The root cause of Duchenne muscular dystrophy is the lack of dystrophin in smooth muscle of blood vessels rather than in skeletal muscle per se [version 1; referees: awaiting peer review]

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Abstract

**Background:** The dystrophin protein is part of the dystrophin associated protein complex (DAPC) linking the intracellular actin cytoskeleton to the extracellular matrix. Mutations in the dystrophin gene cause Duchenne and Becker muscular dystrophy (D/BMD). Neuronal nitric oxide synthase associates with dystrophin in the DAPC to generate the vasodilator nitric oxide (NO). Systemic dystrophin deficiency, such as in D/BMD, results in muscle ischemia, injury and fatigue during exercise as dystrophin is lacking, affecting NO production and hence vasodilation. The role of neuregulin 1 (NRG) signaling through the epidermal growth factor family of receptors ERBB2 and ERBB4 in skeletal muscle has been controversial, but it was shown to phosphorylate α-dystrobrevin 1 (α-DB1), a component of the DAPC. The aim of this investigation was to determine whether NRG signaling had a functional role in muscular dystrophy.

**Methods:** Primary myoblasts (muscle cells) were isolated from conditional knock-out mice containing lox P flanked ERBB2 and ERBB4 receptors, immortalized and exposed to CRE recombinase to obtain Erbb2/4 double knock-out (dKO) myoblasts where NRG signaling would be eliminated. Myotubes, the in vitro equivalent of muscle fibers, formed by fusion of the lox P flanked Erbb2/4 myoblasts as well as the Erbb2/4 dKO myoblasts were then used to identify changes in dystrophin expression.

**Results:** Elimination of NRG signaling resulted in the absence of dystrophin demonstrating that it is essential for dystrophin expression. However, unlike the DMD mouse model mdx, with systemic dystrophin deficiency, lack of dystrophin in skeletal muscles of Erbb2/4 dKO mice did not result in muscular dystrophy. In these mice, ERBB2/4, and thus dystrophin, is expressed in the smooth muscle of blood vessels allowing normal blood flow through vasodilation during exercise.

**Conclusions:** Dystrophin deficiency in smooth muscle of blood vessels, rather than in skeletal muscle, is the main cause of disease progression in DMD.

**Keywords**
Dystrophin, DAPC, Duchenne muscular dystrophy, Smooth muscle, Blood vessels, Neuregulin, ERBB2/4, HER2/4
The author declares that the figure and corresponding data reported in this paper was used as part of the information for filing a patent application on ERBB4 receptor modulators based on the discovery that NRG signaling through ERBB2/4, via the cleaved ERBB4 intracellular domain, stimulated dystrophin expression.

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**Introduction**

Signaling from neuregulin 1 (NRG), through its epidermal growth factor (EGF) family of receptors ERBB1-4, has major functions in several organs such as heart, breast, and nervous system including central and peripheral synapses. The role of NRG signaling in skeletal muscle has been controversial. To investigate signaling events in muscle fibers, myotubes formed by fusion of myoblasts, are routinely used as the in vitro equivalent of muscle fibers. We already reported that in myotubes, formed from C2C12 myoblasts, NRG signaling through ERBB2/4 heterodimeric receptors phosphorylated α-dystrobrevin 1 (α-DB1)\(^1\), one of the components of the dystrophin associated protein complex (DAPC). DAPC links the intracellular actin cytoskeleton to the extracellular matrix and is thereby thought to provide structural stability during muscular activity. DAPC, apart from containing dystrophin which is a 427 kDa protein, consists of several other proteins such as α- and β-dystrobrevins, dystroglycans, sarcoglycans, sarcospan, syntrophins, and laminins. At the neuromuscular synapse, the DAPC is also formed with utrophin, also a 427 kDa protein, instead of dystrophin. The phosphorylation of α-DB1 through NRG/ERBB signaling stabilized acetylcholine receptors (AChRs) at the neuromuscular synapse\(^3\). Duchenne and Becker muscular dystrophy (D/BMD) patients have mutation(s) in the dystrophin gene, resulting in the expression of a truncated dystrophin protein\(^4,5\). Taken together, the main body of research on DMD argues for the lack of dystrophin in skeletal muscle as the cause for DMD.

In mice, apart from muscular dystrophy, absence of dystrophin causes neuromuscular junction (NMJ) fragmentation similar to the NMJ fragmentation associated with a loss of NRG/ERBB signaling\(^1\). Lack of dystrophin, besides causing muscular dystrophies, results in cardiomyopathy\(^6\) and is also responsible for several disease states in the brain\(^7\). The importance of NRG signaling for normal cardiac development in mice was firmly established by the fact that ablation of NRG, ERBB4, or ERBB2 resulted in premature death during midgestation\(^8,10\). In cardiac muscle NRG/ERBB4 signaling is sufficient for cardiomyocyte proliferation and repair of heart injury\(^9,10\), but knowledge of the detailed signaling mechanisms and the target proteins through which this was achieved are lacking. The aim of this investigation was to identify the function of NRG/ERBB signaling in muscle and, as it phosphorylated α-DB1 in the DAPC complex, determine if it had a functional role in muscular dystrophy by identifying downstream signaling targets.

**Methods**

**Cell culture, cell lines**

erbb2/4 dKO and l oxP flanked erbb2/4 myoblasts (a kind gift from M. Courtet, and previously described\(^1\)) were cultured on laminin-coated dishes (Roche) and upon reaching 70–80% confluency, were allowed to form myotubes by changing to laminin-coated dishes (Roche) and upon reaching 70–80% confluency, were allowed to form myotubes by changing to differentiation media (2% horse serum, 1% penicillin/streptomycin (Sigma-Aldrich), DMEM (Sigma-Aldrich)).

**Western blotting**

Myotubes from 10-cm culture dishes were harvested in 600 μl lysis buffer and protein complexes were immunoprecipitated as described previously\(^1,11\) with modifications. In brief, myotubes harvested in ice-cold lysis buffer (10 mM Na\(_2\)PO\(_4\), pH 7.8, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease inhibitor mixture (Roche), and phosphatase inhibitors Pic1 and Pic2 (Sigma-Aldrich)) were homogenized in a Dounce homogenizer and incubated for 3 h at 4°C with protein G-coupled mouse monoclonal syntrophin antibody 1351 (4 μl/80 μl protein G beads, Abcam, catalog number ab11425). Beads were then washed in lysis buffer containing protease inhibitors, but without Triton X-100, resuspended in 3 x SDS loading buffer (150 mM Tris-HCl [pH 6.8], 300 mM dithiothreitol [added just before use], 6% SDS, 0.3% bromophenol blue, and 30% glycerol), and denatured (94°C, 3 min) before loading on an 6–8% gradient/0.8% bis-acrylamide SDS-PAGE gels buffered with Tris-glycine.

Gels were transferred onto PVDF membranes (Millipore) and subject to ECL (Thermo Fisher Scientific) development after incubation with primary and secondary antibodies. BSA (3%) was used as a blocking reagent. The following primary antibodies were used: rabbit anti-dystrophin (H300) polyclonal (diluted 1:400, catalog number sc-15376) and mouse anti-utrophin (55) monoclonal (diluted 1:400, catalog number sc-136116) were from Santa Cruz Biotechnology, Inc., mouse monoclonal anti-syntrophin 1351 (4 μl antibody/80 μl protein G beads for lystate from a 10 cm culture dish of myotubes, catalog number ab11425) from abcam and rabbit anti-α-syntrophin 259 (5 μg/ml for Westerns; a kind gift from Stanley C. Froehner and Marvin Adams, University of Washington, Seattle, WA). Goat anti-mouse IgG-HRP (catalog number sc-2005) and goat anti-rabbit IgG-HRP (catalog number sc-2004) secondary antibodies (Santa Cruz Biotechnology, Inc.) were used at a 1:5,000 dilution.

**RNA isolation and qPCR**

RNA isolation and qPCR were performed as previously described\(^12\) and the 2\(^−\Delta\Delta\_Ct\) method was used to analyze relative changes in gene expression. RNA from myotube cultures was isolated with TRIzol (Invitrogen) according to their protocol. DNase I (Promega) treatment and reverse transcription was performed on 1 μg total RNA with random primers and superscript reverse transcriptase from Invitrogen according to their protocol. cDNA was diluted 1:5 before use in qPCR, which was performed with Sybr Green mix (Applied Biosystems) using the Applied Biosystems StepOne machine with two-step PCR (60°C, 1 min and 95°C 15 s) for 40 cycles using the standard program. The quantitative PCR mix was prepared as follows: 12.5 μl SyBR Green mix, 2.5 μl of a 3 μM solution each of forward and reverse primer, 1 μl of diluted cDNA and made up to 25 μl total volume with sterile water. Each sample for real time PCR was done in triplicate and the mean of the resulting three values were taken. The following primers, designed to recognize exons with at least one intron in between for each primer pair, were used for dystrophin, utrophin, and rL8 amplifications: dystrophin forward, 5′-GATGATGAAACATTGTTAATCCAGC-3′ and reverse, 5′-CATATTCTGCTTGCAGATCTCTCG-3′; utrophin forward, 5′-CTAAACTCTGTCCGCGACACG-3′ and reverse, 5′-GTGTCAAGTGTAGTCATGCTATGC-3′ and rL8 (normalization gene) forward, 5′-ACTGGACAGTTCGTGTACTG-3′ and reverse, 5′-GCTTCACTCGAGTCTTCTTG-3′.
Results
We reported previously that on western blots following immunoprecipitation, there were two isoforms of α-dystrobrevin1 associated with DAPC, a 75 kDa and 89 kDa protein. We also demonstrated that ablation of ERBB2/4 receptors resulted in a lack of phosphorylation of the 75 kDa protein. In addition, the amount of the 75 kDa protein detected on western blots, compared to the 89 kDa protein, was reduced in Erbb2/4 dKO myotubes. However in myotubes with intact ERBB2/4 receptors, such as in C2C12 myotubes, it was the other way around i.e. less 89 kDa protein. As absence of dystrophin in the DMD mouse model mdx, also resulted in a reduction in the 75 kDa protein compared to wild-type mice, it was possible that the reduction in the 75 kDa protein in the Erbb2/4 dKO myotubes was due to a reduction in dystrophin. Furthermore the AChR fragmentation observed in mdx mice, paralleled those seen in Erbb2/4 dKO mice raising the possibility that another reason for the observed destabilization of the AChR cluster in Erbb2/4 dKO myotubes could be due to the reduced levels of dystrophin in these myotubes. These observations taken together suggest that one of the targets of NRG/ERBB signaling is dystrophin. To investigate this, Erbb2/4 dKO myotubes derived from immortalized Erbb2/4 dKO myoblasts and myotubes formed from myoblasts, before transfection of myoblasts with Cre recombinase, containing loxP flanked Erbb2/4 genes were used. Both, ERBB2 and ERBB4 receptors were ablated to eliminate NRG signaling through these receptors in muscle, because ERBB4 receptors, apart from forming heterodimers, can also form homodimers and ERBB2 can heterodimerize with ERBB3. Furthermore, as cultured myotubes secrete NRG, the external addition of NRG was not necessary, as demonstrated for phosphorylation of α-dystrobrevin 1 by NRG/ERBB.

Three independent experiments (Figure 1A) each using myotubes formed from a different myoblast clone containing loxP-flanked Erbb2/4 genes, clearly detected dystrophin (lanes 1–3). However dystrophin was not detected in Erbb2/4 dKO myotubes (lanes 4–6) where ERBB2/4 receptors were ablated after CRE recombination of loxP flanked Erbb2/4 genes. Utrophin on the other hand was detected in myotubes with and without

Figure 1. Dystrophin, and utrophin levels in erbb2/4 dKO myotubes. (A and B), Western blots of immunoprecipitated proteins from myotubes with loxP flanked exons of Erbb2 and Erbb4 gene (lanes 1 to 3) and cre mediated knock-out of Erbb2 and Erbb4 genes (lanes 4 to 6) detected with dystrophin (A) and utrophin (B) antibodies. Lanes 1 to 3 and 4 to 6 each represent the same experiment performed independently and loaded on the same gel. The lower panel shows detection of syntrophin that served as a loading control. Immunoglobulin G (IgG) detected is the syntrophin antibody used for the immunoprecipitation. As the western blot in (A) was stripped of antibodies and used in (B), the loading control in (B) applies to both A and B. (C and D) qPCR data of dystrophin and utrophin levels in C2C12 myotubes, relative to Erbb2/4 dKO (Erbb2/4 dKO) myotubes. Expression levels were normalized to ribosomal protein L8 (rL8) expression. This experiment was performed at least twice with similar results. This figure was previously published in a patent (Patent Link: WO 2017/036852 A1), but the copyright is the author’s own.
ERBB2/4 receptors (Figure 1B) demonstrating that NRG/ERBB signaling selectively regulates dystrophin expression (Figure 2).

The qPCR estimation of dystrophin and utrophin levels (Figure 1C, D) confirmed that dystrophin expression is absent in Erbb2/4 dKO (shown in Figure 1 as erbb2/4<sup>−/−</sup>) myotubes, as there was no detectable mRNA and signals in qPCR were only observed above threshold at about 34 cycles which is essentially detection of non-specific amplification or background signal, whereas detection of ribosomal protein L8, used to normalize expression of dystrophin and utrophin, was above threshold at about 22 cycles in Erbb2/4 dKO and C2C12 samples. Detection of dystrophin mRNA in C2C12 cells by qPCR confirmed that dystrophin is present in C2C12 and the level cannot be estimated since the level of dystrophin in C2C12 was relative to that in Erbb2/4 dKO myotubes, for which essentially background non-specific values were obtained in qPCR due to the absence of dystrophin mRNA. Utrrophin detection (Figure 1D), using the same RNA/cDNA preparation used for dystrophin detection, confirmed that the cDNA preparation from Erbb2/4 dKO myotubes used for dystrophin detection was intact. Utrrophin expression was reduced to only less than half the amount (Figure 1D) in Erbb2/4 dKO myotubes compared to C2C12 myotubes which may be due to the lack of NRG signaling since NRG stimulates utrophin expression to some extent<sup>8</sup>. Myotubes formed from C2C12 myoblasts were used as a control for qPCR instead of myotubes containing loxP flanked Erbb4 genes (used for the western blots in Figure 1A, B) as the loxP-flanked Erbb4 gene<sup>18</sup> is a hypomorph due to the insertion of the neo selection cassette. Hence C2C12 myotubes were used instead of Erbb2/4 dKO myotubes to exclude the possibility that levels of dystrophin mRNA may have been affected (NRG signals through ERBB2/4 to stimulate dystrophin expression and reduced Erbb4 expression may have affected this). This is not a problem for dystrophin protein detection (not estimation) in immunoprecipitated samples from myotubes containing loxP flanked Erbb2/4 genes. qPCR on Erbb2/4 dKO confirmed the absence of dystrophin expression (Figure 1C) as observed on the western blot (Figure 1A). Hence NRG/ERBB signaling is necessary for dystrophin expression.

Discussion

Even though dystrophin is lacking in skeletal muscles of Erbb2/4 dKO mice, they do not show dystrophic symptoms<sup>20</sup>. The promoter used for CRE expression in generating Erbb2/4 dKO mice, the human skeletal actin promoter (HSA), is expressed in the striated muscles, skeletal and heart muscle<sup>19,21,22</sup>. Therefore ERBB2/4 and dystrophin levels in smooth muscle of blood vessels would not be affected, as CRE is not expressed in smooth muscle of Erbb2/4 dKO mice, allowing the formation of a normal functional DAPC. Hence smooth muscle of blood vessels in these mice allows for increased blood flow to skeletal and cardiac muscle during exercise. This is because neuronal nitric oxide synthase (nNOS) associating with dystrophin and generating nitric oxide (NO) that signals to soluble guanylate cyclase, generating cyclic guanosine 3',5'-monophosphate (cGMP) in smooth muscle of blood vessels, causes vasodilation enabling exercise-induced increase of blood flow and thereby prevents muscle ischemia<sup>21</sup>. Thus the absence of obvious dystrophic symptoms in Erbb2/4 dKO skeletal muscle where there is a lack of dystrophin strongly suggests that the main cause of muscular dystrophy is not the lack of dystrophin in skeletal muscle per se but systemic lack of functional dystrophin, especially in smooth muscle of blood vessels, resulting in impaired sympatholysis and muscle ischemia during exercise<sup>23</sup>. This hypothesis is consistent with published data using phosphodiesterase type 5 (PDE5) inhibitors, which interfere

Figure 2. Schematic drawing of neuregulin (NRG) signaling to stimulate dystrophin expression. Under normal circumstances, NRG signaling through ERBB2/4 (HER2/4) receptors stimulates dystrophin expression, allowing the formation of a normal dystrophin-associated protein complex (DAPC). If either the dystrophin gene contains mutations or NRG signaling is blocked, then in the absence of functional dystrophin, a normal DAPC is not formed, resulting in various disease states such as dilated cardiomyopathy, Duchenne/Becker muscular dystrophy (DBD), and neuromuscular synapse instability.
with breakdown of NO by PDE5 and thereby prolong the half-life of cGMP, the target of NO\textsuperscript{4}. This was demonstrated in mdx mice where PDE5 inhibition alleviates the dystrophic phenotype\textsuperscript{23} and also in DMD patients where PDE5 inhibition with either tadalafil or sildenafil treatment in Duchenne muscular dystrophy boys restored normal blood vessel function and blood flow during exercise\textsuperscript{24}. Thus the muscle has an extensive vasculature to provide more oxygenated blood through vasodilation the absence of which would result in muscle ischemia, injury and fatigue during exercise.

We previously reported that the blockade of NRG signaling through ERBB2/4 receptors prevented phosphorylation of α-dystrobrevin1 and hence affected NMJ stability\textsuperscript{1}. However ablation of ERBB2/4 receptors, and thus elimination of NRG signaling through them, results in a lack of dystrophin expression (Figure 1). Thus NRG/ERBB signaling maintains NMJ stability through at least two pathways, one where it phosphorylates α-dystrobrevin\textsuperscript{1,2} and the other where it stimulates dystrophin expression and thereby allowing the formation of a DAPC that stabilizes acetylcholine receptors (Figure 2).

NRG/ErbB signaling also induces cardiomyocyte proliferation and repairs heart injury\textsuperscript{9} and is essential for normal cardiac development\textsuperscript{9,10}, whereas dystrophin deficiency does not impair cardiac development but does result in dilated cardiomyopathy (DCM)\textsuperscript{3}. Since ERBB4 can heterodimerize with ERBB2 and a function blocking ERBB2 antibody treatment results in DCM in mice\textsuperscript{11} and DCM in cancer patients with a function blocking HER2 antibody treatment\textsuperscript{12}, this is consistent with NRG/ERBB signaling stimulating dystrophin expression, since DMD patients and mdx mice, both lacking functional dystrophin, develop DCM (Figure 2). Hence the data presented here provides a mechanism for the reported beneficial effects of ERBB4 in repairing heart injury whereby NRG/ERBB2/4 signaling stimulates dystrophin expression. Thus NRG/ERBB carries out different functions in cardiac development and maintenance through different signaling targets, in the latter case through regulating dystrophin expression.

Increasing NRG signaling through ERBB2/4, especially in the smooth muscle of blood vessels, could be a way to increase truncated dystrophin expression in D/BMD patients. This would ameliorate dystrophic symptoms in those patients where the mutation in dystrophin does not affect association of nNOS\textsuperscript{26} and thereby enabling normal blood flow during exercise. Furthermore, as dystrophin is also present in all regions of the brain, being most abundant in the cerebellum\textsuperscript{27}, and since NRG/ERBB signaling regulates dystrophin expression, levels of dystrophin could be the underlying cause of some of the disease states such as schizophrenia, associated with NRG/ERBB function in the brain.

Conclusions
NRG signaling through ERBB2/4 receptors is necessary for stimulation of dystrophin expression. However, when ERBB2/4 receptors are lacking in skeletal muscle but expressed in smooth muscle, mice do not exhibit dystrophic symptoms demonstrating that lack of dystrophin expression in smooth muscle is the root cause of the onset of D/BMD.

Data availability
Dataset 1. Uncropped western blot images and raw Ct values from qPCR. DOI: https://doi.org/10.5256/f1000research.15889.d214628\textsuperscript{2}.

Competing interests
The author declares that the figure and corresponding data reported in this paper was used as part of the information for filing a patent application on ERBB4 receptor modulators based on the discovery that NRG signaling through ERBB2/4, via the cleaved ERBB4 intracellular domain, stimulated dystrophin expression.

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