily assigned a value of 1.
Muscle dissection and histology

Muscles were excised and weighed before being flash frozen in isopentane (cooled in liquid nitrogen at -160°C) and stored at -80°C. All muscle masses were represented as a proportion of total body mass. For histology, serial frozen transverse muscle sections (8 µm) were cut from the midbelly of muscles, stained with haematoxylin and eosin using standard procedures and viewed using an Olympus Provis Ax70 microscope fitted with an Olympus DP70 color camera (Monash Microimaging, Monash University, Australia). Histopathological analysis of muscle sections was conducted on n ≥ 5 mice per genotype (wild type, mdx and mdx/FHL1-Tg littermates) and histopathological features were confirmed by an experienced clinical neuropathologist.

Quantitative analysis of muscle was performed using the open-access Image J program (V1.45b, National Institutes for Health, USA), following the digitization of images. Degenerating (necrotic) fibers were identified on H & E stained transverse muscle sections, by the presence of fragmented sarcoplasm, basophilic and/or hypercontracted muscle fibers and quantified according to the Standard Operating Procedure (SOP) for “Quantification of histopathology in Haemotoxylin and Eosin stained muscle sections in mdx mice” (Miranda Grounds, 17th June 2012, Treat-NMD Neuromuscular Network, http://www.treat-nmd.eu/research/preclinical/dmd-sops) and also in (31, 32). Non-overlapping images of the transverse muscle section were, tiled together to provide a single digital image of the entire muscle cross-sectional area, from which the number of fibers positive for features of degeneration were counted and expressed as a percentage of total fibre number. Mean fiber diameter (µm) was determined by measuring the shortest axis of individual transversely sectioned fibers (minimal Feret’s diameter) as previously described (33). The percentage of fibers with centralized nuclei as a marker of regeneration, and the percentage of fibers exhibiting degeneration, was also quantified. For each parameter quantified n ≥ 5 mice were analyzed per genotype (wild type, mdx, mdx/FHL1-Tg littermates), with ~1000-3000 fibers analyzed per muscle from 10-12 consecutive fields at x 200 magnification. Data represent the mean ± SEM.
**Western blot analysis of skeletal muscle lysates**

Muscles were excised, minced and homogenized on ice using a TissueRuptor rotor-stator homogenizer (Qiagen) in 1% NP-40, Tris saline lysis buffer pH 8, containing protease inhibitors (Roche protease inhibitor cocktail tablets). Protein concentration was determined using DC protein assay reagents (Bio-Rad-Laboratories) according to the manufacturer’s protocol. For low molecular weight proteins (FHL1 32kDa, HA-FHL1 34 kDa and β-tubulin 55 kDa), 10 µg of lysate was separated by SDS-PAGE using standard methods and immunoblotted for FHL1 (1:1000, Abcam, ab23937), HA (1:5000, Covance) and β-tubulin (1:5000, Zymed Laboratories Inc. – loading control). For high molecular weight proteins (dystrophin, utrophin and dMHC), 50 µg of protein was separated on 6% SDS-PAGE at 100 v and 100 mamps for 2 hours and transferred to PVDF membranes at 250 v and 250 mamps for 2 hours in pre-cooled transfer buffer (25 mM Tris, 192 mM glycine, 10 % methanol), containing 0.05 % SDS. Immunoblots were probed with antibodies specific for dystrophin (1:200, Abcam, ab15277), utrophin (1:500, BD transduction laboratories or 1:200 sc33700 Santa Cruz Biotechnology), dMHC (1:100, Novo Castra) and vinculin (1:5,000, Sigma Aldrich, V9131 – loading control). Immunoblots were developed by chemiluminescence (ECL, Western Lightning ECL Pro for dMHC immunoblots, Perkin Elmer). Immunoblot band intensities were quantified by densitometry using ImageQuant TL analysis (Amersham Biosciences). Protein expression was corrected for protein loading using β-Tubulin/Vinculin densitometry values and expression in mdx/FHL1-Tg mice presented as the fold difference relative to mdx littermates.

**Immunohistochemistry**

Transverse muscle sections were prepared as described above for muscle histology. For IgM staining muscle sections were fixed for 2 min in ice cold acetone, washed in PBS and blocked (1 % BSA, 5% horse serum in PBS) for 1 hour at room temperature. Sections were stained for 1 hour with goat anti-mouse IgM-FITC conjugated (1:100, Sigma Aldrich, F9259), rinsed with PBS and mounted with coverslips using Slowfade Gold anti-fade reagent (Molecular Probes). The Vector®
M.O.M.™ (mouse on mouse) immunodetection kit (Vector Laboratories) was utilized for all staining of muscle sections using mouse monoclonal antibodies according to the manufacturer’s protocol; dMHC (1:15, Novo Castra), Mac-1 (1:100, Novo Castra), Pax-7 (1:100, R&D Systems Inc. MAB1675), syntrophin (1:300, Abcam, ab11425), α-dystroglycan (1:50, Abcam ab106110), β–dystroglycan (1:200, Abcam ab49515), and collagen (1:500, Sigma Aldrich, C1926). For staining with rabbit polyclonal antibodies sections were fixed and blocked as described above for IgM staining and incubated overnight with primary antibody diluted in PBS containing 1 % BSA; dystrophin (1:200, Abcam ab15277), laminin (1:25, Abcam ab11575), β-sarcoglycan (1:50, Novus Biologicals NBP1-19782), and γ-sarcoglycan (1:100, Santa Cruz Biotechnology sc28280). Sections were washed with PBS and incubated with goat anti-rabbit (H + L) – Alexa Fluor 488-conjugated secondary antibody (1:600, Molecular Probes) for 1 hour at room temperature, washed again in PBS and coverslips mounted using Slowfade gold anti-fade reagent (Molecular Probes).

Utrophin immunostaining of muscle sections using a rabbit polyclonal antibody was performed as above (utrophin antibody 1:300, Santa Cruz Biotechnology sc15377) with DAPI (Molecular Probes) co-staining to identify nuclei (1:100). To examine the localization of utrophin at the neuromuscular junction, samples were co-stained with α-Bungarotoxin Alexa Fluor 555-Conjugate (1:4000, Molecular Probes) for 1 hour. To examine the sarcolemmal localization of utrophin, sections were co-stained with a mouse monoclonal vinculin antibody (1:600, Sigma Aldrich, V9131) followed by anti-mouse (H+L)-Alexa Fluor 568 secondary antibody (1:400, Molecular probes). For utrophin co-staining with transgenic expressed HA-FHL1 sections were also stained with a mouse monoclonal HA antibody (1:1000, Covance) followed by goat anti-mouse (H+L)-Alexa Fluor 567 (1:400, Molecular probes) secondary antibody. For utrophin and NFATc1 co-staining, serial sections of muscle were stained individually with the rabbit polyclonal utrophin and NFATc1 (1:100, Santa Cruz Biotechnology sc13033) antibodies, followed by goat anti-rabbit (H+L)-Alexa Fluor 488 or 567 (1:400, Molecular probes) secondary antibodies. Images of NFATc1 and utrophin staining of serial muscle sections were overlayed using the MacBiophotonics Image J software (V1.43m, National Institutes for Health, USA).
Muscle sections were viewed using either an Olympus Provis Ax70 fluorescence microscope fitted with an Olympus DP70 color camera, or a Nikon C1 inverted confocal microscope (Monash Microimaging, Monash University, Australia) at 20x/0.4 NA or 40x/0.65 NA with oil immersion at room temperature. Images were captured using SIS Analysis Software. Only linear adjustments (altering brightness and contrast) were made to microscopy images and was applied equally to every pixel.

All analyses were manually performed using MacBiophotonics Image J software. For all quantification 10-12 consecutive fields at x 200 magnification was examined for each muscle section (~2000-3000 muscle fibers). Quantification of the proportion of fibers exhibiting sarcolemmal localization of utrophin was limited to adult muscle fibers with peripherally located nuclei as reported previously, and regenerating fibers with central nuclei were excluded (41). The percentage area of collagen staining was measured using the entire transverse diaphragm section, which was traced to avoid the inclusion of non-muscle area in the analysis. The area of collagen staining was averaged from n = 3 serial sections. For calibration purposes, a minimum fluorescence threshold for collagen staining was selected from the analysis of wildtype muscle sections and remained constant for mdx and mdx/FHL1-Tg image analysis. Results are presented as the mean ± SEM for n ≥ 5 for each muscle group and genotype.

Quantitative Real Time-PCR analysis

Total RNA was isolated from skeletal muscle by homogenisation using a TissueRuptor rotor-stator homogeniser (Qiagen) and purified using the Qiagen RNeasy Fibrous Tissue Mini kit (Qiagen) according to the manufacturer’s instructions. 50 ng RNA was reverse transcribed using the Affinity script QPCR cDNA synthesis kit (Agilent Technologies). qRT-PCR was performed in triplicate using a 72 well-format using the Corbett Real Time PCR Rotorgene 3000 system (Qiagen). Validated murine utrophin A primers were used; sense 5’-ATGGCCAAGTATGGGGACCTTG-3’ and anti-sense 5’- GTCAACGTCCTCAACTTCACCAC-3’ (37) and primers for murine MyoD (sense 5’-GCCCGCGCTCCAAGTCTCTGAT-3’ and anti-sense 5’-...
CCTACGGGTGTCGCCCCCTGTGC-3'), myogenin (sense 5’- GGGCCCTGGAAGAAAG-3’ and anti-sense 5’- AGGAGGCCTGTTGGGAGT-3’) (59). mRNA transcript levels were normalised to the housekeeping gene GAPDH (Mm GAPDH QuantiTect primer assay QT01658692, Qiagen) using the comparative $2^{-\Delta \Delta Ct}$ method and expressed in mdx/FHL1-Tg mice relative to mdx and wildtype littermates. Each amplicon was analyzed in triplicate in a 72 well rotor in three independent experiments. Data is presented as the mean ± SEM from $n\geq3$ mice for each muscle group.

**Muscle functional analyses**

Mice were anaesthetised with sodium pentobarbitone (Nembutal, Sigma-Aldrich, NSW, Australia; 60 mg/kg i.p.) such that they were unresponsive to tactile stimuli. Contractile properties of diaphragm muscle strips were assessed *in vitro* as described in detail previously (33). Briefly, strips of diaphragm muscle were bathed in oxygenated Krebs solution at 25°C in a custom-made organ bath. Preparations were stimulated by supramaximal 0.2-ms square wave pulses of 450 ms duration delivered via platinum plate electrodes flanking both sides of the muscle. Contractile properties of the TA muscle were assessed *in situ* (45). Briefly, TA muscles maintained at 37°C were stimulated by supramaximal (14 V) 0.2 ms square wave pulses of 350 ms in duration delivered via two wire electrodes resting on the nerve. All muscles were adjusted to optimum muscle length ($L_o$), determined from maximum isometric twitch force ($P_t$). Maximum isometric tetanic force ($P_o$) was recorded from the plateau of the frequency-force relationship, and normalized to muscle cross-sectional area (specific force; $sP_o$), for comparisons between groups where appropriate (46, 47). The susceptibility of TA and diaphragm muscles to contraction-induced injury was determined from the protocols that described in detail previously (33). Isolated muscles were maximally activated to produce isometric force and then stretched to perform an eccentric contraction (at a velocity of 2 $L_f$/s) at progressively increasing magnitudes of stretch. Maximum isometric force was determined prior to each eccentric contraction (33). All statistical comparisons were made using a two-way ANOVA and considered significant at $p < 0.05$. 
**Growth of C2C12 myoblasts**

C2C12 myoblasts were obtained from ATCC and cultured in DMEM supplemented with 20% fetal calf serum, and 2 mM L-glutamine. Myoblast cultures were maintained at subconfluent levels (<50%) and low passage (<25) to avoid spontaneous differentiation.

**C2C12 myoblast Luciferase Assays**

For luciferase assays, myoblasts were plated into 12-well dishes at a density of $2.5 \times 10^4$ cells/well and were transfected using 4 µl lipofectamine 2000 (Invitrogen); Lac-Z utrophin promoter reporter construct (2000 ng) (13), HA-vector or HA-FHL1 (1000ng), Myc-vector or Myc-NFATc1(1000 ng) (26) and pRL-Tk (Renilla luciferase, transfection efficiency control; Promega) (20 ng). 48 hours post-transfection myoblasts were lysed using Passive Lysis Buffer (Promega) and 50 µl of lysate assayed for β-galactosidase (LacZ reporter) activity using the Beta Glo Assay System (Promega). Renilla luciferase activity was assayed using 50 µl of lysate and the Dual-Luciferase Assay reporter system (Promega). β-Galactosidase activity for each sample was normalized to the corresponding renilla luciferase activity. Results were expressed as fold increase from Myc-vector/HA-vector transfected cells and are the mean ± SEM of n=4.

**Expression of utrophin in C2C12 myoblasts**

Utrophin protein and mRNA were examined in C2C12 myoblasts over- and under-expressing FHL1. For FHL1 overexpression myoblasts (2 x 10^5 plated in 60 mm dish) were transiently transfected with 4 µg of pCGN-FHL1 (HA-FHL1) or pCGN vector (HA-Vector) (cloned previously (60), using 4 µl of lipofectamine 2000 according to manufacturer’s instructions. A subset of HA-FHL1 expressing myoblasts were also treated with the cell-permeable NFAT peptide inhibitor VIVIT (2 µM, Calbiochem) or the calcineurin inhibitor cyclosporine A (50µM, Calbiochem) or vehicle (DMSO) overnight as previously(61).
For under-expression studies validated Mission Lentivirus shRNA particles specific for mouse FHL1 (or control) were purchased from Sigma Aldrich (FHL1 coding sequence TRCN0000113521; pLKO.1-puro Non-target shRNA control plasmid SHC016). C2C12 myoblasts (1.6 x 10^4 cells/well, 96-well dish) were stably transduced with lentivirus particles (MOI5) using hexadimethine bromide according to manufacturer’s protocol, with selection of stably transduced cells using 8000 ng/ml puromycin.

qRT-PCR analysis of utrophin A mRNA or murine FHL1 (Mm FHL1 QuantiTect primer assay QT00107569, Qiagen) were performed as above following extraction of RNA using RNeasy Mini Kit (Qiagen). For Western blot analysis cell lysates were prepared in buffer containing 1% Triton X-100 and tris saline pH 7.4 as previously (26) and immunoblotted as above for skeletal muscle lysates.

**RNA stability assay**

Utrophin mRNA stability assays were performed as reported in (40). Briefly, C2C12 myoblasts (2 x 10^5 plated in 60 mm dish) were transiently transfected with 4 µg of pCGN-FHL1 (HA-FHL1) or pCGN vector (HA-Vector) (60), using 4 µl of lipofectamine 2000 according to manufacturer’s instructions. Myoblasts were left untreated or treated with actinomycin D (5 µg/ml, Sigma Aldrich) for 0, 3, 6 or 9 hours. RNA was extracted using RNeasy Mini Kit (Qiagen) and qRT-PCR analysis undertaken for utrophin mRNA as above. Data is represented as the percentage of utrophin mRNA remaining in actinomycin-treated myoblasts relative to untreated control myoblasts (n = 4 independent experiments).

**Statistical analysis**

All statistical analysis was performed using the GraphPad Prism 5 for windows program (version 5.04, GraphPad Software Inc.) or Microsoft Excel. All results are presented as the mean ± the
standard error of the mean (SEM). Statistical significance was assessed using the unpaired student’s
t-test comparing data from *mdx* and *mdx*/FHL1-Tg mice, p-values of <0.05 were considered
significant.
ACKNOWLEDGEMENTS

This work was supported by the Muscular Dystrophy Association USA (grant number 89973) (C.A.M) and in part by a Muscular Dystrophy Association grant to G.S.L. (grant number 175821). C.E.D. was supported by an Australian Postgraduate Research Scholarship. We thank Paul Kennedy and Veronica Gazdik from the State Neuropathology Service (Anatomical Pathology, Alfred Hospital, Australia) for their technical support with skeletal muscle histology. We also thank the Monash Microimaging Facility (Monash University) for provision of instrumentation, training and support. Our gratitude also to Rajendra Gurung (Monash University) for preparation of the model.

CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest for this study.
REFERENCES


FIGURE LEGENDS

Figure 1. Generation of mdx/FHL1-Tg mice.

Western blot analysis of skeletal muscle lysates from wildtype (WT), mdx and mdx/FHL1-Tg mice immunoblotted with antibodies specific for dystrophin (A), endogenous (FHL1) and transgenic HA-FHL1 (HA) (B). Lysates were also immunoblotted with vinculin or β-tubulin-specific antibodies as a loading control. (C) Relative FHL1 protein expression in mdx versus mdx/FHL1-Tg mice was determined by densitometric analysis and standardized for β-tubulin loading. Relative FHL1 expression is shown as mean ± SEM, n=5 experiments per muscle and mouse genotype. (D) Whole body mass. Masses of individual muscles presented relative to body mass at 4-weeks (E) and 16-weeks (F) of age. Data shown in (D-F) represent the mean ± SEM from n=9 mice analyzed per genotype.

Figure 2. Expression of FHL1 results in reduced muscle degeneration in mdx mice.

(A) Low magnification images of H & E-stained transverse muscle sections taken from the gastrocnemius, tibialis anterior (TA) (4 weeks of age) and diaphragm (16 weeks of age) of wildtype (WT), mdx and mdx/FHL1-Tg mice showing degenerating fibers (black arrows). Scale bars, 100µm.

(B) High magnification images of H & E stained transverse sections taken from the gastrocnemius muscles (arrows show degenerating fibers). Scale bars, 100µm, x400 magnification. Higher magnification shown inset. Representative of n ≥ 5 mice analyzed per genotype. (C-E) Mean myofiber diameter and (F-H) the percentage of degenerating (necrotic) fibers from indicated muscles. Necrotic muscle fibers were identified on H & E stained transverse muscle sections by the presence of infiltrating mononucleated cells, fragmented sarcoplasm, basophilic and/or hypercontracted muscle fibers. Data represent mean ± SEM, from n = 5 mice per genotype, *p≤0.05. (I) Serum creatine kinase (CK) levels were measured in WT, mdx and mdx/FHL1-Tg mice following the collection of whole blood post-mortem at 16 weeks of age. Data represent the mean±SEM of n ≥ 16 mice analyzed per genotype, *p≤0.02.
Figure 3. Expression of FHL1 results in improved muscle membrane integrity in mdx mice.

(A) IgM immunofluorescence staining was performed on frozen transverse sections of muscle at 4 weeks and 16 weeks from mdx and mdx/FHL1-Tg mice to detect serum penetration of damaged myofibres due to decreased membrane integrity (arrows). Scale bars, 100µm. The percentage of IgM positive fibers in the TA (B), gastrocnemius (C) and diaphragm (D) muscles were quantified by counting the total number of IgM positive fibers per section. Data represent the mean±SEM from n ≥ 5 mice analyzed per genotype, *p≤0.05.

Figure 4. Expression of FHL1 results in reduced macrophage infiltration in muscles of mdx mice.

(A) Immunofluorescence analysis using a Mac-1-specific antibody for detection of macrophages was performed on transverse sections of TA, gastrocnemius and diaphragm muscles from mdx and mdx/FHL1-Tg mice at 4- and 16 weeks of age. Representative images are shown for the gastrocnemius muscle demonstrating a reduction in macrophage infiltration (arrows) in mdx/FHL1-Tg muscle. Scale bars 100 µm. Higher magnification images shown inset. The total number of Mac-1 positive macrophages was quantified per section in TA (B), gastrocnemius (C) and diaphragm (D) muscles at both 4- and 16 weeks of age from mdx and mdx/FHL1-Tg mice. Data represent the mean±SEM from n ≥ 5 mice analyzed per genotype, *p<0.05.

Figure 5. Muscle regeneration in mdx and mdx/FHL1-Tg mice.

(A) Regenerating myofibres with internal nuclei (black arrows) were identified in H & E stained transverse sections from TA, gastrocnemius and diaphragm muscles in mdx and mdx/FHL1-Tg mice aged 4- and 16-weeks of age. Shown are representative images from the gastrocnemius muscles. Scale bars 100µm. Higher magnification images shown inset. (B) Representative images of dMHC immunofluorescence in the gastrocnemius to detect newly regenerated fibers (white arrows). (C) Representative images of immunofluorescence staining of Pax-7 positive satellite cells (white
arrows) in the gastrocnemius. The percentage of muscle fibers with centralized nuclei (D-F) or expressing the regeneration marker dMHC was quantified. (36) The number of Pax-7 positive satellite cells was also counted per muscle section (J-L). Data represent the mean ± SEM from n ≥ 5 mice analyzed per genotype, *p<0.01.

**Figure 6. Expression of FHL1 reduces fibrosis in the diaphragm of older mdx mice**

(A) H & E stained transverse sections from the diaphragm of older (9 months old) wildtype, mdx and mdx/FHL1-tg mice. Upper and lower panels represent low and high magnification images respectively, scale bars 100 µm. (B) Collagen immunostaining to detect fibrosis in the diaphragm of young (16-weeks) versus older (9 months) wildtype, mdx and mdx/FHL1-Tg mice. Fibrosis is shown by white arrows. (C) The percentage area of collagen staining was quantified from n = 5 mice aged 9 months. Data represents the mean ± SEM, *p<0.0004.

**Figure 7. FHL1 coactivates transcription of the Utrophin A promoter and increases utrophin protein expression in muscles of mdx mice.**

(A) Muscle lysates (Tibialis anterior; TA and diaphragm) from 16-week old mdx and mdx/FHL1-Tg mice were immunoblotted with a utrophin-specific antibody. Vinculin was used as a loading control. Densitometry analysis of utrophin protein expression is shown in (B) and represents the mean utrophin protein expression in mdx/FHL1-Tg mice relative to mdx littermates from n = 4 mice per genotype. (C) Utrophin A mRNA was analyzed using qRT-PCR in muscle from mice aged 16 weeks (n=3 per genotype for the TA muscle, n=10 per genotype for the diaphragm, *p<0.05). (D) Utrophin protein expression in C2C12 myoblasts overexpressing FHL1. Myoblasts were transiently transfected with HA-vector, HA-FHL1 or left untransfected as indicated. Cell lysates were immunoblotted with antibodies specific for HA-tag (detect HA-FHL1 expression), FHL1, utrophin or vinculin (loading control). (E) qRT-PCR analysis of utrophin A mRNA in C2C12 myoblasts overexpressing FHL1. Myoblasts were transfected as in (D) and a subset of cells were also treated with the NFAT inhibitor VIVIT, the calcineurin inhibitor cyclosporine A (CSA) or vehicle. Data
represents the mean from n = 3 independent experiments, *p <0.05, **p<0.001. (F) Utrophin protein expression in C2C12 myoblasts with shRNA knockdown of endogenous FHL1 expression. C2C12 myoblasts were stably transduced with control shRNA or FHL1 shRNA lentivirus as indicated and lysates immunoblotted with FHL1, utrophin and vinculin (loading control)-specific antibodies. (G) qRT-PCR analysis of FHL1 (left panel) or utrophin A (right panel) mRNA in control shRNA or FHL1 shRNA knockdown myoblasts. Data represents the mean from n = 3 independent experiments, *p<0.05. (H) C2C12 myoblasts were transiently co-transfected with combinations of HA-vector or myc-vector, HA-FHL1, myc-NFATc1 as indicated, along with the Lac-Z Utrophin A promoter-reporter and the pRLTK renilla luciferase reporter as a control for transfection efficiency. 48-hours post transfection lysates were prepared and assays for β-galactosidase (LacZ) activity as a measure of utrophin A promoter activation. β-Galactosidase values were normalized to renilla luciferase activity and standardized to myc-vector/HA-vector control expressing samples (defined arbitrarily as 1 relative luciferase unit). Data are mean ± SEM of n=4 experiments, *p=0.03. (I) Utrophin mRNA stability assay. The half-life of utrophin A mRNA was examined by qRT-PCR in myoblasts over-expressing HA-FHL1 or HA-vector, following a timecourse of actinomycin D treatment, which inhibits transcription. Data are mean ± SEM of n=3 experiments, *p<0.05.

Figure 8. Utrophin is recruited to the sarcolemma of mature muscle fibers in mdx/FHL1-Tg mice.

(A) Utrophin immunofluorescence was performed on transverse sections of TA, muscle from 4 week old wildtype, mdx and mdx/FHL1-Tg mice. Muscle fibers were co-stained with DAPI to identify nuclei and distinguish between mature (peripheral nuclei) and regenerating (centralized nuclei) fibers. Muscle sections were also co-stained with α-bungarotoxin to identify the neuromuscular junction and vinculin to highlight the sarcolemma. Utrophin localization at the NMJ is shown (closed arrows). Mature fibers with peripherally located nuclei exhibiting sarcolemmal utrophin localization are also shown (open arrows). Scale bars, 50µm. (B) The percentage of mature
myofibres (distinguished by peripherally located nuclei) exhibiting sarcolemmal utrophin staining per section was quantified. (C) Co-immunostaining of transverse muscle sections from mdx and mdx/FHL1-Tg mice for utrophin and dMHC, the latter a marker for regenerating muscle fibers. TOPRO3-iodide was used to detect nuclei. Open arrows indicate utrophin staining at the sarcolemma. Scale bars, 50µm. (D) Co-immunostaining of transverse muscle sections from mdx/FHL1-Tg mice for utrophin and HA-tagged transgenic FHL1 (HA antibody). (E) Quantification of the proportion of mature myofibres (excluding regenerating fibers) co-stained for transgenic HA-FHL1 and sarcolemmal localization of utrophin. (F) Quantification of the proportion of myofibres with nuclear localization of NFATc1 and (G) both nuclear NFATc1 and sarcolemmal utrophin immunostaining. All quantification represents the mean ± SEM of n=5 experiments per genotype, *p<0.05.

Figure 9. FHL1-mediated expression of utrophin induces formation of a compensatory UGC complex in dystrophin-deficient muscles.

(A) The localization of several dystrophin-associated proteins was analyzed in TA muscle from wildtype, mdx and mdx/FHL1-Tg mice via immunofluorescence. Antibodies specific for laminin, dystrophin, utrophin, α-dystroglycan (α-DG), β-dystroglycan (β-DG), β-Sarcoglycan (β-Sarc), γ-Sarcoglycan (γ-Sarc) were applied to transverse sections of the TA muscle in 4 week old mice. (B) Transverse TA muscle sections from mdx/FHL1-Tg mice co-stained for utrophin and either α- or β-dystroglycan or syntrophin as indicated. Nuclei were detected using TOPRO3-iodide staining. Asterix indicates muscle fibers which exhibit strong sarcolemmal localization of utrophin and also α- or β-dystroglycan or syntrophin. Hash symbol indicates low level sarcolemmal localization for both utrophin and also α- or β-dystroglycan or syntrophin. (C) Transverse muscle sections from the diaphragm of wildtype, mdx and mdx/FHL1-Tg mice immunostained for laminin, dystrophin, utrophin or β-dystroglycan. Scale bars for all images, 50µm.
Figure 10. FHL1 expression enhances skeletal muscle strength and confers protection from contraction-induced damage in mdx mice

Susceptibility of diaphragm muscle strips (A) and TA muscles (B) from 16-week old wildtype, mdx and mdx/FHL1-Tg mice to contraction-induced injury measured in vitro and in situ respectively, *p<0.05 (two-way ANOVA, main effect for TA of mdx versus mdx/FHL1-Tg). Specific force (force normalized for muscle cross sectional area) was also quantified from diaphragm muscle strips (C) and TA muscles (D), *p<0.03 (for diaphragm of mdx versus mdx/FHL1-Tg). Data represents the mean ± SEM from n ≥ 8 mice per genotype. (E) Model showing; FHL1 ameliorates the dystrophic pathology in mdx mice by enhancing NFATc1-dependent utrophin expression and sarcolemmal localization.
ABBREVIATIONS

DMD, Duchenne muscular dystrophy; DGC, dystrophin-glycoprotein complex; FHL1, four and a half LIM protein 1; FHL1-Tg, skeletal muscle-specific FHL1-transgenic mouse; LIM, acronym from Lin11, Isl-1 and Mec-3; mdx/FHL1-Tg, mdx mouse with increased skeletal muscle-specific expression of FHL1; NFATc1, nuclear factor of activated T cells 1; TA, tibialis anterior; UGC, utrophin-glycoprotein complex.
Figure 1

A) Gastrocnemius

<table>
<thead>
<tr>
<th>kD</th>
<th>WT</th>
<th>mdr</th>
<th>mdr/FHL1-Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>427</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>116</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dystrophin
Vinocul

B) Gastrocnemius

<table>
<thead>
<tr>
<th>kD</th>
<th>WT</th>
<th>mdr</th>
<th>mdr/FHL1-Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HA-FHL1
FHL1
β-Tubulin

C) Relative FHL1 protein expression

D) Whole body mass (g)

E) Muscle mass relative to body mass (g)

F) Muscle mass relative to body mass (g)

187x189mm (300 x 300 DPI)
Figure 2. Expression of FHL1 results in reduced muscle degeneration in mdx mice.
167x293mm (300 x 300 DPI)
Figure 3

A

Tibialis Anterior

Gastrocnemius

B

C

D

Tibialis anterior

Gastrocnemius

Diaphragm

166x185mm (300 x 300 DPI)
Figure 4

A

B  Tibialis anterior  C  Gastrocnemius  D  Diaphragm

150x140mm (300 x 300 DPI)
Figure 5. Muscle regeneration in mdx and mdx/FHL1-Tg mice.

161x233mm (300 x 300 DPI)
Figure 6. Expression of FHL1 reduces fibrosis in the diaphragm of older mdx mice.

163x233mm (300 x 300 DPI)
Figure 7

A

B

C

D

E

F

G

H

I

257x329mm (300 x 300 DPI)
Figure 8. Utrophin is recruited to the sarcolemma of mature muscle fibers in mdx/FHL1-Tg mice.

215x227mm (300 x 300 DPI)
Figure 9

Tibialis Anterior (4 weeks)

A

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>mdx</th>
<th>mdx/FHL1-Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dystrophin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-DG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-DG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Sarc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-Sarc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syntrophin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

124x213mm (300 x 300 DPI)
Figure 9. FHL1-mediated expression of utrophin induces formation of a compensatory UGC complex in dystrophin-deficient muscles.

164x287mm (300 x 300 DPI)
Figure 10

A Diaphragm

B Tibialis anterior

C Diaphragm

D Tibialis anterior

E

Specific force (sPo)

Specific force (kN/m²)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>mdc</th>
<th>mdc/FHL1-Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphragm</td>
<td>150</td>
<td>200</td>
<td>250</td>
</tr>
<tr>
<td>Specific force (kN/m²)</td>
<td>100</td>
<td>150</td>
<td>200</td>
</tr>
</tbody>
</table>

155x146mm (300 x 300 DPI)