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General Article

GENERAL ARTICLE

The potential of utrophin and dystrophin combination therapies for Duchenne muscular dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is a lethal neuromuscular disorder caused by loss of dystrophin. Several therapeutic modalities are currently in clinical trials but none will achieve maximum functional rescue and full disease correction. Therefore, we explored the potential of combining the benefits of dystrophin with increases of utrophin, an autosomal paralogue of dystrophin. Utrophin and dystrophin can be co-expressed and co-localized at the same muscle membrane. Wild-type (wt) levels of dystrophin are not significantly affected by a moderate increase of utrophin whereas higher levels of utrophin reduce wt dystrophin, suggesting a finite number of actin binding sites at the sarcolemma. Thus, utrophin upregulation strategies may be applied to the more mildly affected Becker patients with lower dystrophin levels. Whereas increased dystrophin in wt animals does not offer functional improvement, overexpression of utrophin in wt mice results in a significant supra-functional benefit over wt. These findings highlight an additive benefit of the combined therapy and potential new unique roles of utrophin. Finally, we show a 30% restoration of wt dystrophin levels, using exon-skipping, together with increased utrophin levels restores dystrophic muscle function to wt levels offering greater therapeutic benefit than either single approach alone. Thus, this combination therapy results in additive functional benefit and paves the way for potential future combinations of dystrophin- and utrophin-based strategies.

Introduction

Duchenne muscular dystrophy (DMD) is a fatal X-linked neuromuscular disorder caused by loss of function mutations in the dystrophin gene (DMD, MIM #310200; 1,2). This disease affects 1 in 5000 newborn males (3), making it one of the most common recessive disorders in the human population. The cytoskeletal dystrophin protein establishes a mechanical critical link between the extracellular matrix and the actin cytoskeleton in myofibres (4). Dystrophin deficiency in DMD or reduction/truncation in the milder Becker muscular dystrophy (BMD, MIM #300376; 5) leads to the rupture of the myofibre membrane during muscle contraction, triggering chronic inflammation and repeated cycles of muscle necrosis and failed regeneration.

Affected boys are generally diagnosed between 2 and 5 years of age with motor developmental delay leading to progressive muscle degeneration and loss of ambulation usually by the age of 12 (6), assisted ventilation typically before the age of 20 and premature death in the second to fourth decade of life due to cardio-respiratory failure (7). At present, there is no cure for DMD, only palliative care. Current interventions can be categorized into the following two groups: (1) strategies targeting the primary defect aiming to restore dystrophin such as exon-skipping (8), stop codon read-through (9), compensation for the lack

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of dystrophin with micro-dystrophin gene (10,11) or utrophin overexpression approaches (12); and (2) approaches to mitigate secondary and downstream pathology (13). These approaches are promising and several clinical trials are on-going or have been conducted, with variable success (14). Each strategy has its own benefits and potential caveats. Dystrophin restoration-based strategies, such as termination codon read-through and exon-skipping, are only applicable to a specific subset of DMD patients, and gene therapy is limited by poor targeting and low efficiency in fibrotic dystrophic muscle and challenges in virus production and systemic delivery (11,15). Truncated micro-dystrophin and utrophin overexpression strategies will not recapitulate the benefit of the full-length dystrophin. It is unlikely that even dystrophin replacement approaches will be 100% effective. An effective treatment may well lie in the application of a combination of these strategies.

There is now an increasing interest in developing combination DMD therapies (16) with the objective to obtain additive or synergistic benefits above individual potencies and efficacies of each drug. Initial proof of principle studies at the pre-clinical level used dual Adeno-associated virus (AAV) delivery of a micro-dystrophin in conjunction with either insulin-like Growth factor-1 (IGF-1; 17) or follistatin (18), an inhibitor of the negative regulator of muscle mass myostatin (19). Both strategies provide greater benefits than each mono-therapy but rely on the administration of two AAVs, one for delivery of micro-dystrophin and the other for mitigating the downstream pathology. In view of the challenges of systemic single AAV delivery, dual AAV solutions are unlikely to become of routine clinical use. Pre-conditioning treatment with a peptide-phosphorodiamidate morpholino (P-PMO) antisense oligonucleotide to temporarily restore dystrophin at the muscle membrane by exon-skipping improves the membrane integrity and reduces the loss of the AAV genome in the *mdx* mouse but this may not be viable in patients where muscle growth and degeneration are significant (20). Exploration of combination therapies is needed to maximize clinical benefit for DMD patients (16).

Utrophin is a structural and functional paralogue of dystrophin (12,21,22), ubiquitously expressed and distributed throughout the sarcolemma in foetal muscle (23–25). Utrophin is progressively replaced by dystrophin at the muscle membrane during late embryonic stages and is restricted to the myotendinous (MTJ) and neuromuscular junctions (NMJ) and blood vessels in normal adult muscle (26). In dystrophic muscle, utrophin is increased by 2–5-fold (27,28) at the sarcolemma of regenerating fibres (27,29) as part of the repair process. Both proteins share a high level of structural identity (21,30). Utrophin occupies the same cortical cytoskeleton area subjacent to the plasma membrane, normally filled by dystrophin (31), and binds the same complement of proteins with only a few functions operating through distinct molecular mechanisms (30). Thus, it was proposed that utrophin could act as a surrogate to compensate for the lack of dystrophin in DMD (22). Transgenic mice overexpressing utrophin showed its localization along the muscle membrane suppresses pathology in a dose-dependent manner (32), without toxicity (33). This paved the way for the development of utrophin-based therapies applicable to all DMD patients regardless of their genetic mutation (12,14).

Studies have shown that utrophin and dystrophin can be co-expressed at the sarcolemma of the same muscle fibre. In quadriceps muscle biopsies of human foetuses, utrophin and dystrophin are co-expressed at the sarcolemma, at 12–22 weeks of foetal development (23). Utrophin and dystrophin can also co-localize in the dystrophic context in 'revertant' fibres arising from subsequent mutations or an exon-splice event (34). In BMD patients and DMD carriers, utrophin is detected at the sarcolemma of both dystrophin positive and negative myofibres (35,36). Interestingly, the intensity of these utrophin signals is not reduced in these dystrophin positive fibres compared to dystrophin negative myofibres. In inflammatory myopathies such as polymyositis and dermatomyositis, dystrophin is positive and utrophin was reported as increased at the sarcolemma (37) showing that these two proteins could co-localize at the muscle membrane. Thus, dystrophin- and utrophin-based strategies could potentially be used in combination in dystrophic muscles.

In the present study, we investigate the possibility of combining the benefits of dystrophin restoration with increased expression of utrophin. We chose to use transgenic wt and *mdx* mice overexpressing utrophin to address this question as they express different stable levels of the full-length utrophin in all skeletal muscle. The current generation of utrophin drugs does not provide this consistent context and delivering truncated utrophin and dystrophin genes using AAV is unlikely to be relevant for the clinic. In contrast to previous combination attempts, utrophin and dystrophin strategies both target the primary defect of DMD and the muscle membrane instability. Skeletal muscle can tolerate supraphysiological levels of dystrophin up to 50–100× (38) but despite excessive amount of dystrophin, skeletal muscle morphology and function are completely normal (39). Whereas the most developed exon-skipping strategies restore 20–30% of dystrophin expression (40), combining overexpression of utrophin and dystrophin restoration approaches could lead to greater benefits and pave the way to future combinatorial treatments for DMD.

Results

Utrophin and dystrophin can co-localize at the muscle membrane

We first evaluated the expression of dystrophin and utrophin in 14-week-old *tibialis anterior* (TA) muscles from wild-type (wt), wt mice expressing moderate (wt-Fergie) and high (wt-Fiona) levels of utrophin, dystrophin-deficient (*mdx*) and transgenic dystrophin-deficient *mdx* overexpressing moderate (*mdx*-Fergie) and high (*mdx*-Fiona) levels of utrophin. As expected in wt muscle, dystrophin is expressed at the muscle membrane and utrophin is restricted to the NMJ, MTJ and blood vessels (Fig. 1A). In *mdx* muscle, dystrophin is absent and utrophin is increased compared to wt muscle and localized at the sarcolemma of regenerating myofibres (Fig. 1A). Using merosin as a mask, co-laminin-α2/dystrophin and co-laminin-α2/utrophin immunofluorescence stains (Supplementary Material, Fig. S1), we measured the intensity of the sarcolemmal-associated dystrophin and utrophin expression in TA muscles. In *mdx* TA muscle, dystrophin is negligible [Recovery score (RS) = 0] and utrophin is significantly increased 2-fold compared to wt (Fig. 1B and D). These results correlate with dystrophin and utrophin western blots showing a significant 1.9-fold increase of utrophin in dystrophin-null *mdx* muscle compared to wt (Fig. 2).

As in the *mdx*, dystrophin is absent in *mdx*-Fergie and *mdx*-Fiona, (Figs 1 and 2). Measurements of the relative sarcolemmal utrophin intensity showed a 3.5-fold increase of utrophin in *mdx*-Fergie compared to wt and a higher level in *mdx*-Fiona, 5.8-fold increase compared to wt (Fig. 1A, B and D). Similar results were obtained by western blots (Fig. 2). As utrophin is increased by 1.9–2.0-fold in *mdx* compared to wt, our results in *mdx*-Fergie

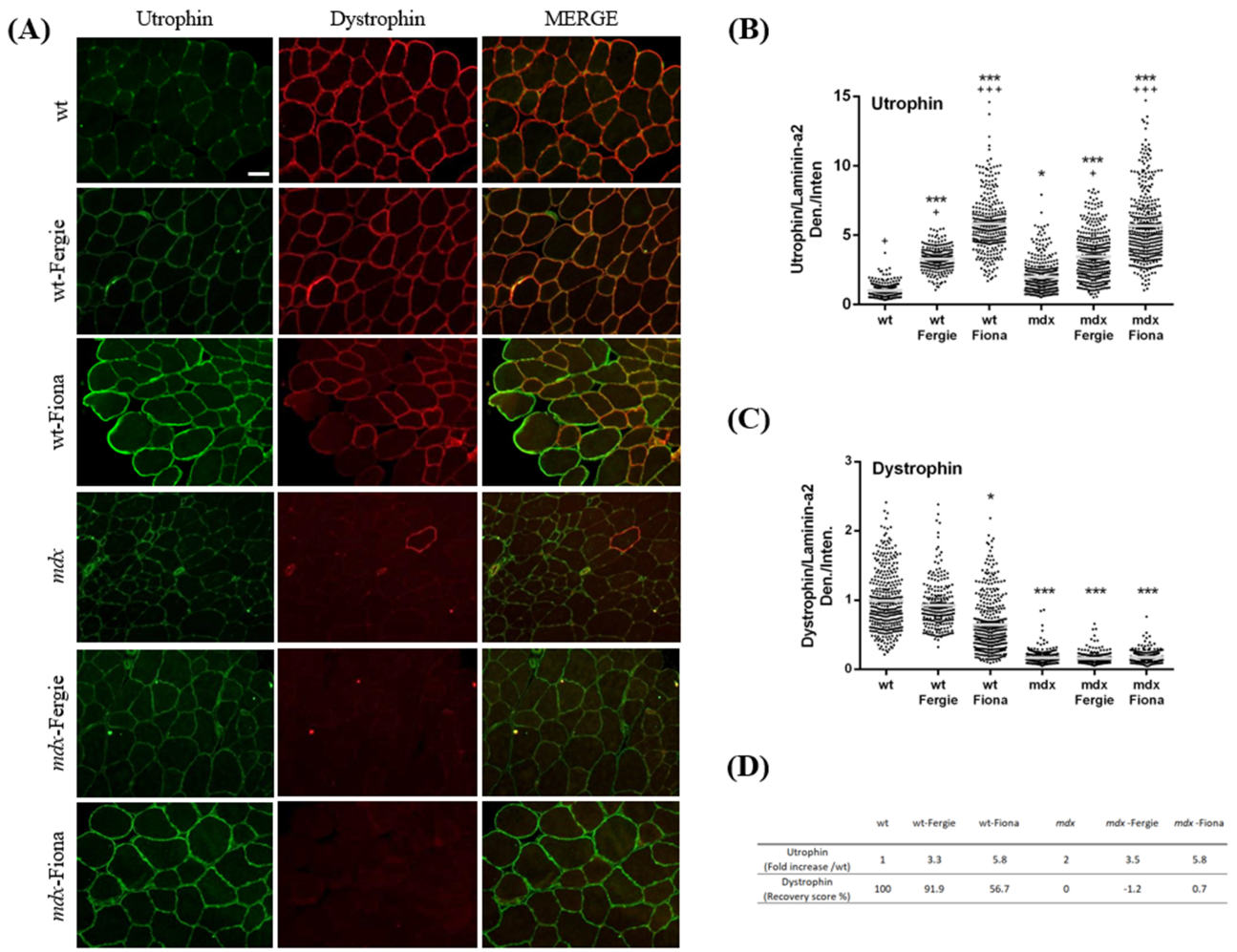


Figure 1. Utrophin and dystrophin can co-localize at the sarcolemma of the same muscle fibre. **(A)** Representative images of co-immunofluorescence of utrophin and dystrophin in wt, wt-Fergie, wt-Fiona, *mdx*, *mdx*-Fergie and *mdx*-Fiona 14 week TA muscle. Scale bar, 100 µm. **(B)** Quantification of utrophin staining relative to control laminin-α2 in 14-week-old muscles. **(C)** Quantification of dystrophin staining relative to control laminin-α2 in 14-week-old muscles. **(D)** Utrophin fold increase and dystrophin recovery score in all mouse strains compared to wt. $n = 4/6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ relative to wt; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.01$ relative to *mdx*. Publisher's note: Two frames in Panel A of Figure 1 were mounted the wrong way round when this paper was first published online. The image for *mdx*-Fergie utrophin has been moved to the *mdx*-Fergie merge location and *vice versa*. The image for *mdx*-Fiona utrophin has been moved to the *mdx*-Fiona merge location and *vice versa*. These changes do not affect the conclusion and an updated figure has been published to replace the original.

(~1.8-fold increase compared to *mdx*) and *mdx*-Fiona (~3.0-fold increase compared to *mdx*) are in line with previous reports using these transgenic animals (32,41,42).

Finally, we studied the dystrophin and utrophin expression in wt-Fergie and wt-Fiona animals. In wt-Fergie, similar utrophin intensity and utrophin protein level were noted compared to *mdx*-Fergie (Figs 1A, B and D and 2). In wt-Fergie, utrophin is therefore expressed at the same level as *mdx*-Fergie despite dystrophin at the sarcolemma. Interestingly, dystrophin is not significantly changed in wt-Fergie (RS = 91.9; Figs 1A, C and D and 2) suggesting that the muscle membrane can tolerate moderate increased levels of utrophin in conjunction with normal levels of dystrophin. In wt-Fiona, utrophin is expressed at the same high level (5.8-fold increase compared to wt) relative to *mdx*-Fiona (Fig. 1A, B and D). Contrary to wt-Fergie, dystrophin is significantly reduced in wt-Fiona with a RS = 56.7 (Fig. 1A, C and D). The immunofluorescence results were confirmed by western blots showing the significant reduction of dystrophin level (RS = 61.3) in a high utrophin level context (Fig. 2). Thus, the sarcolemma cannot contain high levels of utrophin and normal levels of dystrophin.

Taken together, these results indicate that utrophin and dystrophin can be expressed and co-localized at the sarcolemma of the same muscle fibre. However, whereas wt dystrophin is not significantly affected by moderate levels of utrophin at the muscle membrane, high levels of utrophin at the sarcolemma result in a significant reduction of the wt dystrophin expression suggesting a finite number of actin binding sites at the muscle membrane.

Overexpression of utrophin offers supra-functional benefits over wt

To investigate functional benefits of moderate and high levels of utrophin in wt and dystrophic contexts, we performed muscle function studies (Fig. 3). Following 10 eccentric contractions (ECs), force is reduced by 13.6% in wt *extensor digitorum longus* (EDL) muscle. In comparison, force drops by 64.2% in *mdx* muscle. Moderate and high levels of utrophin in *mdx* muscle

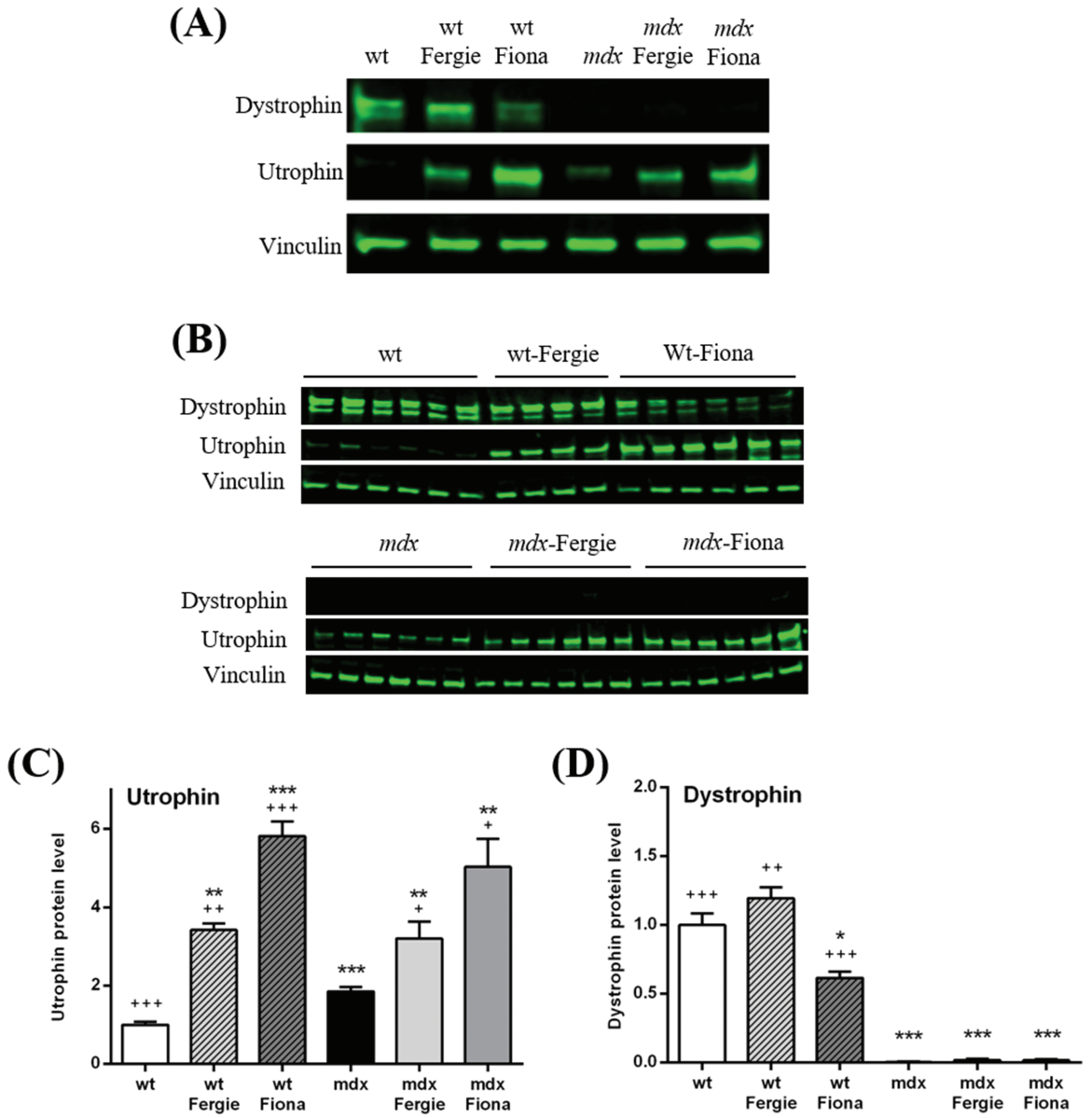
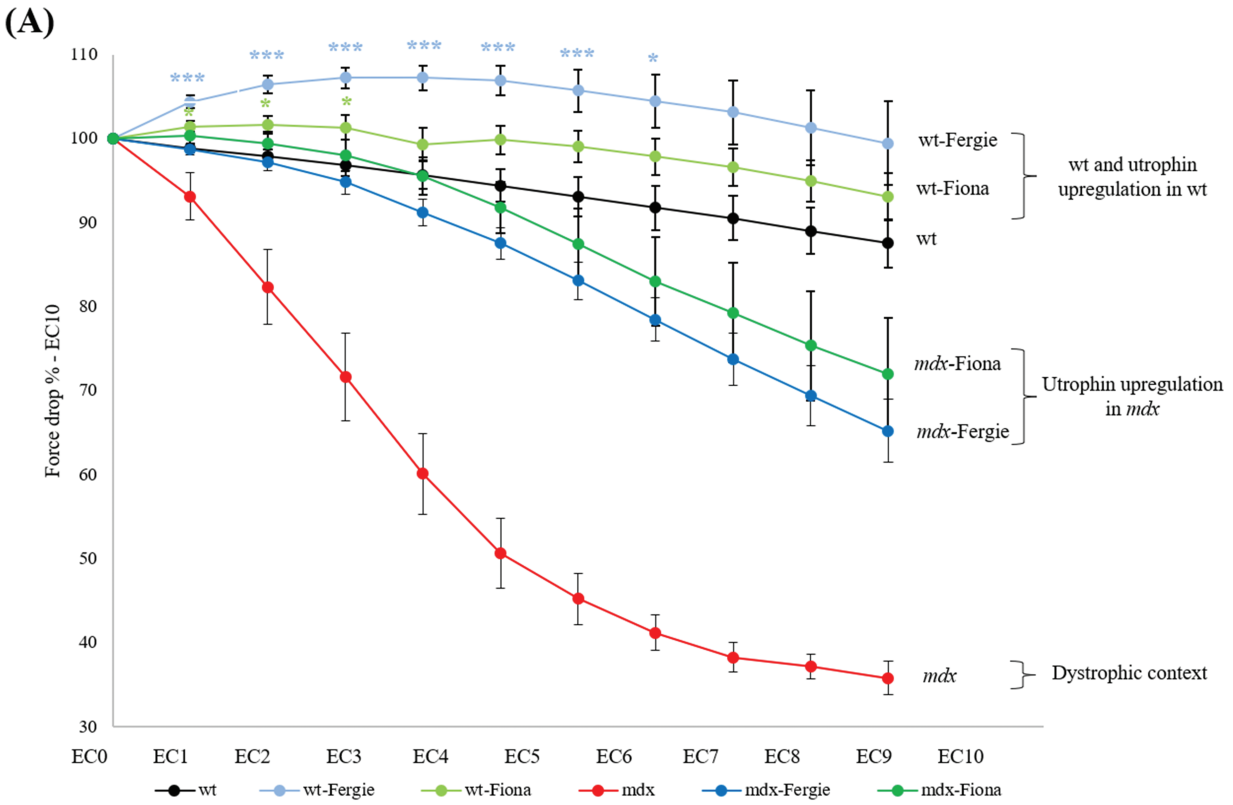


Figure 2. Utrophin and dystrophin can be co-expressed simultaneously at the protein level. **(A)** Relative utrophin and dystrophin protein levels with pooled samples from wt, wt-Fergie, wt-Fiona, *mdx*, *mdx*-Fergie and *mdx*-Fiona 14 week TA detected via western blot. **(B)** Protein immunoblot of all individual samples for all mouse lines involved. **(C)** Utrophin protein quantification relative to wt. **(D)** Dystrophin protein quantification relative to wt. $n = 4/6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ relative to wt; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.01$ relative to *mdx*.

significantly rescue the muscle function in *mdx*-Fergie (34.7% force drop) and *mdx*-Fiona (28.0% force drop). Importantly it should be noted that, in agreement with previously published results (32,42), force production in *mdx*-Fergie (RS = 84.4) and *mdx*-Fiona (RS = 94) is significantly restored to wt level after 5 EC (Fig. 3A and B and Supplementary Material, Fig. S3). Nevertheless, after 10 EC, muscle function is not fully rescued to wt level in *mdx*-Fergie or *mdx*-Fiona. Interestingly, there is no significant difference between these two mouse strains indicating that a moderate level of utrophin has already reached a maximal potential as measured by force drop experiments (Fig. 3A and B and Supplementary Material, Fig. S3). These results highlight the benefit of utrophin overexpression in dystrophic muscle.

As previously described, the wt-Fiona mice express high levels of utrophin and 56% of the normal wt dystrophin. This specific utrophin/dystrophin balance provides a significant 12% functional benefit at EC5 and an 11% improvement over wt after 10 EC (Fig. 3 and Supplementary Material, Fig. S3). Interestingly, the wt-Fergie which expresses moderate levels of utrophin and normal wt level of dystrophin demonstrates greater benefit with a highly significant 29% improvement over wt in this functional assay at EC5. After 10 EC, a 23% benefit in wt-Fergie over wt was observed (Fig. 3 and Supplementary Material, Fig. S3). It should be noted that EDL muscle function in wt-Fergie is significantly improved at EC5 and EC10 compared to *mdx*-Fergie and that wt-Fiona presents a significant improvement relative to *mdx*-Fiona at EC10, illustrating the benefits of the utrophin/dystrophin combination (Supplementary Material, Fig. S3). Overall, the muscle function benefits are greater in wt-Fergie than in wt-Fiona. No difference in absolute force was observed and



(B)

	wt	wt-Fergie	wt-Fiona	*mdx*	*mdx* -Fergie	*mdx* -Fiona
EC5 - Recovery score %	100	128.7	112.5	0	84.4	94.0
EC10 - Recovery score %	100	123.0	110.7	0	56.9	70.0

Figure 3. Combining utrophin and dystrophin results in supra-functional benefits over wt. **(A)** Force drop in wt, wt-Fergie, wt-Fiona, *mdx*, *mdx*-Fergie and *mdx*-Fiona 14 week EDL after 10 ECs. $n = 4/6$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.01$ relative to wt. **(B)** Recovery score at EC5 and EC10.

specific force was normalized to wt levels without supra-benefits in wt-Fergie, wt-Fiona, *mdx*-Fergie and *mdx*-Fiona (data not shown).

Whereas increased dystrophin in wt animals does not offer functional benefit, these results demonstrated for the first time that overexpression of utrophin in wt animals results in significant supra-functional improvements over wt mice. These findings highlight an additive functional benefit between utrophin and dystrophin.

Dystrophin restoration and utrophin overexpression in dystrophin-deficient muscles

We next assessed the consequence of dystrophin restoration using an exon-skipping approach with P-PMO treatment in *mdx*, *mdx*-Fergie and *mdx*-Fiona mice. A previous study reported that a 2 week treatment with a single intravenous dose of P-PMO results in a 37% dystrophin restoration and fully rescues the muscle function in *mdx* TA muscle (40).

Following a similar protocol with a single intravenous injection, a homogeneous sarcolemmal dystrophin expression was observed throughout the whole of the *mdx* treated TA muscle (Fig. 4A). In agreement with previously published results, we quantified a significant 32.6% recovery of the sarcolemmal dystrophin expression (Fig. 4C and D) in *mdx* treated TA muscle using co-laminin-α2/dystrophin immunostaining (Supplementary Material, Fig. S2). The restoration of the dystrophin protein was confirmed by western blot analysis (Fig. 5). Interestingly, utrophin expression was not altered after dystrophin restoration (Figs 4A, B and D and 5).

Following P-PMO treatment in *mdx*-Fergie and *mdx*-Fiona, sarcolemmal dystrophin expression was restored to 34.2% and 33.9%, respectively, relative to wt animals (Fig. 4D). The restoration of dystrophin in *mdx*-Fergie and *mdx*-Fiona is comparable to the dystrophin rescue in P-PMO treated *mdx* (Fig. 4A, C and D). Thus, moderate and high levels of utrophin do not influence the efficacy of the P-PMO treatment which is similar in *mdx*, *mdx*-Fergie and *mdx*-Fiona. Furthermore, respective moderate and high utrophin protein levels in P-PMO treated *mdx*-Fergie and *mdx*-Fiona were unchanged compared to untreated animals (Fig. 4A, B and D). Western blot analysis confirmed these results (Fig. 5).

Taken together, these results demonstrate that the muscle membrane can fully support 30–35% of wt dystrophin levels in combination with moderate and high levels of utrophin.

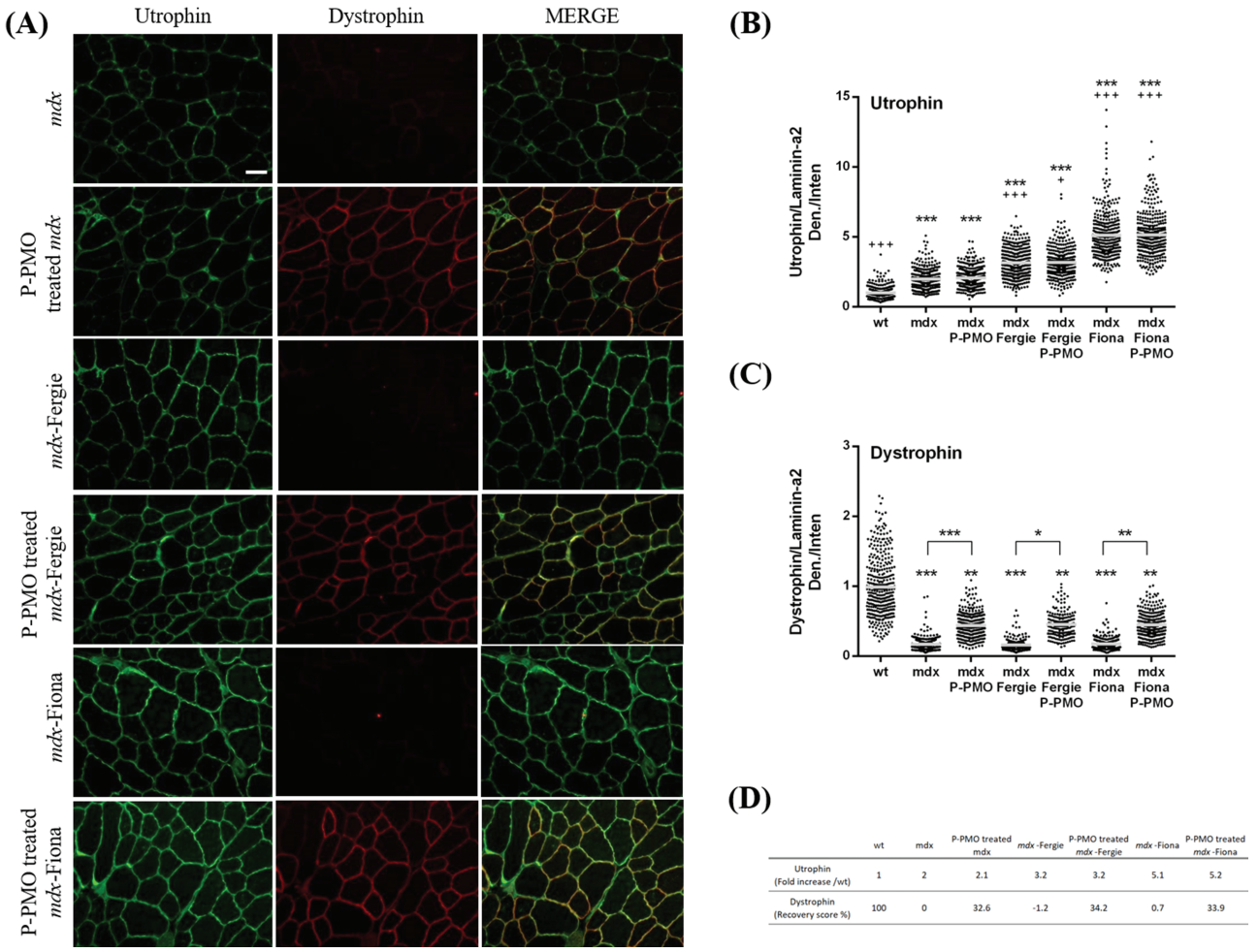


Figure 4. Restoration of dystrophin using exon-skipping is compatible with moderate and high level of utrophin in dystrophic muscle. **(A)** Representative images of co-immunofluorescence of utrophin and dystrophin in untreated and P-PMO treated *mdx*, *mdx*-Fergie and *mdx*-Fiona 14 week TA muscle. Scale bar, 100 µm. **(B)** Quantification of utrophin relative to control laminin-α2 in 14-week-old muscles. **(C)** Quantification of dystrophin immunohistochemical staining relative to control laminin-α2 counter-stain in 14-week-old muscles. **(D)** Utrophin fold increase and dystrophin recovery score in all mouse strains compared to wt. $n = 4/6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ relative to wt; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.01$ relative to *mdx*.

Additive functional effect of dystrophin restoration and utrophin overexpression

P-PMO is a well-characterized exon-skipping strategy providing significant functional benefits in the dystrophic *mdx* model (40,43). Knowing that this treatment is equally efficient in different increased utrophin contexts raises the question of whether restored dystrophin/overexpressed utrophin combination could be functionally beneficial.

As expected, restoration of dystrophin using P-PMO significantly protects skeletal muscle from EC damage. After 5 EC, P-PMO treatment results in a 71.63 recovery score and in a 67.8 recovery score at EC10 (Fig. 6). Similarly, moderate and high levels of utrophin in untreated *mdx*-Fergie (RS-EC5 = 84.4; RS-EC10 = 56.9) and *mdx*-Fiona (RS-EC5 = 94.0; RS-EC10 = 70.0) provide equivalent muscle function protection (Fig. 6). No significant difference between P-PMO treated *mdx* and untreated *mdx*-Fergie and *mdx*-Fiona were noted (Supplementary Material, Fig. S3C and D). Despite these functional benefits, none of these mice shows a fully rescued muscle function compared to the wt condition, notably after 10 EC (Fig. 6 and Supplementary Material, S3D). By contrast, P-PMO treatment in *mdx*-Fergie and *mdx*-Fiona results in a complete rescue of the muscle function to wt levels with a respective RS of (EC5) = 107.9; (EC10) = 97.2 and (EC5) = 103.8; (EC10) = 103.3 (Fig. 4B and Supplementary Material, Fig. S3D). Importantly, the muscle function in P-PMO treated *mdx*-Fergie is significantly improved from untreated *mdx*-Fergie. A significant benefit was also noted between P-PMO treated *mdx*-Fiona and untreated *mdx*-Fiona, respectively (Fig. 6A and Supplementary Material, Fig. S3D). These results highlight the benefits of P-PMO in moderate and high utrophin level contexts and illustrate the additive functional improvement of combining dystrophin and utrophin. Importantly, it should be noted that, in this context, P-PMO treated *mdx*-Fergie and P-PMO treated in *mdx*-Fiona are not significantly different. The combination of a 30–35% of wt dystrophin level due to P-PMO treatment and a moderate level of utrophin already provides a full protection to the EDL muscle similar to the wt condition. As illustrated in Figure 6, the following three distinct groups emerged: (1) the dystrophic *mdx* muscle, (2) the single therapies allowing a significant but incomplete muscle function rescue and (3) the combination of these approaches which fully restored muscle function to wt level.

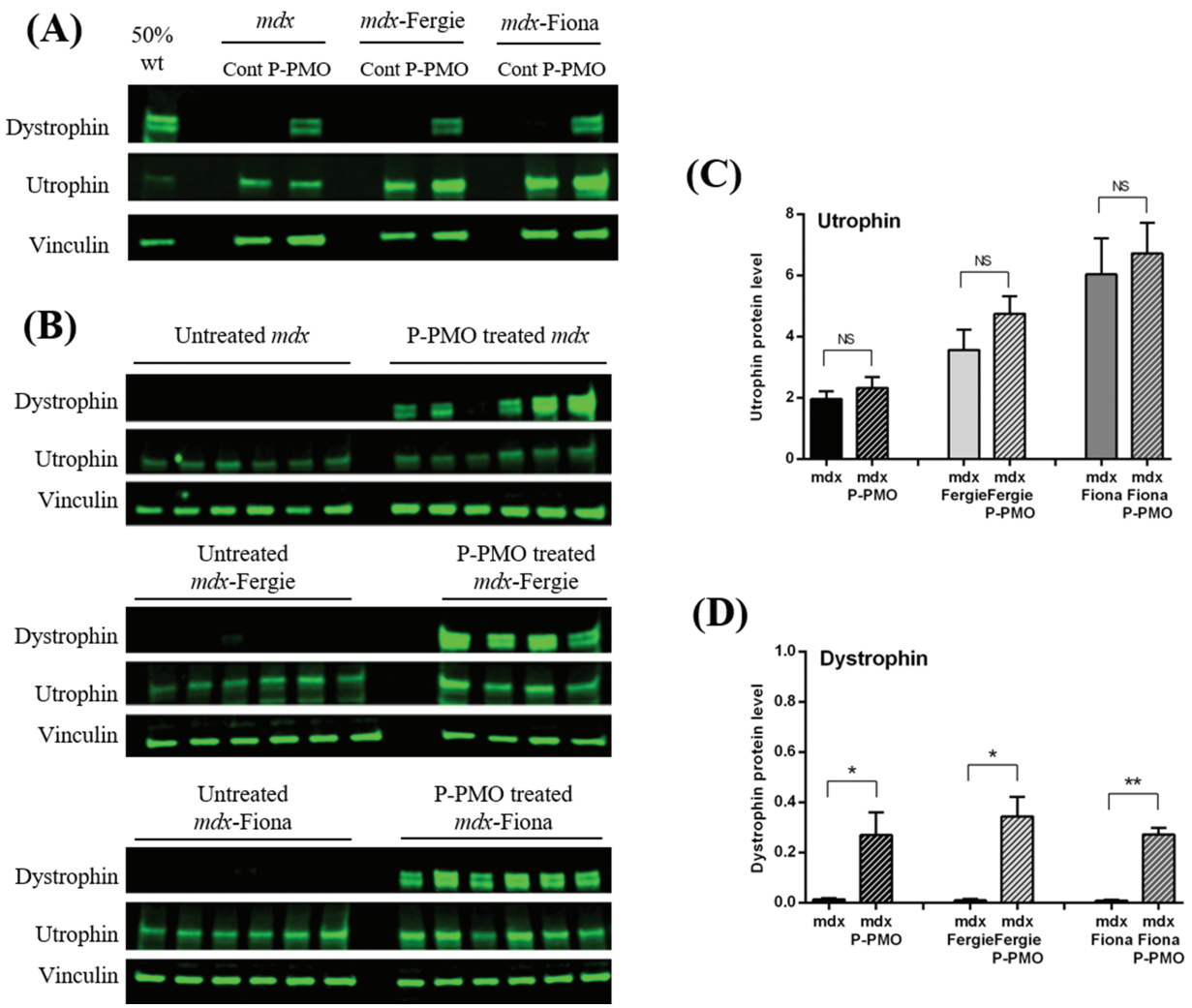


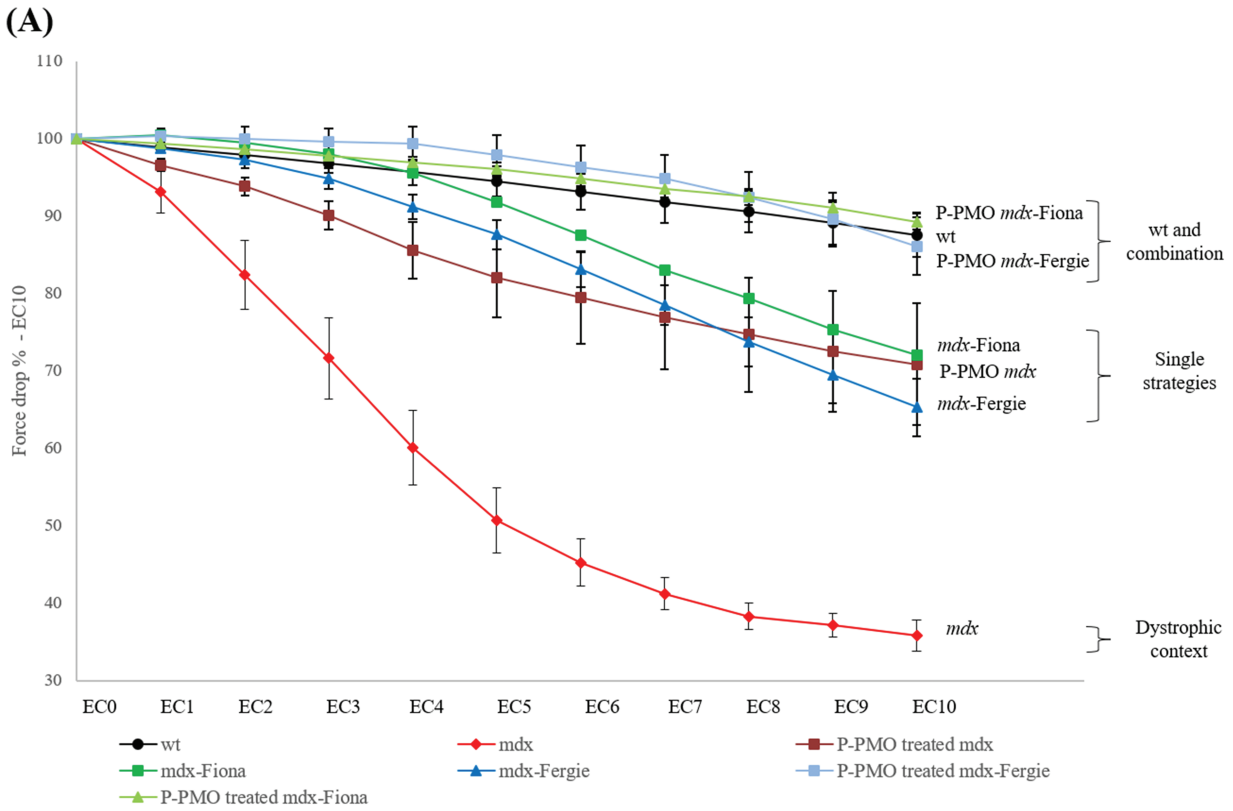
Figure 5. Dystrophin and utrophin protein levels after P-PMO treatment in *mdx*, *mdx*-Fergie and *mdx*-Fiona animals. **(A)** Relative utrophin and dystrophin protein levels in pooled samples from untreated and P-PMO treated *mdx*, *mdx*-Fergie and *mdx*-Fiona 14 week TA. **(B)** Protein immunoblot of all individual samples for all mouse lines involved. **(C)** Utrophin protein quantification. **(D)** Dystrophin protein quantification. $n = 4/6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$.

Discussion

While several promising mono-therapies are currently being evaluated for DMD, the probability of one treatment being able to reverse or completely arrest the progression of all the pathophysiological consequences of the lack of dystrophin in all patients is low (44). Clinical development of single approaches is a critical and necessary step in the DMD field but a one-size-fits-all treatment of all DMD boys does not seem likely. Personalized/combinatorial therapies represent the future of effective DMD treatments.

In this manuscript, we explore the potential of combining the benefits of dystrophin with an increase of utrophin, an autosomal paralogue of dystrophin. We used transgenic mice overexpressing utrophin as they offer different and stable full-length utrophin overexpression that current utrophin modulators drugs and truncated mini-gene AAV strategy cannot provide. To our knowledge, our 'proof of principle' study is the first attempt to combine two strategies for DMD targeting the primary defect of the disease and aiming to restore the sarcolemma stability using two different complementary approaches. As previously described in BMD patients (34), DMD carriers (35,36) or in inflammatory myopathy (37), we confirmed that utrophin and dystrophin can co-localize at the sarcolemma of the same myofibre. Importantly, we show that wt levels of dystrophin are not significantly affected by a moderate increase of utrophin whereas higher levels of utrophin reduce levels of wt dystrophin. Thus, there is an interconnected balance between the utrophin and dystrophin levels in normal muscle. An explanation previously mentioned (31) is that there is a finite number of actin binding sites at the sarcolemma. However, other studies have demonstrated that the muscle membrane can accept a 50-fold dystrophin overexpression (38,39). Therefore, our data suggest that whereas the number of binding sites at the DAPC is finite, utrophin and dystrophin expression could be intrinsically linked and coordinated. Such an idea is reinforced by the observation that in early development, utrophin is highly expressed but progressively silenced after birth by the Ets-2 repressor factor (45) to be replaced at the muscle membrane of adult myofibres by dystrophin (23,46).

Whereas an excessive amount of dystrophin does not improve the function of wt muscle (39), we demonstrated for the first time that utrophin and dystrophin, expressed at the same



(B)

	wt	*mdx*	P-PMO treated *mdx*	*mdx* -Fergie	P-PMO treated *mdx* -Fergie	*mdx* -Fiona	P-PMO treated *mdx* -Fiona
EC5 - Recovery score %	100	0	71.6	84.4	107.9	94.0	103.7
EC10 - Recovery score %	100	0	67.8	56.9	97.2	70.0	103.3

Figure 6. Dystrophin restoration and utrophin overexpression combination results in synergistic functional effect. **(A)** Force drop in wt and untreated or P-PMO treated *mdx*, *mdx*-Fergie and *mdx*-Fiona 14 week EDL after 10 ECs. $n = 4/6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ relative to wt. **(B)** Recovery score at EC5 and EC10.

localization and targeting the primary cause of DMD, can be used in combination and offer additive and extra functional benefits over the normal condition. Importantly, higher functional benefits were obtained in wt-Fergie animals since the combination of a moderate utrophin level together with a wt dystrophin level offers a better overall utrophin/dystrophin balance than high levels of utrophin associated with significantly reduced dystrophin level in wt-Fiona mice. These supra-functional benefits imply that utrophin could fulfil some roles that dystrophin cannot. In wt-Fergie and wt-Fiona mice, utrophin is uniformly distributed at the sarcolemma throughout the muscle under the control of the human skeletal α-actin (HSA) regulatory region. Thus, the potential benefits from utrophin can only come from a specific area where the HSA promoter drives the utrophin transgene expression (47). Whereas utrophin may act as a component of the postsynaptic cytoskeleton, contributing to the development or maintenance of the postsynaptic folds (48), it was recently shown that low dystrophin levels are insufficient to normalize the neuromuscular synaptic abnormalities of *mdx* mice (49). As utrophin and dystrophin show different spatial expression at the NMJ (50), an essential component for force production and transmission, one hypothesis is that utrophin could perform different and complementary roles to dystrophin at the synaptic basal lamina of the NMJ. Therefore, overexpression of utrophin could provide unique benefits at the synapse junction.

In the present study, we also demonstrate the potential of combining utrophin overexpression and dystrophin restoration using exon-skipping in dystrophic muscle. Moderate as well as high levels of utrophin are fully compatible with the 30% of restored dystrophin from P-PMO treatment and the muscle membrane fully accommodates these utrophin and dystrophin levels without saturation of the binding sites. The efficiency of P-PMO to restore dystrophin is the same in the different utrophin contexts and moderate as well as high utrophin expression is unaffected by the restored dystrophin. Importantly, whereas neither utrophin upregulation nor dystrophin restoration by P-PMO fully restores the muscle function, the combined strategy provides a complete recovery of the muscle physiology. The combination is the only therapy able to fully reach the wt functional level. Interestingly, these additive benefits are functionally similar with either a moderate or high level of utrophin suggesting that, in association with a 30% restored dystrophin, a moderate increase of utrophin is sufficient to obtain maximal benefits and normalize muscle function. It will be interesting in future experiments to study the synergistic potential of small amounts of dystrophin restoration with utrophin modulators. These results provide the first pre-clinical data to support the

development of combination strategies based on utrophin modulation and truncated dystrophin restoration approaches such as micro-dystrophin gene therapy or exon-skipping strategies (43,51).

In BMD muscles, the level of mutated dystrophin expression is highly variable from less than 10% to as high as 90% of the full-length expression of normal muscles, with an average of 33% of the healthy muscle (52). In DMD carriers, dystrophin levels are similar or lower than in BMD (35,36). It has been a concern that expression of utrophin might displace the mutant dystrophin from the membrane. Our data demonstrate that a moderate level of utrophin can increase the stability of the membrane in the presence of dystrophin. These data provide important evidence that utrophin-based strategies such as utrophin modulation using small drugs (53) or utrophin micro-gene delivery with rAAV (54) could be applicable to BMD patients. It is unlikely that a moderate increase in utrophin will displace the truncated dystrophin present in BMD patients. These utrophin approaches would also be applicable to DMD carriers with lower dystrophin levels (28).

In summary, our study demonstrates that utrophin and dystrophin can co-localize at the sarcolemma of the same myofibre and that overexpression of utrophin in wt animals results in significant supra-functional benefit over wt. Our data suggest that utrophin fulfils unique roles as well as similar roles to dystrophin and that utrophin upregulation approaches may be applied to BMD patients and other DMD carriers with reduced dystrophin levels. We also demonstrate the additive physiological benefits of combining overexpression of utrophin and restoration of dystrophin-based strategies over mono-therapies. These data pave the way for future combination therapies which represent the future of effective DMD treatment.

Materials and Methods

Mice

All animal procedures were performed in accordance with UK Home Office regulations which conform with the European Community Directive published in 1986 (86/609/ EEC). The work was performed under certificate of designation number XEC303F12 and project license number 30/3104 following the approval by the University of Oxford Departments of Physiology, Anatomy & Genetics and Experimental Psychology Joint Departmental Ethics Review Committee. Wild-type C57BL/10ScSnOlaHsd (wt), wild-type C57BL/10ScSnOlaHsd-Tg (ACTA1-Utrn)1Ked (wt-Fergie), wild-type C57BL/10ScSnOlaHsd-Tg (ACTA1-Utrn)2Ked (wt-Fiona), dystrophin-deficient C57BL/1010ScSn-*Dmdmdx/J* (*mdx*), dystrophin-deficient/utrophin overexpressing C57/Bl10ScSn-*Dmdmdx*/J-Tg (ACTA1-Utrn)1Ked (*mdx*-Fergie) and dystrophin-deficient/utrophin over-expressing C57/Bl10ScSn-*Dmdmdx*/J-Tg (ACTA1-Utrn)2Ked (*mdx*-Fiona) mice were bred in the Biomedical Services facility, University of Oxford.

P-PMO synthesis, preparation and administration

Pip9b2 was synthesized by standard solid phase Fmoc chemistry and purified by HPLC, as previously described (43). The PMO sequence (5′-GGCCAAACCTCGGCTTACCTGAAAT-3′) was purchased from Gene Tools LLC, Philomath, Oregon, United States. For P-PMO treatment, 12-week-old *mdx* mice were administered a single intravenous tail-vein dose of Pip9b2-PMO (10 mg/kg). After 2 weeks of treatment, mice were sacrificed by CO_2 asphyxiation. Muscles were immediately excised and snap frozen in liquid nitrogen or embedded in Optimal cutting temperature (OCT) and frozen in thawing isopentane. Samples were stored at −80°C until further analysis.

Immunofluorescence

Frozen transverse TA muscle sections (10 µm thick) were fixed in acetone for 10 min and blocked for 30 min with PBS1X 10%FBS. Sections were incubated overnight with the following primary antibodies at 4°C: goat anti-utrophin (1:500, URD40), rabbit anti-dystrophin (1:2000, ab15277, Abcam, Cambridge, United Kingdom) and rat anti-laminin-α2 (1:50, sc-59854, Santa Cruz Biotechnology, Dallas, Texas, United States). Sections were next incubated for 2 h at room temperature with the appropriate secondary Alexa Fluor® 488 or 594 antibodies according to the co-immunofluorescence performed and examined under an Axioplan 2 Microscope System (Carl Zeiss, Germany).

Immunohistological intensity measurements

Sarcolemmal utrophin and dystrophin were quantified relative to laminin-α2 co-stain using ImagePro software (MediaCybernetics, Rockville, Maryland, USA) as previously described (40). Six random images were taken from six TA sections and from each mouse. Following the Arechavala-Gomeza approach (28), 10 regions of interest were randomly placed on the laminin-α2 image which was overlaid on the corresponding dystrophin or utrophin image to attain the minimum and maximum fluorescence intensity. Each individual spot on the graphs represents the utrophin mean intensity normalized to the mean intensity of laminin-α2 at one point of the muscle membrane. Approximately 360 areas and individual spots were obtained per mice group.

The percentage recovery score was calculated as described on the TREAT-NMD website (TREAT-NMD SOP M.1.1_001: http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD__M.1.1_001.pdf). All counting was performed blinded and no fibres were specifically selected or removed to generate the results.

Protein analyses

Muscles samples were homogenized on ice in Radioimmunoprecipitation assay (RIPA) buffer (R0278-50 ml, Sigma-Aldrich, St. Louis, Missouri, United States) supplemented with protease inhibitors (P8340, Sigma-Aldrich, St. Louis, Missouri, United States). Following Bicinchoninic acid assay (BCA) quantification, 10 µg of total protein were heat-denatured for 5 min at 100°C before loading onto NuPAGE 3–8% TRIS Acetate Midi Gel (Novex, Life Technologies, Carlsbad, California, United States) and transferred to Polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, Massachusetts, United States). Membranes were blocked for 1 h with Odyssey Blocking buffer (926–41090; LI-COR; USA) and then incubated with the following primary antibodies for 2 h at room temperature: mouse anti-utrophin [1:50, MANCHO3(84A)] and rabbit anti-dystrophin (1:200, ab15277, Abcam, Cambridge, United Kingdom). The Odyssey Imaging System and the Image Studio Lite software (LI-COR Biosciences; USA) were used to quantify target proteins relative to vinculin.

Isolated muscle function analysis

Peak force, specific force and force drop from the EDL muscle were measured as previously described (42). In brief, isolated EDL were attached to a lever arm connected to a force transducer and stimulator; the equipment was controlled using the signal interface and the DMC software (Aurora Scientific, Bristol, United Kingdom). After determination of the optimum length (L_o), the optimum fibre length (L_f) was calculated by multiplying L_o by the fibre length to muscle length ratio of 0.44. A force–frequency curve was generated and the maximum isometric force calculated. Absolute force (P_o) are normalized to specific force (sP_o; mN/cm^2) using the equation (muscle mass/L_f $\times 1.06$). Percentage force drop was calculated by comparing maximum force between the first (EC0) and fifth (EC5) or tenth (EC10) eccentric (EC5) contractions, expressed as a percentage of EC0. All data were digitized and analysed using the DMC software.

Statistics

Results were analysed using Prism (GraphPad Software Inc, La Jolla, CA). Comparison of animal groups was performed using one-way Analysis of variance (ANOVA) with *post-hoc* Tukey test. Statistical difference between two groups was performed using a Student's *t*-test with a two-tailed distribution assuming equal or unequal sample variance depending of the equality of the variance. Data are presented as mean ± SEM with *n* indicating the number of independent biological replicates used in each group. Differences were considered significant at (*) $P < 0.05$; (**) $P < 0.01$ and (***) $P < 0.001$.

Supplementary Material

Supplementary Material is available at *HMG* online.

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