

# Linker molecules between laminins and dystroglycan ameliorate laminin- $\alpha$ 2-deficient muscular dystrophy at all disease stages

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**M**utations in laminin- $\alpha$ 2 cause a severe congenital muscular dystrophy, called MDC1A. The two main receptors that interact with laminin- $\alpha$ 2 are dystroglycan and  $\alpha$ 7 $\beta$ 1 integrin. We have previously shown in mouse models for MDC1A that muscle-specific overexpression of a miniaturized form of agrin (mini-agrin), which binds to dystroglycan but not to  $\alpha$ 7 $\beta$ 1 integrin, substantially ameliorates the disease (Moll, J., P. Barzaghi, S. Lin, G. Bezakova, H. Lochmüller, E. Engvall, U. Müller, and M.A. Ruegg. 2001. *Nature*. 413:302–307; Bentzinger, C.F., P. Barzaghi, S. Lin, and M.A. Ruegg. 2005. *Matrix Biol.* 24:326–332.). Now we show that late-onset expression of mini-agrin still prolongs life

span and improves overall health, although not to the same extent as early expression. Furthermore, a chimeric protein containing the dystroglycan-binding domain of perlecan has the same activities as mini-agrin in ameliorating the disease. Finally, expression of full-length agrin also slows down the disease. These experiments are conceptual proof that linking the basement membrane to dystroglycan by specifically designed molecules or by endogenous ligands, could be a means to counteract MDC1A at a progressed stage of the disease, and thus opens new possibilities for the development of treatment options for this muscular dystrophy.

## Introduction

Congenital muscular dystrophies (CMDs) represent a clinically and molecularly heterogeneous group of autosomal recessive neuromuscular disorders with a typical early onset of symptoms. Estimates in Italy suggest an incidence rate of  $4.65 \times 10^{-5}$  (Mostacciolo et al., 1996). Thus, after Duchenne muscular dystrophy (DMD), CMDs represent the second most frequent neuromuscular disorder. Laminin- $\alpha$ 2-deficient CMD, classified as MDC1A, accounts for ~30–40% of all CMD patients. MDC1A is a severe progressive muscle-wasting disease that leads to death in early childhood (Miyagoe-Suzuki et al., 2000; Muntoni and Voit, 2004; Ruegg, 2005). It shows a rather homogenous clinical picture, with severe neonatal hypotonia associated with joint contracture and inability to stand or walk.

Moreover, MDC1A is accompanied by a peripheral neuropathy that is caused by demyelination in the peripheral and central nervous system. However, no mental retardation is observed in most patients.

Laminins are cruciform-like molecules formed by  $\alpha$ ,  $\beta$ , and  $\gamma$  chains (Fig. 1 A). There are 5  $\alpha$ , 3  $\beta$ , and 3  $\gamma$  chains described so far that give rise to 15 isoforms (Aumailley et al., 2005). The central role of laminins can be explained by their dual function in organizing a structured basement membrane through interaction with other basement membrane proteins and connecting basement membranes to adjacent cells via cell surface receptors. Inactivation of different laminin chains in mice causes distinct phenotypes (for review see Miner and Yurchenco, 2004). The laminin- $\alpha$ 2 chain assembles to laminin-211 (LM-211;  $\alpha$ 2,  $\beta$ 1, and  $\gamma$ 1) and LM-221. LM-211 is the main isoform in the basement membrane of muscle and peripheral nerve, whereas laminin-221 is restricted to neuromuscular junctions (Patton et al., 1997). In the basement membrane, LM-211 and -221 bind to other laminins, to nidogen (which in turn binds to collagen IV and perlecan), and to agrin (Fig. 1 A). The self-polymerization activity of LM-211 is thought to be particularly

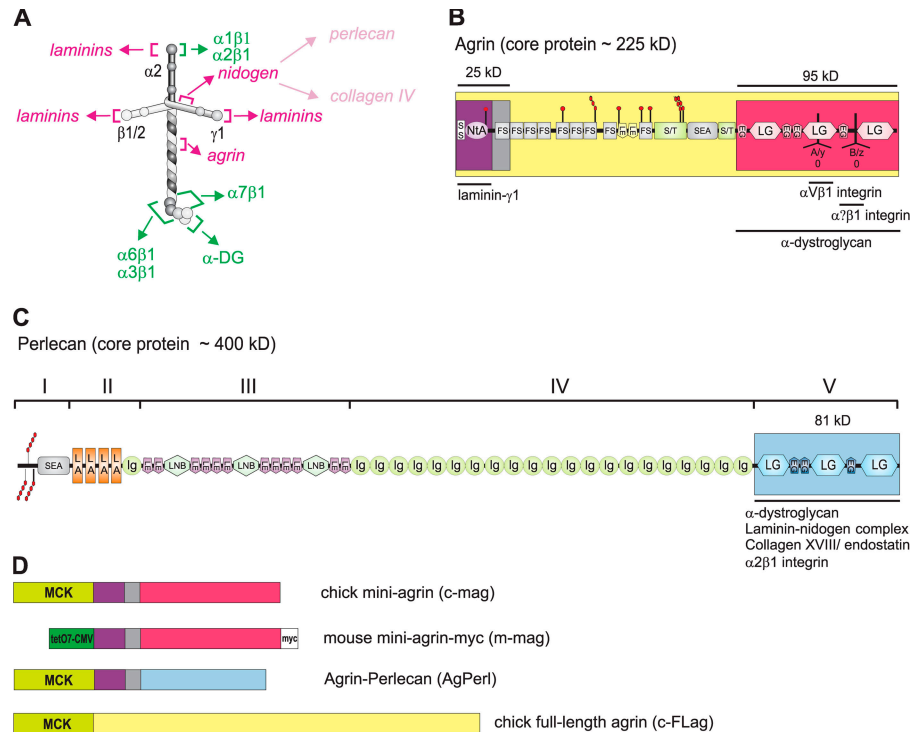
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Abbreviations used in this paper: c-Flag, chick full-length muscle agrin; c-mag, chick mini-agrin; CK, creatine kinase; CMD, congenital muscular dystrophy; DGC, dystrophin-glycoprotein complex; DMD, Duchenne muscular dystrophy; dMyHC, developmental myosin heavy chain; HE, hematoxylin and eosin; MCK, muscle CK; m-mag, mouse mini-agrin; tTA, tetracycline-dependent transcription activator; WT, wild-type.

The online version of this article contains supplemental material.

Figure 1. **Interactions of laminin-211 and scheme of constructs used in the study.**

(A) Structure and binding sites of LM-211. Laminins form by coiled-coil interactions of  $\alpha$ ,  $\beta$ , and  $\gamma$  chains. Interactions of LM-211 and -221 with extracellular matrix components are indicated in red and italics. The main receptors are indicated in green and include different integrins and  $\alpha$ -dystroglycan. (B and C) Schematic representation of nonneuronal agrin (B) and perlecan (C). Domain structures and abbreviations are adopted from previous studies (Bezakova and Ruegg, 2003; Iozzo, 2005). The domains included in the constructs used in this study are color-coded, and relevant binding partners are indicated. (D) Schematic presentation of constructs used in the study. Promoters (green), domains (see color code in B and C), and tags are indicated for each construct. MCK represents the 1.3-kb fragment of the human MCK promoter. TetO7-CMV represents the tetracycline-responsive promoter (Fig. S1 A). Fig. S1 is available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>.

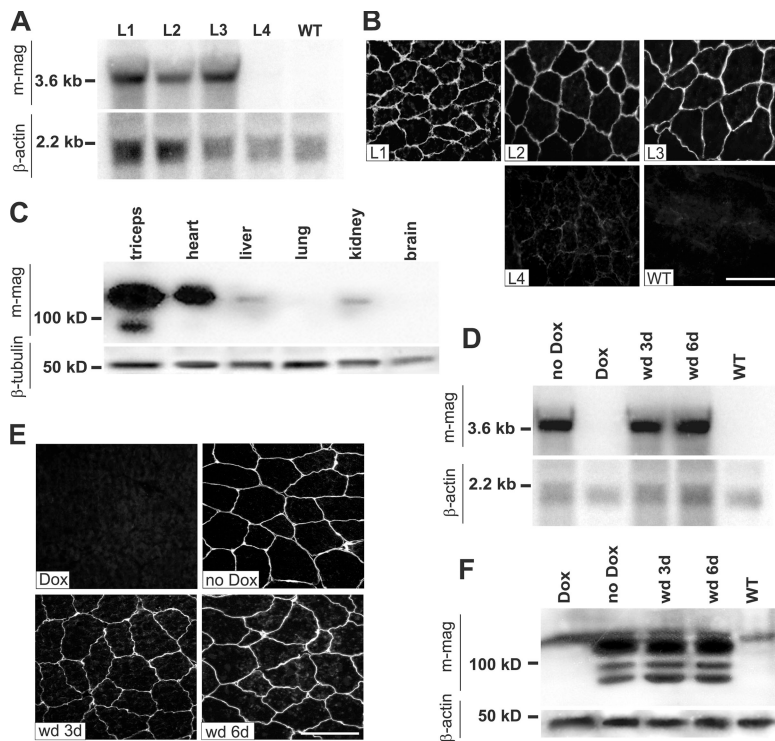


important for the formation of a proper muscle basement membrane. The main receptors for laminin- $\alpha$ 2 in adult muscle are dystroglycan and  $\alpha$ 7 $\beta$ 1 integrin (Fig. 1 A, green). Dystroglycan is cleaved into the peripheral  $\alpha$ -dystroglycan and the transmembranous  $\beta$ -dystroglycan. In the membrane, dystroglycan associates with the sarcoglycans and sarcospan and intracellularly binds to dystrophin, which in turn links the complex to the f-actin cytoskeleton. The complex between LM-211, dystroglycan, sarcoglycans, and dystrophin, which is called the dystrophin-glycoprotein complex (DGC), has been shown to be of utmost importance for the maintenance of muscle integrity, as mutations in these components cause different types of muscular dystrophies (for review see Davies and Nowak, 2006). Similarly, mice or humans that are deficient of  $\alpha$ 7 integrin display a mild muscular dystrophy (Mayer et al., 1997; Hayashi et al., 1998), and muscle-specific inactivation of  $\beta$ 1 integrins has a major impact on muscle development (Schwander et al., 2003). Thus, the evidence is strong that both receptor systems contribute to the linking of basement membrane to the f-actin cytoskeleton, and it is likely that the two systems act synergistically.

MDC1A is among the most severe muscle dystrophies, which may be based on the observation that the absence of laminin- $\alpha$ 2 leaves both receptor systems unoccupied by its ligand. As a compensatory mechanism, muscle fibers of MDC1A patients and laminin- $\alpha$ 2-deficient mice increase synthesis of laminin- $\alpha$ 4 (Patton et al., 1997; Ringelmann et al., 1999; Moll et al., 2001; Bentzinger et al., 2005). However, LM-411 is truncated at the N-terminal end, which prevents its self-polymerization, and it also does not bind to  $\alpha$ -dystroglycan or  $\alpha$ 7 $\beta$ 1 integrin with high affinity (Kortessmaa et al., 2000; Talts et al., 2000). There is

also evidence that muscle fiber membranes of MDC1A patients, and mice models thereof, contain significantly lower levels of  $\alpha$ 7 $\beta$ 1 integrin (Vachon et al., 1997) and  $\alpha$ -dystroglycan (Moll et al., 2001; Bentzinger et al., 2005). In addition, the ability of muscle to regenerate is greatly impaired (Kuang et al., 1999; Bentzinger et al., 2005). These deficiencies lead to the dystrophic phenotype characterized by high levels of creatine kinase (CK) in the blood, large variation in fiber size, successive replacement of muscle by fibrous tissue, and infiltration of adipose tissue. Good models for the disease are *dy<sup>w</sup>/dy<sup>w</sup>* mice generated by homologous recombination (Kuang et al., 1998). Like human patients, *dy<sup>w</sup>/dy<sup>w</sup>* mice have an early onset and severe dystrophic phenotype, which is often lethal between 6 and 16 wk. They grow at a slow rate, the histology of muscles is very similar to that of human patients, and they have a prominent peripheral neuropathy based on defective myelination of the peripheral nerve.

There is no curative treatment for MDC1A. However, a "replacement therapy" using a miniaturized form of the basement membrane component agrin (mini-agrin) was shown to markedly lower muscle degeneration and mortality in *dy<sup>w</sup>/dy<sup>w</sup>* mice (Moll et al., 2001). This is caused by both increasing the tolerance to mechanical load and improving the regenerative capability of the muscle (Bentzinger et al., 2005). These studies left several questions unanswered that were addressed in the current study. First, an efficacious treatment also needs to work after the onset of the disease. Second, a requisite to envisage pharmacological treatment options that aim at increasing synthesis of endogenous agrin is to show that full-length agrin can also have a beneficial effect. Finally, although it is highly suggestive that the beneficial effect of mini-agrin is based on the linking of the up-regulated LM-411 with  $\alpha$ -dystroglycan



**Figure 2. Expression of m-mag in transgenic lines.** (A) Northern blot analysis of quadriceps and (B) immunostaining of triceps brachii cross sections from transgenic mouse lines 1–4 (L1–L4) and WT mice. Highest expression of m-mag mRNA (~3.6 kb) and protein was detected in L3. (C) Western blot analyses from different tissues of L3. High levels of m-mag (~130 kD) were detected in skeletal muscle (triceps brachii) and the heart. Very low levels of m-mag were observed in liver and kidney, but not in lung or brain. (D–F) Expression of m-mag in mouse line L3 is regulated by doxycycline. 5 mg/l doxycycline (Dox) in the drinking water suppresses the expression of m-mag at the transcriptional (D) and protein level (E and F). After withdrawal of doxycycline for 3 d (wd 3d), m-mag is detected, and levels similar to nontreated transgenic mice (no Dox) can be reached after 6 d (wd 6d). Note the lower molecular weight protein bands in (C and F), which are indicative of proteolytic degradation/processing. For normalization, probes for β-actin were used in Northern blot analyses (A and D) and antibodies against β-tubulin and -actin in Western blots (C and F). For quantification see Table I. Bars, 50 μm.

(Moll et al., 2001), other mechanisms (e.g., via integrins) may also contribute.

In an attempt to answer these open questions, we prepared a panel of constructs to generate different transgenic mouse lines (Fig. 1, B–D). First, we used the tet-off system (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>) to generate  $dy^W/dy^W$  mice in which expression of mini-agrin can be temporally controlled (Gossen and Bujard, 1992). Second, we generated transgenic  $dy^W/dy^W$  mice that overexpress chick full-length muscle agrin (c-FLag) in muscle (Fig. 1, B and D). Third, we generated  $dy^W/dy^W$  mice that overexpress a fusion construct in which we replaced the α-dystroglycan binding region of chick mini-agrin (c-mag) with that of mouse perlecan (AgPerl; Fig. 1, C and D). Domain V of perlecan (also called endoreppelin; Iozzo, 2005) binds to α-dystroglycan (Talts et al., 1999), but not to integrins that are expressed in muscle. In this study, we show that mini-agrin can slow down the progression of MDC1A at any stage of the disease, full-length agrin is capable of improving muscle function, and the fusion construct between agrin and perlecan also counteracts the disease. In summary, our results are conceptual proof that linkage of laminin isoforms with α-dystroglycan is a means to treat MDC1A also at progressed stages of the disease.

## Results

The most important questions for developing a treatment are to determine the efficacy of therapy at a progressed stage of the disease, to establish a molecular understanding of how the treatment interferes with disease progression, and to establish possible routes of applying the treatment. To this end, we generated a set of transgenic animals that overexpress artificially designed

proteins in skeletal muscle. All the constructs, including their promoters, are listed in Fig. 1 D.

### Tight spatial and temporal regulation of mini-agrin expression

To test whether mini-agrin is also capable of ameliorating the disease when the phenotype is already apparent, we generated mice in which expression of mini-agrin can be controlled by removal of doxycycline (Gossen and Bujard, 1992). To minimize immune responses and for detection, we constructed mini-agrin from mouse cDNA and fused a c-myc tag to its C terminus (Fig. 1 D). Like c-mag, the tagged mouse mini-agrin (m-mag) bound to LM-111 and α-dystroglycan (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>). Double transgenic mice in which expression of m-mag could be controlled by doxycycline (Gossen and Bujard, 1992; Ghersa et al., 1998) were generated (for details see Fig. 1 D, Fig. S1, and Materials and methods). When they were examined for the expression of m-mag, the highest levels on the mRNA and protein level were detected in line L3 (Fig. 2, A and B; Table I for quantification) which was used in all further experiments. Expression of m-mag was highest in skeletal muscle and heart, whereas only little or no m-mag was detected in liver, lung, kidney, or brain (Fig. 2 C). Next we determined the concentration of doxycycline needed to suppress expression of m-mag throughout embryonic development and to allow fast induction. We found that 5 μg/ml doxycycline in the drinking water of pregnant and gestating females was sufficient to completely inhibit m-mag transcription (Fig. 2 D) and translation (Fig. 2, E and F). 3 d after withdrawal of doxycycline, m-mag was already expressed at high levels, and it reached a maximum after 6 d (Fig. 2, D–F; Table I for quantification).

Table 1. **Quantification of mRNA and protein levels of m-mag in different transgenic lines, and induction by withdrawal of doxycycline**

Line	Dox	NB	WB	IHC
L1		65	63 ± 12	70 ± 8
L2		40	57 ± 14	68 ± 3
L3		100	100 ± 7	100 ± 4
L4		1	3 ± 1	5 ± 2
L3	Dox	1	0	1 ± 1
L3	wd 3d	72	92 ± 6	56 ± 7
L3	wd 6d	102	96 ± 9	97 ± 6

Quantification of Northern blot analysis (NB), immunohistochemistry (IHC), and Western blot analysis (WB). Values show expression levels of m-mag in skeletal muscles of mouse lines L1–L4. Data of line L3 after suppression of expression by doxycycline (Dox) and subsequent withdrawal for 3 (wd 3d) or 6 d (wd 6d) are relative to levels measured in mouse line L3. Values represent the mean ± the SEM.  $n \geq 3$ .

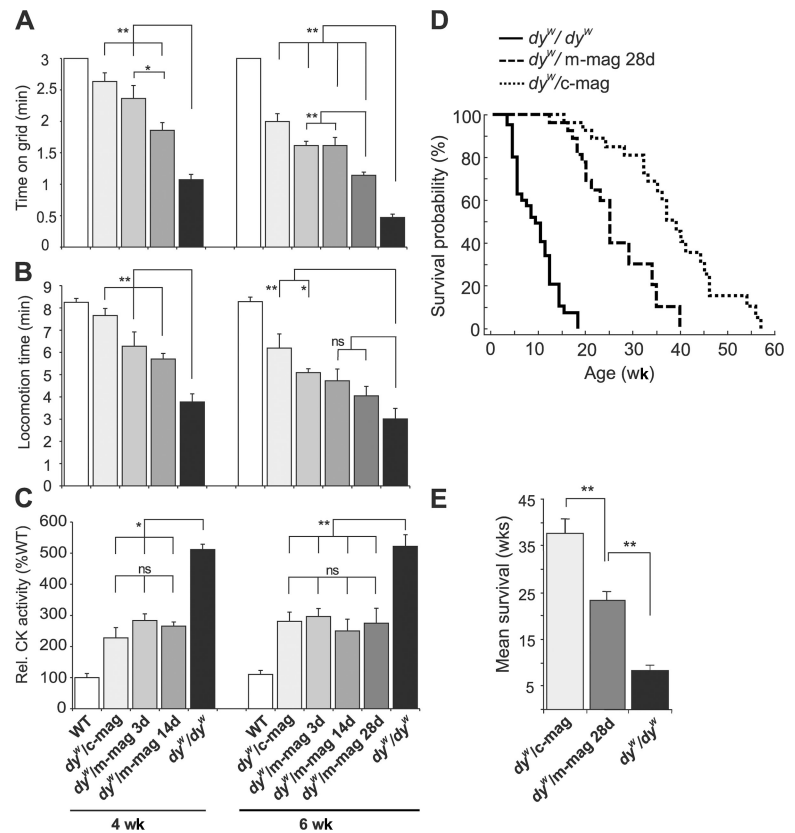
### Late onset of mini-agrin expression ameliorates disease progression in $dy^W/dy^W$ mice

To generate laminin- $\alpha 2$ -deficient mice that allowed controlling expression of m-mag, we mated line L3 with mice heterozygous for the laminin- $\alpha 2$  mutation. This breeding eventually resulted in  $dy^W/dy^W$  mice that contained all the necessary genetic elements (see Fig. S1 B for the breeding scheme). In such mice, which are called  $dy^W/m$ -mag, we removed doxycycline at birth, which resulted in expression of m-mag at postnatal day 3 (henceforth called  $dy^W/m$ -mag 3d), at day 11 ( $dy^W/m$ -mag 14d), or at day 25 ( $dy^W/m$ -mag 28d). Muscular dystrophy was always evaluated in 4- and 6-wk-old mice. In a grip test,  $dy^W/m$ -mag

mice always performed better than  $dy^W/dy^W$  controls, irrespective of when expression of m-mag was started (Fig. 3 A). In the 4- and 6-wk-old animals the improvement was less pronounced if m-mag expression was started late ( $dy^W/m$ -mag 28d). In an open field test, locomotory activity in 4-wk-old m-mag transgenic mice was significantly higher than in  $dy^W/dy^W$  mice, whereas in 6-wk-old mice, statistical significance could only be reached in mice expressing the transgene early (Fig. 3 B). To get a direct measure for ongoing muscle fiber damage, we measured the CK activity in the blood (Fig. 3 C). CK activity was  $\sim 5$  times higher in  $dy^W/dy^W$  than in wild-type (WT) mice. Expression of m-mag lowered CK activity in  $dy^W/dy^W$  mice by approximately half (Fig. 3 C). In contrast to the behavioral tests, the lowering of the CK activity was not dependent on when mini-agrin expression started. Although the improvement in the capability of moving is certainly important to determine the benefit of a treatment, the strongest endpoint in such a severe disease is life expectancy. As shown in Fig. 3 D, treatment that started only after 4 wk substantially increased survival probability. In accordance with this, the mean lifespan of  $dy^W/m$ -mag 28d mice was approximately tripled compared with  $dy^W/dy^W$  mice (Fig. 3 E).

Another hallmark of the severe muscular dystrophy in  $dy^W/dy^W$  mice is the presence of many small fibers and of fibrotic tissue as visualized by hematoxylin and eosin (HE, Fig. 4 A) and Masson's Trichrome staining (Fig. 4 B) of cross sections from triceps brachii. In 6-wk-old  $dy^W/dy^W$  mice, muscle showed strong signs of degeneration and replacement of muscle with nonmuscle tissue (Fig. 4 A, top right). The nonmuscle cells

Figure 3. **Mini-agrin improves overall performance, lowers muscle damage, and prolongs lifespan.** Parameters were measured in 4- or 6-wk-old WT, laminin- $\alpha 2$ -deficient ( $dy^W/dy^W$ ), and laminin- $\alpha 2$ -deficient mice expressing m-mag ( $dy^W/m$ -mag) or c-mag ( $dy^W/c$ -mag). (A) In grip strength, all mini-agrin-expressing mice show a significant improvement compared with  $dy^W/dy^W$  mice. The improvement is less in mice expressing mini-agrin late ( $dy^W/m$ -mag 14d or  $dy^W/m$ -mag 28d). (B) Locomotive activity within 10 min. In 4-wk-old mice, all mini-agrin-expressing mice show a significant improvement compared with  $dy^W/dy^W$  mice. In 6-wk-old mice, only early treatment ( $dy^W/c$ -mag and  $dy^W/m$ -mag 3d) is significant. (C) CK levels in the blood. All values are normalized to WT mice. CK activity is reduced to approximately half of that measured in  $dy^W/dy^W$  mice in all mini-agrin-expressing mice, irrespective of the onset of expression. (D and E) Survival curves of mice with different genotypes. Late start of mini-agrin expression ( $dy^W/m$ -mag 28d) increases the survival probability (D;  $n \geq 29$ ) and the mean survival (E;  $n \geq 16$ ) more than twice in comparison to  $dy^W/dy^W$  mice. Note that late expression of mini-agrin is significantly less effective than constitutive expression of c-mag ( $dy^W/c$ -mag mice). All values represent the mean ± the SEM.  $n \geq 3$ . P-values (t test) are as follows: \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns (not significant),  $P > 0.05$ .

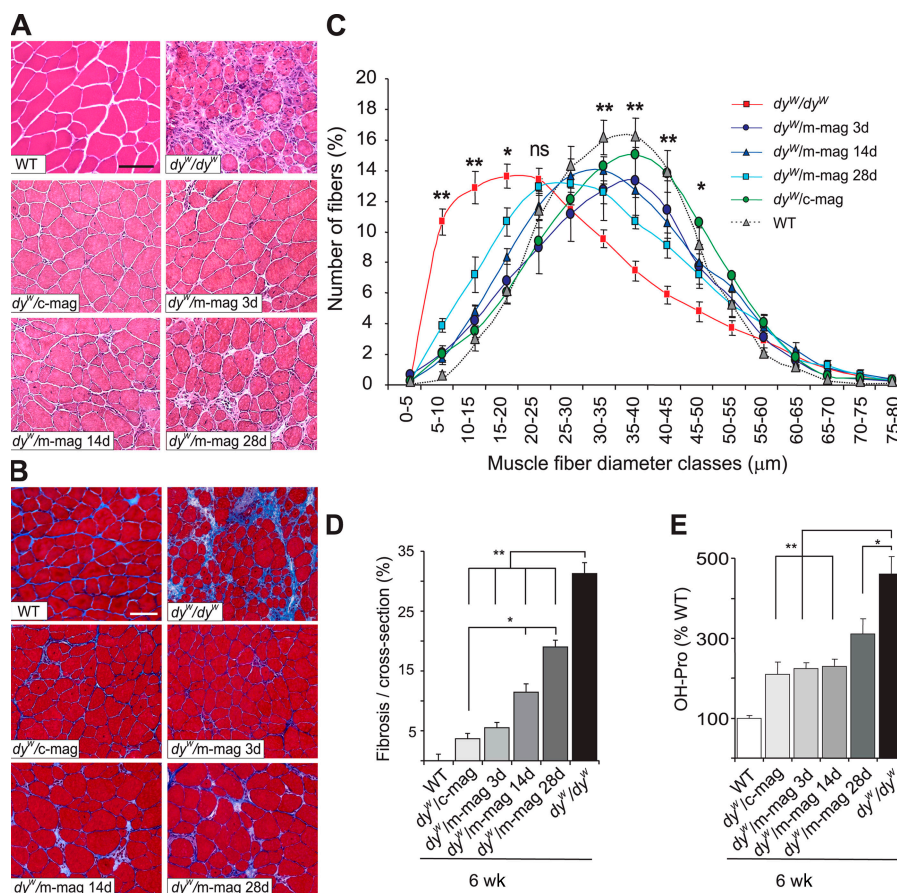




represented mainly fibrotic tissue, as suggested by the blue color in the Masson's Trichrome staining (Fig. 4 B). Moreover, muscle fibers in  $dy^W/dy^W$  mice often lost their characteristic polygonal shape, which is indicative of impaired nerve conduction. Although expression of the m-mag transgene prevented much of the fibrosis (Fig. 4, A and B), it did not affect the shape of the muscle fibers. The extent of fibrosis depended on the time point of the transgene expression. It was, however, compelling that a treatment of only 2 wk was still sufficient to improve the histological picture of the muscle. To measure these parameters more quantitatively, we determined the muscle fiber size distribution in 4-wk- (not depicted) and 6-wk-old mice (Fig. 4 C). Compared with WT mice, the fiber size distribution was obviously shifted toward smaller fibers in  $dy^W/dy^W$  mice, as many did not exceed a minimal diameter of 15  $\mu\text{m}$  (Fig. 4 C). This shift was prevented by the expression of m-mag. To quantify fibrosis, we determined first the relative percentage of the area covered by nonmuscle tissue in a series of muscle cross sections. As shown in Fig. 4 D, expression of the m-mag transgene prevented the fibrotic phenotype of  $dy^W/dy^W$  muscle to a great extent. As an independent measure of fibrosis, we also determined the amount of hydroxylated proline in muscles of the different genotypes (Fig. 4 E). Hydroxyproline is a main constituent of collagens whose expression is high in fibrotic tissue. This quantification also showed the beneficial effect of m-mag. In contrast to the counting of nonmuscle tissue, the amount of hydroxyproline was at least twice as high in all mini-agrin trans-

genic  $dy^W/dy^W$  mice compared with WT controls, and this increase was independent of the time point of expression (Fig. 4 E).

Several lines of evidence strongly indicate that muscles of MDC1A patients, and animal models thereof, have a reduced capacity of regenerating upon damage (Miyagoe et al., 1997; Kuang et al., 1999). In  $dy^{3K}/dy^{3K}$  mice, another mouse model for MDC1A, this pathology is reversed by constitutive expression of mini-agrin (Bentzinger et al., 2005). To test whether the onset of expression of m-mag influences the outcome of the regeneration process, we induced degeneration by injection of notexin into the tibialis anterior muscle of  $dy^W/m\text{-mag}$  28d mice 1 wk after induction of the mini-agrin. Muscles were then examined 6, 14, and 28 d after injection and compared with WT and  $dy^W/dy^W$  mice. As shown in Fig. 5 A, 6 d after injection, many muscle fibers had already reformed in both WT and  $dy^W/m\text{-mag}$  28d mice. Indicative of ongoing regeneration, these muscle fibers expressed high levels of developmental myosin heavy chain (dMyHC; Fig. 5 B). In contrast, muscle of  $dy^W/dy^W$  mice contained mainly cells with a very small cytoplasmic surround and only a few dMyHC-positive fibers (Fig. 5, A and B). The difference between  $dy^W/dy^W$  and WT or  $dy^W/m\text{-mag}$  28d mice was highly significant in the quantitative assessment of the fiber size distribution (Fig. 5 C). 14 d after notexin injection, the muscle fiber size had further increased, and fibers no longer expressed dMyHC in both WT and  $dy^W/m\text{-mag}$  28d mice (Fig. 5 A). Myonuclei still had a central position, which was indicative of the recent regeneration (Fig. 5 A, arrows).

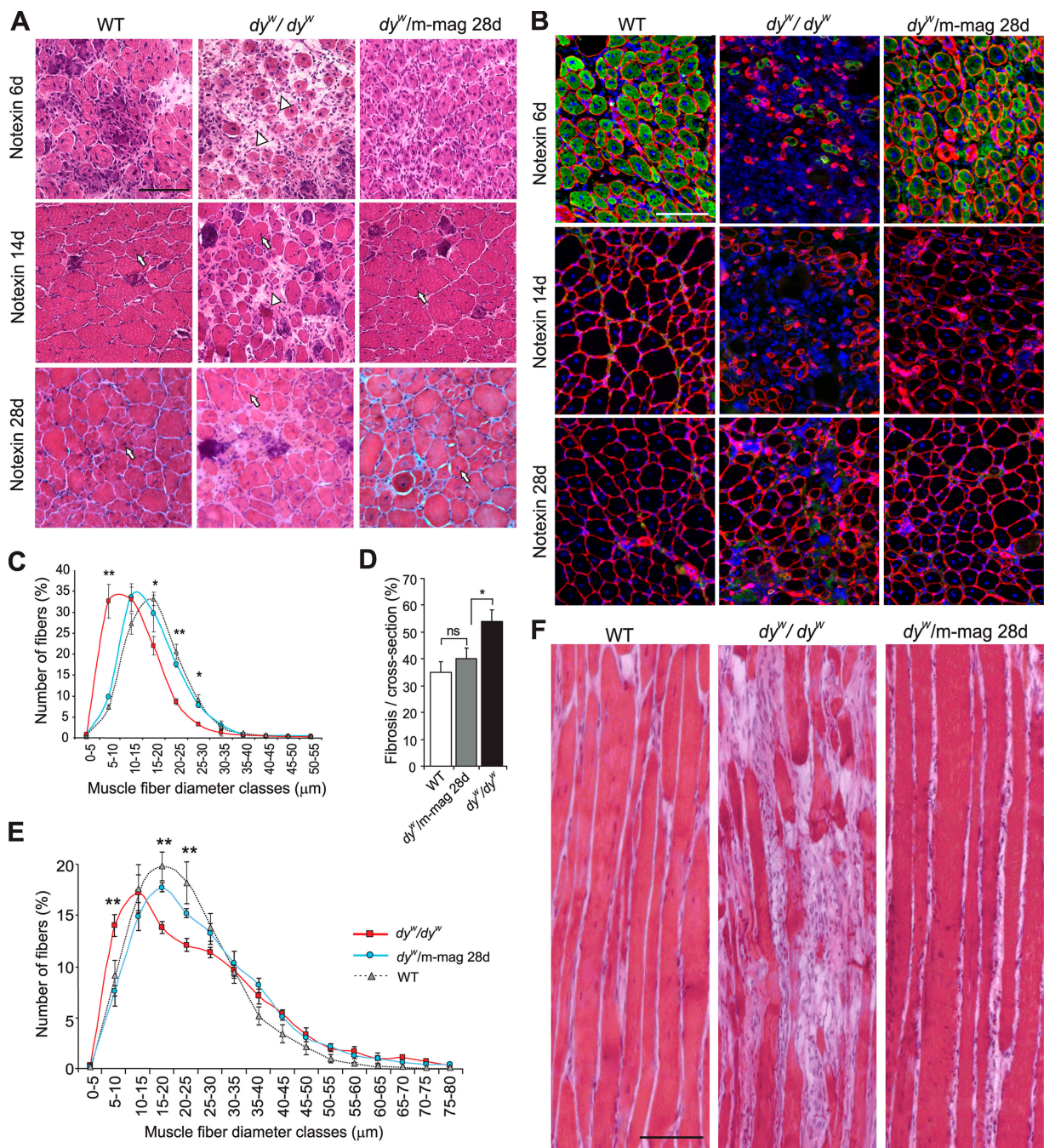


**Figure 4. Phenotype analysis in triceps brachii muscle of 6-wk-old mice.** HE (A) and Masson's Trichrome (B) staining of cross sections. Pathological changes in the muscle of  $dy^W/dy^W$  mice, i.e., fibrosis, variation in muscle fiber diameters, infiltration of nonmuscle tissue, and collagen-containing tissue (blue in B), are less pronounced in mice expressing mini-agrin, but are dependent on the time point of mini-agrin expression. Note that mini-agrin expression does not prevent appearance of polygonally shaped muscle fibers. (C) Muscle fiber size distribution. Values represent relative numbers of fibers in a given diameter class. Muscle fibers of  $dy^W/dy^W$  mice are significantly smaller than age-matched fibers of  $dy^W/dy^W$  mice expressing mini-agrin. (D) Relative contribution of fibrotic regions to the total area in cross sections. In 6-wk-old  $dy^W/dy^W$  mice, the fibrotic tissue represents >30% of the entire muscle. In all the mini-agrin transgenic  $dy^W/dy^W$  mice, the fibrosis is significantly reduced. (E) Relative amount of hydroxyproline (OH-Pro) in muscles of the different genotypes. The amount of OH-Pro is significantly reduced by mini-agrin ( $dy^W/m\text{-mag}$  3d and 14d, >50%;  $dy^W/m\text{-mag}$  28d, >30%). Values represent the mean  $\pm$  the SEM.  $n = 3$ . P-values (t test) are as follows: \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns,  $P > 0.05$ . Bar, 50  $\mu\text{m}$ .



In contrast, cross sections from  $dy^W/dy^W$  mice contained large regions with mononucleated cells (Fig. 5 A, arrowheads), and the few muscle fibers did not express dMyHC (Fig. 5 B). This

deficiency in regenerative capacity of  $dy^W/dy^W$  mice was also eminent in the fiber size distribution (not depicted). 4 wk after notexin injection, muscles had almost completely recovered.



**Figure 5. Expression of mini-agrin enhances regeneration of skeletal muscle after notexin-induced injury of tibialis anterior muscle.** Notexin injection was performed in 5-wk-old mice in which mini-agrin expression had been started 1 wk before. Muscles were analyzed 6 (A and B, notexin 6d; C), 14 (A and B, notexin 14d), and 28 d (A and B, notexin 28d; D–F) after injection. (A) HE-stained cross sections. (B) Staining with the nuclear marker DAPI (blue), antibodies to dMyHC (green), and laminin- $\gamma$ 1 (red) were used to determine the state of regeneration. 6 d after notexin injection, muscle fibers are regenerating in WT and  $dy^W/m\text{-mag 28d}$  mice, while regeneration is poor in  $dy^W/dy^W$  mice; this is indicated by large regions containing mononucleated cells (arrowheads). 14 d after notexin injection,  $dy^W/dy^W$  muscle still contains many mononucleated cells (arrowheads) and little dMyHC is expressed. In WT and  $dy^W/m\text{-mag 28d}$  mice, regeneration has progressed and most of the muscle fibers have lost expression of dMyHC. 28 d after notexin injection, muscle is restored in WT and  $dy^W/m\text{-mag 28d}$ , although centralized nuclei are still present. In  $dy^W/dy^W$  mice, large regions of the muscle fail to regenerate and are replaced by nonmuscle tissue. (C) Fiber size distribution 6 d after notexin injection. Quantification of fibrosis (D) and fiber size distribution 28 d after injection (E). There is a significant difference between  $dy^W/dy^W$  mice and the other two genotypes. (F) HE staining of longitudinal sections of muscles 28 d after notexin injection.  $dy^W/dy^W$  muscle is characterized by extensive fibrosis, and most of the remaining muscle fibers are smaller and thinner than in the other two genotypes. Values represent the mean  $\pm$  the SEM.  $n \geq 3$ . P-values ( $t$  test) are as follows: \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns,  $P > 0.05$ . Bars, 50  $\mu\text{m}$ .

Table II. **Quantification of laminin- $\alpha$ 5 and  $\alpha$ -dystroglycan detected at the membrane of triceps brachii**

Strain	Laminin- $\alpha$ 5	$\alpha$ -Dystroglycan
WT	100 $\pm$ 17	100 $\pm$ 13
$dy^W/dy^W$	138 $\pm$ 9	27 $\pm$ 6
$dy^W/c\text{-mag}$	373 $\pm$ 33	71 $\pm$ 14
$dy^W/m\text{-mag}$ 3d	365 $\pm$ 17	64 $\pm$ 18
$dy^W/m\text{-mag}$ 14d	409 $\pm$ 17	61 $\pm$ 10
$dy^W/m\text{-mag}$ 28d	368 $\pm$ 16	63 $\pm$ 17

Expression levels of laminin- $\alpha$ 5 and  $\alpha$ -dystroglycan were comparable in  $dy^W/c\text{-mag}$  and  $dy^W/m\text{-mag}$  mice starting expression in advanced stages of the disease. Values are relative to WT mice and represent the mean  $\pm$  the SEM.  $n \geq 3$ .

Muscle of  $dy^W/dy^W$  mice still contained large regions that were reminiscent of fibrotic tissue (see Fig. 5 D for quantification), and the diameter of the muscle fibers was often  $<15 \mu\text{m}$  (Fig. 5 E). To see whether the regenerated muscle fibers spanned the entire length of the muscle, we also examined longitudinal sections of tibialis anterior muscle. In contrast to WT mice, most of the regenerated muscle fibers were rather short and thin, and large parts of the muscle of  $dy^W/dy^W$  mice still contained mononucleated cells (Fig. 5 F). Muscle from  $dy^W/m\text{-mag}$  28d and WT mice showed a homogenous fiber size distribution and only little fibrosis. These experiments show that mini-agrin is sufficient to restore the regenerative capacity of muscle from  $dy^W/dy^W$  mice to almost WT levels. Importantly, 1 wk of m-mag expression is sufficient for this effect.

We have previously shown that constitutive overexpression of mini-agrin in  $dy^W/dy^W$  mice leads to increased levels of laminin- $\alpha$ 5 and  $\alpha$ -dystroglycan (Moll et al., 2001), and that this is based on posttranscriptional effects (Bentzinger et al., 2005). The protein levels of laminin- $\alpha$ 5 were also increased in all  $dy^W/m\text{-mag}$  mice, irrespective of the onset of m-mag expression

(Fig. S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>; and Table II). Similarly, using antibodies directed to the core protein (Herrmann et al., 2000), we found increased levels of  $\alpha$ -dystroglycan in all the transgenic mice (Fig. S3 A; Table II). In contrast, we could not detect any changes in the levels of  $\alpha$ 7 integrin (not depicted), which is in agreement with earlier findings (Moll et al., 2001). Because recent experiments showed that transgenic expression of laminin- $\alpha$ 1 is highly beneficial in  $dy^{3K}/dy^{3K}$  mice (Gawlik et al., 2004), we also stained for this laminin chain. Basement membranes surrounding skeletal muscle did not contain detectable levels of laminin- $\alpha$ 1 (Fig. S3 B), which is consistent with published results (Patton et al., 1997). Our data thus show that an increase in the amount of laminin- $\alpha$ 1 is unlikely the mechanism of how mini-agrin ameliorates the disease in  $dy^W/dy^W$  mice.

### Full-length agrin or an agrin-perlecan fusion protein ameliorate disease progression

Another treatment option for MDC1A patients is the up-regulation of the expression of endogenous agrin in muscle, similar to what has been proposed for utrophin in DMD patients (for review see Miura and Jasmin, 2006). Because full-length agrin is a large, highly glycosylated protein, its efficacy in ameliorating the disease might differ from mini-agrin. To test this, we generated transgenic mice that overexpress c-FLag in muscle (Fig. 1, B and D). In another set of experiments, we wanted to test our initial hypothesis that the beneficial effect of mini-agrin is based on the linking of the up-regulated laminin isoforms containing laminin- $\alpha$ 4 to  $\alpha$ -dystroglycan (Moll et al., 2001) and not to the integrins. To this end, we generated a fusion construct in which we replaced the 95-kD, C-terminal half of agrin with domain V/ endorepellin of mouse perlecan (Fig. 1, C and D). Like mini-agrin, this fusion protein (AgPerl) bound to  $\alpha$ -dystroglycan (Fig. S2 C). These data are consistent with the finding that

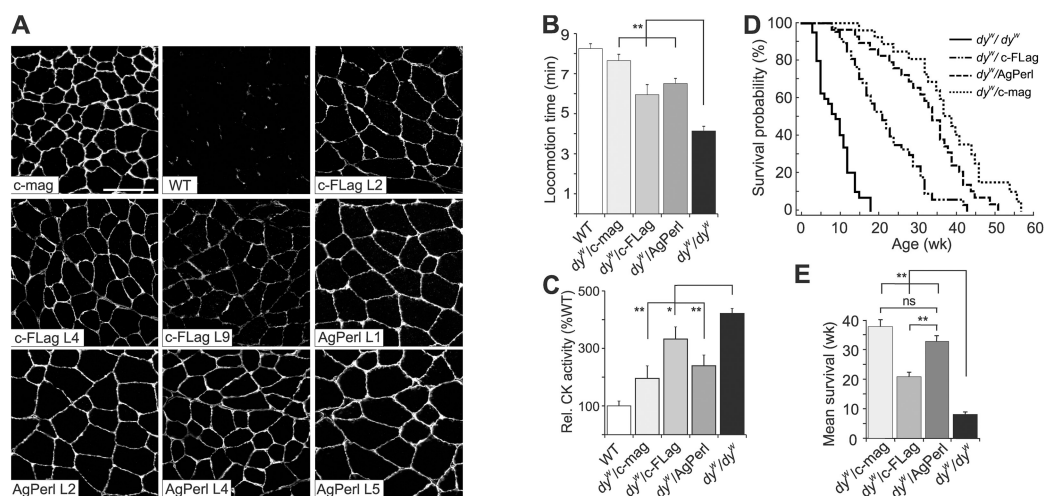


Figure 6. **Transgenic expression of c-Flag and an AgPerl fusion protein improves muscle function and lifespan in  $dy^W/dy^W$  mice.** (A) Immunostaining of transgenes in triceps brachii muscle of transgenic mouse lines for c-Flag (L2, L4, and L9) or AgPerl (L1, L2, L4, and L5). L4 (c-Flag L4) and L1 (AgPerl L1) express the highest levels for c-Flag and AgPerl fusion construct, respectively. For quantification see Table III. Improvement in locomotion (B) or CK values in the blood (C) is evident for all the transgenic mice at an age of 4 wk. (D and E) Life expectancy. The survival probability (D;  $n \geq 29$ ) and the mean survival (E;  $n \geq 23$ ) are more than doubled in the transgenic compared with the  $dy^W/dy^W$  mice. Note that in most of the parameters measured there is a trend that the amelioration is less pronounced in  $dy^W/c\text{-FLag}$  mice than in  $dy^W/c\text{-mag}$  or  $dy^W/\text{AgPerl}$  mice. Values in all quantifications represent the mean  $\pm$  the SEM.  $n \geq 3$ . P-values (t test) are as follows: \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns,  $P > 0.05$ . Bar, 50  $\mu\text{m}$ .



Table III. **Quantification of mRNA levels by Northern blot analysis (NB) and protein levels by immunohistochemistry (IHC) or Western blot analysis (WB) in different transgenic lines expressing c-Flag or AgPerl**

Strain	Line	NB	WB	IHC
c-mag		100	100 ± 6	100 ± 5
c-Flag	L2	57	89 ± 19	84 ± 6
	L4	63	97 ± 10	87 ± 5
	L9	42	67 ± 24	78 ± 9
AgPerl	L1	210	79 ± 2	84 ± 5
	L2	72	70 ± 5	78 ± 6
	L4	42	69 ± 1	87 ± 3
	L5	50	60 ± 9	85 ± 6

Values show the expression in skeletal muscles of c-Flag in mouse lines L2, L4, and L9 and of AgPerl in mouse lines L1, L2, L4, and L5. Data are relative to levels measured in mice transgenic for c-mag (Moll et al., 2001). Values represent the mean ± the SEM.  $n \geq 3$ .

domain V/endorepellin binds to  $\alpha$ -dystroglycan with similar affinity as laminin- $\alpha$ 2 (Talts et al., 1999) or agrin (Gesemann et al., 1998).

Between the different mouse lines, the mRNA levels of the transgenes varied substantially, whereas the amount of protein detected in muscle was similar (Fig. 6 A; Table III). To assess the capability of the transgenes to ameliorate the disease in  $dy^W/dy^W$  mice, the mouse lines with the highest expression levels were crossbred and analyzed (i.e., c-Flag L4 and AgPerl L1). In the locomotory test, all the transgenic lines showed a highly significant improvement compared with  $dy^W/dy^W$  mice (Fig. 6 B). Moreover, CK levels in the blood were significantly lower (Fig. 6 C). Most importantly, the survival probability and the mean survival of the transgenic mice were higher than in  $dy^W/dy^W$  mice (Fig. 6, D and E).

Muscle histology was substantially improved as shown by HE staining of triceps brachii from 4-wk-old mice (Fig. 7 A), and the size distribution of the muscle fibers was shifted to larger fibers (Fig. 7 B). Consistent with the hypothesis that the mechanism of amelioration by the transgenes is the same as in mini-agrin transgenic mice, protein levels for both laminin- $\alpha$ 5 and  $\alpha$ -dystroglycan were elevated (Fig. 7 C; Table IV for quantification). Our experiments thus show that full-length agrin and a fusion protein of agrin and perlecan ameliorate the disease phenotype in  $dy^W/dy^W$  mice. In most measurements, mice expressing the AgPerl transgene showed a better improvement than those expressing c-Flag.

If human patients were to be treated, an appropriate route of application must be defined. Such routes for mini-agrin could be viral vectors (Qiao et al., 2005), but also injection of recombinant protein, as done for other muscle diseases (Bogdanovich et al., 2002; Raben et al., 2003). To test the feasibility of protein application, we determined the turnover rate of mini-agrin in our mouse model. To this end, mice were raised in the presence of doxycycline (i.e., m-mag not expressed), followed by 1 wk without doxycycline (m-mag expressed). Thereafter, doxycycline was reapplied and m-mag expression was followed on the mRNA and the protein level over time. 1 d after readdition of doxycycline, the mRNA encoding mini-agrin had already dropped to  $\sim 10\%$ , and it could not be detected anymore after 2 d (Fig. 8 A). Concomitantly, with the repression of transcription, m-mag protein steadily declined, as determined by Western blot analysis (Fig. 8 B) and immunohistochemistry (Fig. 8 C).

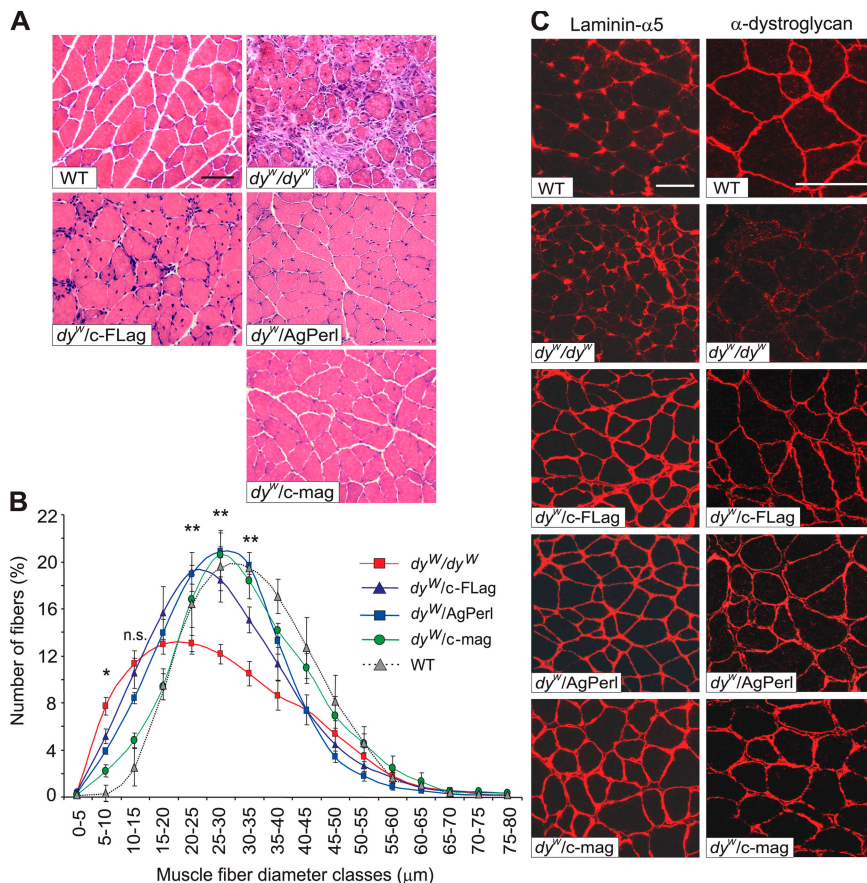
Quantification of the amount of mini-agrin after suppression of its transcription indicates a half-life of 4.5 d (Fig. 8 D).

To get an estimate of how high the levels of agrin must be to achieve an improvement, we compared the levels of endogenous mouse agrin found in other tissues to the levels of the transgenes expressed in muscles of our mice. We first compared the levels of the transgenic protein for mini- and full-length agrin using antibodies that recognize chick, but not mouse, agrin (Fig. 8 E, left column). The amount of c-mag was  $\sim 20\%$  higher than c-Flag (Fig. 8 F; left column). We then compared staining intensity of the transgenic m-mag in muscle with that for endogenous agrin in kidneys using antibodies directed to mouse agrin (Fig. 8 E, right column). Expression levels of the transgenic m-mag were 13% lower than the levels of endogenous agrin detected in kidney (Fig. 8 F, right columns). To compare levels of endogenous agrin in kidney and the amount of c-Flag in muscle, we assumed that the amount of c- and m-mag were the same. This assumption is based on the fact that the overall improvement in the phenotype is the same in  $dy^W/c$ -mag and  $dy^W/m$ -mag mice. Expression levels of c- and m-mag transgenes were therefore set as being equal. Based on this, the protein level of the transgenic c-Flag (81% of c-mag) is substantially lower than the amount of endogenous agrin found in kidney (113% of m-mag). Thus, expression levels of endogenous agrin in kidney are even substantially higher than the levels of the transgenic c-Flag in the muscle. Thus, agents that increase the amount of agrin in muscle to the amount in kidney are sufficient to be of benefit for  $dy^W/dy^W$  mice.

## Discussion

In our previous work (Moll et al., 2001; Bentzinger et al., 2005), we provided in vivo evidence that mini-agrin could be a means to prevent muscular dystrophy in MDC1A patients. The work described in this study approaches both therapeutic and mechanistic aspects of how mini-agrin ameliorates the phenotype in  $dy^W/dy^W$  mice. It provides strong evidence that mini-agrin also decelerates disease progression when applied at late stages, and it shows that full-length agrin, if expressed at a level similar to that in kidney, is capable of ameliorating the disease. Finally, our evidence that the fusion construct between the laminin-binding domain of agrin and the  $\alpha$ -dystroglycan-binding domain of perlecan has the same ameliorating activity in  $dy^W/dy^W$





**Figure 7. Characterization of muscles from mice transgenic for c-Flag or the AgPerl fusion protein.** Animals were analyzed at 4 wk of age. (A) HE staining of triceps brachii cross sections. (B) Muscle fiber size distribution. Values are given as relative number of fibers in each diameter class. Muscles of *dy<sup>W</sup>/dy<sup>W</sup>* mice contain a significantly higher percentage of small fibers. (C) Levels of laminin- $\alpha$ 5 (left column) and  $\alpha$ -dystroglycan (right column) are increased in muscles of *dy<sup>W</sup>/c-Flag* or *dy<sup>W</sup>/AgPerl* mice relative to *dy<sup>W</sup>/dy<sup>W</sup>* mice. See Table IV for quantification. Values in all quantifications represent the mean  $\pm$  the SEM.  $n \geq 3$ . P-values (*t* test) are as follows: \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns,  $P > 0.05$ . Bars, 50  $\mu$ m.

mice as mini-agrin clearly indicates that the amelioration is based on the linking of muscle basement membrane to the DGC, and not to integrins.

#### Mini-agrin slows down MDC1A disease progression

In MDC1A patients, the disease is often diagnosed in the first year of life because of the floppy appearance of the infants. However, muscular dystrophy has already started to manifest at the time of diagnosis, and treatment of infants faces difficulties. Therefore, it is important to evaluate the potential of mini-agrin treatment at progressed stages of the disease. To test this, we generated *dy<sup>W</sup>/dy<sup>W</sup>* mice that allow the temporal control of the expression of mini-agrin in muscle using the tet-off system (Gossen and Bujard, 1992; Ghersa et al., 1998). We show that mini-agrin is of clear, but attenuated, benefit when applied at progressed disease stages. Importantly, expression of mini-agrin after 4 wk, when the disease is already far progressed, still tripled the mean survival. Our evidence indicates that mini-agrin mainly acts on the tissue that has not yet been destroyed in the course of the disease. This is best manifested by the finding that late expression of mini-agrin seems not to affect already existing fibrosis (Fig. 4), and that early treatment is superior in the behavioral tests (Fig. 3, A and B). There is also evidence that the time point of transgene expression is not relevant for parameters that measure acute responses, such as CK activity in the blood (Fig. 3 C) or the regeneration upon injury (Fig. 5). Our experiments are thus evi-

dence that even late application of mini-agrin is highly beneficial, but that treatment is most successful if initiated early.

#### Mini-agrin combines several advantages for a feasible treatment of MDC1A patients

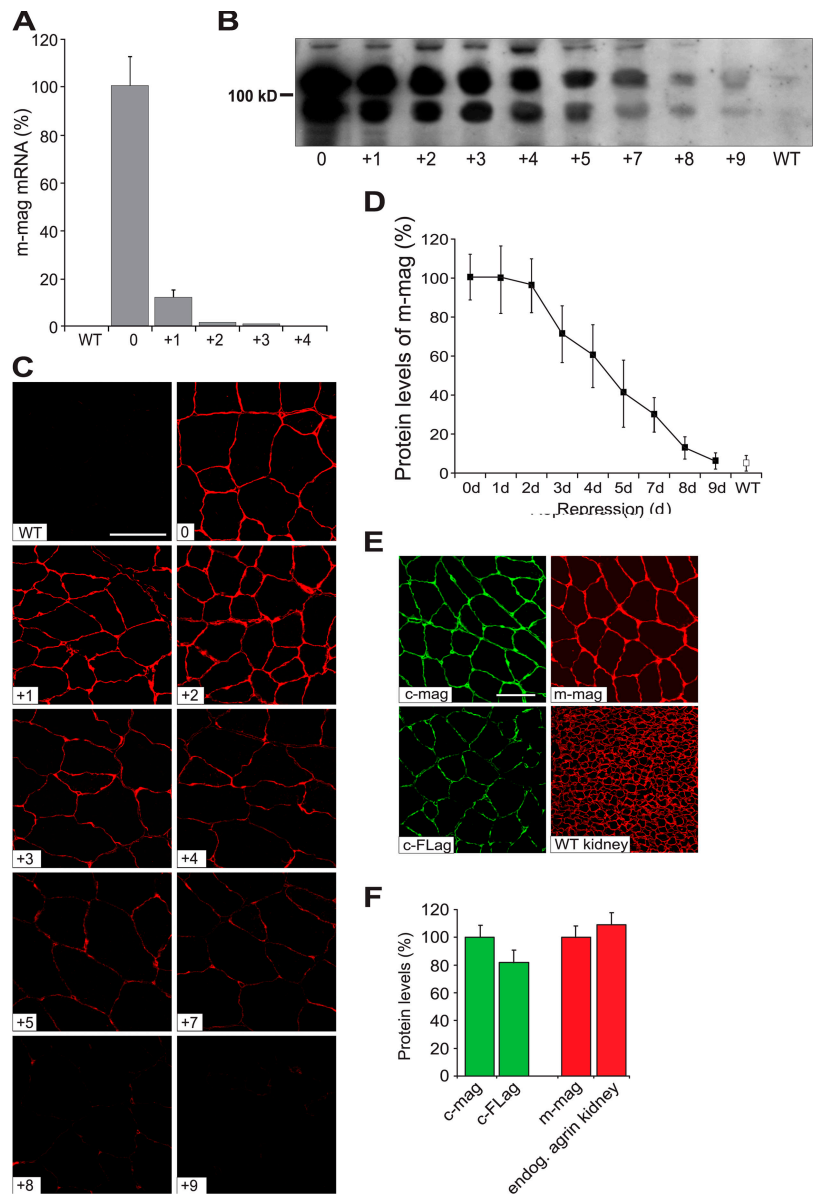
Our proof-of-concept experiment using transgenic mice is a crucial step toward devising ways of treating MDC1A patients. Similar to mini-agrin, transgenic expression of laminin- $\alpha$ 1 also improves muscle function (Gawlik et al., 2004). Although this approach may be interesting for therapy, the use of laminin- $\alpha$ 1 seems less feasible than that of mini-agrin. First, the size of its cDNA (>9 kb) prevents its packaging into AAV vectors. Second, laminin- $\alpha$ 1 must also become incorporated into the laminin heterotrimer to be functional, which makes it difficult to generate a miniaturized version of laminin- $\alpha$ 1 because

**Table IV. Quantification of laminin- $\alpha$ 5 and  $\alpha$ -dystroglycan in triceps brachii of *dy<sup>W</sup>/dy<sup>W</sup>* mice expressing *dy<sup>W</sup>/c-Flag* or *dy<sup>W</sup>/AgPerl***

Strain	Laminin- $\alpha$ 5	$\alpha$ -Dystroglycan
WT	100 $\pm$ 16	100 $\pm$ 11
<i>dy<sup>W</sup>/dy<sup>W</sup></i>	138 $\pm$ 20	28 $\pm$ 3
<i>dy<sup>W</sup>/c-Flag</i>	263 $\pm$ 64	60 $\pm$ 6
<i>dy<sup>W</sup>/AgPerl</i>	266 $\pm$ 39	61 $\pm$ 6
<i>dy<sup>W</sup>/c-mag</i>	264 $\pm$ 40	69 $\pm$ 12

Both transgenes increase the concentration of laminin- $\alpha$ 5 and  $\alpha$ -dystroglycan in *dy<sup>W</sup>/dy<sup>W</sup>* mice. Similar values were observed in *dy<sup>W</sup>/c-mag* mice. Values are relative to WT mice and represent the mean  $\pm$  the SEM.  $n \geq 3$ .

**Figure 8. Feasibility for the use of mini-agrin or up-regulation of endogenous agrin expression as a treatment option.** (A–D) Stability of mini-agrin in skeletal muscle of 4-wk-old transgenic mice. (A) Time course of m-mag transcripts in triceps brachii after repression by 50 mg/liter doxycycline in the drinking water, as determined by quantitative real-time PCR. After 2 d, m-mag transcripts cannot be detected anymore. Concomitantly, mini-agrin protein is lost from the muscle basement, as determined by Western blot analysis from quadriceps (B) and quantitative immunostaining of cross sections from triceps brachii muscle (C). (D) Quantitative immunohistochemistry indicates a half-life of mini-agrin protein of  $\sim 4.5$  d. Values are expressed as percentages of staining relative to values before suppression by doxycycline. (E) Staining of transgenic c-mag and c-Flag (left) in triceps brachii muscle using antibodies recognizing chick, but not mouse agrin. Staining using antibodies against mouse agrin in triceps brachii muscle of mice transgenic for m-mag or in kidneys of WT mice (WT kidney; right). Note that staining in green (left) detects only the transgenes, whereas staining in red (right) can detect both the m-mag and endogenous agrin. (F). Quantification of staining intensity. Protein expression levels of c-Flag reach 81% of c-mag (left). Endogenous agrin expression in kidney is 13% higher than the levels of transgenic m-mag in muscle (right). For details see the text and the Materials and methods. Values represent the mean  $\pm$  the SEM.  $n \geq 3$ . Bar, 50  $\mu\text{m}$ .



several domains contribute to its functionality. In contrast, mini-agrin combines several advantages, and thus might be a promising strategy for the treatment of MDC1A patients. As the next step will be the defining of a route of application, we determined the half-life of mini-agrin in the transgenic mice. We found that it was  $\sim 4.5$  d, which is substantially less than what has been estimated for full-length agrin when injected into rat muscle (Bezakova et al., 2001). One of the reasons for this difference could be the lack of any *O*-glycosylation in mini-agrin. Moreover, mini-agrin also seems to be targeted by proteases of unknown identity, as the protein displays distinct bands in Western blots (Moll et al., 2001; this study). Nevertheless, the good stability of mini-agrin, in combination with the fact that it acts extracellularly, makes it a valuable candidate gene for gene therapy. Indeed, recent experiments in *dy<sup>W</sup>/dy<sup>W</sup>* mice showed that transduction of skeletal and heart muscle by recombinant adeno-associated virus that express mini-agrin restored muscle function (Qiao et al., 2005; Meinen and Ruegg, 2006).

An alternative way of treating patients might also be the use of recombinant protein and its targeting to the affected tissue. Examples for the successful targeting of recombinant enzymes and antibodies to muscle are the treatment of lysosomal storage diseases (Desnick, 2004) and muscle wasting (Bogdanovich et al., 2002), respectively. The major obstacle for mini-agrin's reaching muscle is, however, its laminin-binding, as laminins line the endothelial wall of blood vessels (Hallmann et al., 2005). Thus, it will be important to reduce the size of the injected protein (e.g., the binding of agrin to  $\alpha$ -dystroglycan requires only two laminin G-like domains) and to apply enhancers of endothelial permeability, such as VEGF or histamine.

#### Up-regulation of endogenous agrin provides an alternative treatment option

An alternative treatment option is the use of molecules that increase the expression of the endogenous agrin protein in

MDC1A patients. In *mdx* mice, which are mouse models of DMD, up-regulation of the endogenous utrophin, which is the autosomal homologue of dystrophin, has been shown to ameliorate the dystrophic phenotype (Miura and Jasmin, 2006). Recently, intraperitoneal injection of a peptide derived from heregulin was shown to increase expression of utrophin and thereby ameliorate the disease in *mdx* mice (Krag et al., 2004). We show that c-FLag, indeed, ameliorates the disease and prolongs lifespan in *dy<sup>w</sup>/dy<sup>w</sup>* mice. This is experimental proof that the aforementioned strategy might be promising for the treatment of MDC1A patients. We found that the improvement with c-FLag is less effective than with mini-agrin. This difference might arise from a distinct orientation of the domains important for laminin and  $\alpha$ -dystroglycan binding caused by the size difference or differences in glycosylation. Full-length agrin is  $\sim$ 95 nm long (Denzer et al., 1998), whereas mini-agrin folds into a globular structure with an estimated length of 20 nm. Moreover, the high carbohydrate content of full-length agrin may impose a different orientation of the two functional domains, and the presence of heparan sulfate glycosaminoglycan side chains may lower its apparent binding affinity to  $\alpha$ -dystroglycan, as binding of agrin to  $\alpha$ -dystroglycan is inhibited by heparin (Gee et al., 1994; Gesemann et al., 1996). Finally, the amount of full-length agrin expressed in our transgenic mice is lower than that of mini-agrin. However, the difference in the protein concentration between the two transgenes became smaller the older the mice were (unpublished data). Thus, it is likely that the slow turnover rate of full-length agrin in the basement membrane allows it to accumulate over time and to eventually reach saturation, despite being less strongly expressed. Accumulation of agrin would be highly desirable in a pharmacological approach because even a moderate increase in agrin transcripts would then result in high concentrations of the protein over time. Moreover, the levels of transgenic agrin necessary for its ameliorative effect are even lower than those found in kidney. Thus, such an approach may indeed be feasible.

#### A mechanistic explanation of agrin's activity

We also provide conceptual proof that mini-agrin's beneficial effect on the disease progression in *dy<sup>w</sup>/dy<sup>w</sup>* mice arises from the reconnection of muscle basement membrane with the cytoskeleton via the DGC, as a chimeric fusion protein between AgPerl has the same efficacy in ameliorating the disease as mini-agrin. Both mini-agrin and AgPerl bind to laminins and  $\alpha$ -dystroglycan, and they compete for the same binding sites (unpublished data). Our experiments therefore indicate that integrins do not contribute to the ameliorating activity because the C-terminal proportions of AgPerl bind to different integrins (Brown et al., 1997; Burgess et al., 2002). In addition, the  $\alpha$ 2 $\beta$ 1 integrin receptor of domain V/endorepellin is not even expressed in muscle. Our model that reconnection of laminins and  $\alpha$ -dystroglycan is the underlying mechanism for the beneficial effect of mini-agrin and AgPerl is also corroborated by the fact that CMDs with phenotypes similar to those of MDC1A are based on mutations in glycosyltransferases that have  $\alpha$ -dystroglycan as their main substrates (Muntoni and Voit, 2004).

One of the most striking findings is that mini-agrin, irrespective of the onset of its expression, increases the regenerative capacity of muscle fibers in *dy<sup>w</sup>/dy<sup>w</sup>* mice. After notexin-induced muscle damage, many fibers in the WT and the *dy<sup>w</sup>/m*-mag mice regenerate within the first week, as indicated by the expression of dMyHC. In contrast, in *dy<sup>w</sup>/dy<sup>w</sup>* mice, dMyHC was expressed only marginally in the early phase, but was also not up-regulated later. Moreover, muscle fibers that had formed in *dy<sup>w</sup>/dy<sup>w</sup>* mice were shorter, and the muscle contained large regions with mononucleated cells (Fig. 5). This is evidence that muscle regeneration in *dy<sup>w</sup>/dy<sup>w</sup>* mice is not simply delayed, but that some of the crucial steps cannot be accomplished. The mechanism behind how LM-211 influences regeneration is not known. For example, expression of LM-211 in satellite cells themselves may improve proliferation or survival. Alternatively, satellite cells may depend on LM-211 bound to muscle basement membrane for adhesion and/or survival, which, in turn, would allow their fusion. These events may even be interdependent, as muscle fibers are known to undergo detachment-induced apoptosis, which is termed anoikis, during regeneration (Kuang et al., 1999). The fact that only 1 wk of mini-agrin expression can restore muscle regeneration to levels indistinguishable from WT mice suggests that its binding to  $\alpha$ -dystroglycan may activate pathways that prevent anoikis. Indeed, disruption of the binding of laminin with  $\alpha$ -dystroglycan induces cell death in cultured muscle cells because of the perturbation of the phosphoinositide 3-kinase–protein kinase B pathway. Thus, we favor a mechanism in which mini-agrin bound to muscle basement membranes allows the survival of satellite cells and early myotubes.

We also find that the level of laminin- $\alpha$ 5, but not laminin- $\alpha$ 1, is increased in all mice that express a transgene. The increase in laminin- $\alpha$ 5 is not based on changes in transcription (Bentzinger et al., 2005), but may be based on its immobilization in the muscle basement membrane. As laminin- $\alpha$ 5 does not bind to  $\alpha$ -dystroglycan (Ido et al., 2004), it probably does not contribute to linking  $\alpha$ -dystroglycan to basement membrane. However, laminin- $\alpha$ 5 is not truncated at the N-terminal end, a site important for the formation of the primary laminin scaffold. Thus, the increased concentration of LM-511 in the transgenic mice may be important for the restoration of muscle basement membrane. We also observed a restoration of the amount of  $\alpha$ -dystroglycan in mice that express the transgene (Fig. 7 and Fig. S3). This change was very striking when we used an affinity-purified antiserum directed against the protein backbone of  $\alpha$ -dystroglycan (Herrmann et al., 2000), but was not seen with the antibody IIIH6 directed to the carbohydrate moiety (Gawlik et al., 2004; unpublished data). It may be possible that alterations in the proteolytic processing of dystroglycan, may lead to the loss of the epitope recognized by the antipeptide antibody, whereas glycosylation is not affected.

#### Future directions in the development of a MDC1A treatment

We and others (Qiao et al., 2005) noticed that mini-agrin does not remove all of the symptoms. Laminin- $\alpha$ 2 deficiency in nonmuscle tissue, particularly in the peripheral nerve, clearly



contributes to the pathology in  $dy^W/dy^W$  mice, and because our transgenes are only expressed in muscle, the pathology in non-muscle cells is not reversed. Interestingly, some symptoms are still present in  $dy^W/dy^W$  mice that express human laminin- $\alpha 2$  in skeletal muscle (Kuang et al., 1998), whereas amelioration is more complete in mice that express laminin- $\alpha 1$  under the control of the ubiquitously expressed chicken  $\beta$ -actin promoter (Gawlik et al., 2004). Thus, the exclusive expression of all our transgenes in muscle does contribute to the incompleteness of the amelioration. In addition, it is also probable that mini-agrin cannot substitute all of the functions of laminin- $\alpha 2$ . For example, mini-agrin is not known to bind to those integrins that are expressed in muscle, and thus, any function mediated by the binding of laminin- $\alpha 2$  to integrins cannot be compensated for. Several lines of evidence strongly suggest that binding of laminin- $\alpha 2$  to  $\alpha 7\beta 1$  integrin is important to prevent anoikis (Vachon et al., 1996, 1997). As recent findings indicate that prevention of apoptosis by genetic manipulation is also beneficial for  $dy^W/dy^W$  mice (Girgenrath et al., 2004; Dominov et al., 2005), it might be possible that antiapoptotic agents act synergistically with mini-agrin. Several apoptosis inhibitors are used in clinical development. Thus, the combination of antiapoptotic drugs with the expression of mini-agrin in muscle, and/or the up-regulation of endogenous agrin, might be a promising approach to help MDC1A patients. Future experiments will be aimed at critically testing such a strategy.

## Materials and methods

### Generation of the constructs and transgenic mice

The m-mag cDNA was obtained by two independent RT-PCRs on mRNA isolated from mouse skeletal muscle. The 0.75-kb cDNA encoding the 25-kD N-terminal agrin and the 2.2-kb cDNA encoding the 95-kD C-terminal half were ligated, and a  $5 \times$  myc-tag (0.25 kb) was added to the 3' end to yield the m-mag-myc (m-mag) construct. The 3.2-kb m-mag construct was sequenced and subcloned downstream of the uni-directional pTRE2 tet-responsive promoter (tetO7-CMV; BD Biosciences; pTRE2, 3.8 kb). A PacI site was inserted into the pTRE2 vector to allow linearization of the construct as a 4.9-kb PacI-Asel fragment for injection into mouse oocytes. All transgenic mouse lines in which the cDNA was stably inserted into the genome were mated with transgenic mice expressing the tetracycline-dependent transcription activator (tTA) under the control of a 3.3-kb fragment of the human muscle CK (MCK) promoter (Fig. S1). MCK-tTA mice were obtained from N. Raben (National Institutes of Health, Bethesda, MD; Ghera et al., 1998) and were shown to drive expression of transgenes in skeletal and heart muscle (Ghera et al., 1998; Raben et al., 2001). The AgPerl fusion protein was created by fusing the cDNA encoding the 0.75-kb 5' region of chick agrin (Mascarenhas et al., 2003) with a cDNA coding for domain V of mouse perlecan (full-length cDNA encoding mouse perlecan was a gift from T. Sasaki, Max-Planck-Institut für Biochemie, Martinsried, Germany). Both the 6.2-kb c-Flag and the 3.1-kb AgPerl were subcloned downstream of the 1.3-kb MCK promoter (Fig. S1, B–D). Constructs were linearized and injected into male pronuclei. C-mag transgenic mice (c-mag) were created as previously described (Moll et al., 2001).  $dy^W/dy^W$  mice (Kuang et al., 1998) containing a LacZ insertion in the LAMA2 gene served as the mouse model for MDC1A.

### Genotyping

Genotyping of heterozygous and homozygous  $dy^W/dy^W$  mice was done as previously described (Kuang et al., 1998). M-mag mice were genotyped by primers designed to amplify a 683-bp-long fragment, including the linker region of the N- and C-terminal parts of the m-mag construct (5'-GCGGATCACTTTGCGGAACC-3' and 5'-TCGAACCTGAAGTGTACATGACC-3'). Both c-Flag and c-mag mice were genotyped with primers amplifying a 591-bp-long fragment coding for the C-terminal part of agrin (5'-ACCTGGATAAGCGTTTGT-3' and 5'-CTCTGTTTTGATGCTCAGC-3').

Genotyping of AgPerl transgenic mice was performed on the chick agrin portion of the construct (5'-GTCCCTTGCTGATGACCTTGA-3', 5'-ACCCAGCCCTCAGTACATGT-3'). To distinguish hemi- from homozygous MCK-tTA mice, we performed quantitative TaqMan PCR (TaqMan PCR core reagent kit; Applied Biosystems) on the genomic DNA. The following primers were used: tTA-transgene, 5'-GCCTACATTGATTGGCATGTA-3', 5'-CAAAAGTGAGTATGGTGCCTATCTAACA-3', and Probe 5'-FAM-CTTGTCTCGACGCCTTAGCCATTGAG-TAMRA 3'. For normalization of copy number, the following probes for  $\beta$ -actin were used: 5'-CCACTGCCGATCCTCTT-3', 5'-GCTCGTTGCCAATAGTGATGAC-3', and Probe 5'-FAM-CCCTGGAGAAGAGCTATGAGCTGCCTG-TAMRA-3'.

### Regulation of the tet-off system

For temporal regulation of m-mag expression under the tetracycline-regulated tet-off expression system (Gossen and Bujard, 1992), 5  $\mu$ g doxycycline (doxycycline hydrochloride; Sigma-Aldrich) per milliliter of drinking water (enriched by 4% sucrose) was administered in dimmed bottles. For repression after transgene expression, 50  $\mu$ g of doxycycline per milliliter of drinking water was applied.

### Protein production

The cDNAs encoding m-mag or AgPerl were subcloned into the pCEP-Pu vector (Kohfeldt et al., 1997) and transfected into HEK 293 EBNA cells. Conditioned medium was collected, and the relative amount of the protein was determined by dot blot assays. Such supernatants were directly used for experiments.

### Solid-phase binding assays

96-well plates were coated with either chick  $\alpha$ -dystroglycan enriched from skeletal muscle as previously described (Gesemann et al., 1998) or with laminin-111 (0.5  $\mu$ g/well), which was a gift from J. Engel (Biozentrum, University of Basel, Switzerland). Proteins were coated in 50 mM sodium carbonate buffer, pH 9.6, and incubated overnight at 4°C. After blocking with PBS containing 0.05% Tween-20, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , and 3% BSA (blocking buffer), wells were incubated with a dilution series (1:6) of supernatant containing m-mag (pure supernatant as the starting concentration) or of purified c-mag (50 nM as the starting concentration). The wells were washed with blocking buffer. Bound protein was detected with polyclonal antibodies raised against the C-terminal, 95-kD part of chick or mouse agrin. Alternatively, the monoclonal antibody 9E10 (Evan et al., 1985) directed against the myc-tag was used. For detection, appropriate horse radish peroxidase-conjugated antibodies, followed by McEvans solution, ABTS, and  $\text{H}_2\text{O}_2$ , were used. The absorbance was measured on an ELISA reader at 405 nm after 15 min.

### Overlay assays

Lysates enriched for  $\alpha$ -dystroglycan were obtained from chick or mouse skeletal muscles, as previously described (Gesemann et al., 1998). Proteins were separated on a 3–15% SDS gel and blotted to nitrocellulose membrane. Blots were blocked for 2 h with PBS containing 0.05% Tween-20, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , and 5% dry milk powder (blocking buffer). Supernatants containing recombinant proteins were added and incubated overnight at 4°C. After several washes with blocking buffer, bound m-mag was detected with the anti-myc antibody 9E10, whereas detection of AgPerl was done using a polyclonal antiserum raised against the N-terminal part of agrin. For detection, appropriate horse radish peroxidase-conjugated antibodies were used, and immunoreactivity was visualized by the ECL detection method (Pierce Chemical Co.).

### Immunoblots

Tissues were homogenized in protein extraction buffer (75 mM Tris-HCl, pH 6.8, 3.8% SDS, 4 M urea, 20% glycerol, and 5%  $\beta$ -mercaptoethanol). Equal amounts of protein were separated on a 3–12% SDS-PAGE and immunoblotted. Protein signals were normalized to  $\beta$ -actin (Santa Cruz Biotechnology; sc-8432) or  $\beta$ -tubulin (BD Bioscience).

### Northern blot analysis and quantitative TaqMan PCR

Northern blot assays were performed on total RNA extracted from skeletal muscles using Northern Max Kit (Ambion). Signals were normalized to corresponding  $\beta$ -actin signals. Quantitative TaqMan PCR was performed on the m-mag transgene (5'-TGTGCCAATGTGACCGCTA-3', 5'-GCTGAAACCCTTGCCAGAA-3', and Probe 5'-FAM-CCCCAAAGTC-CTGTGATTC-3') and was normalized to  $\beta$ -actin (5'-CCACTGCCGATCCTCTT-3', 5'-GCTCGTTGCCAATAGTGATGAC-3', and Probe 5'-FAM-CCCTGGAGAAGAGCTATGAGCTGCCTG-TAMRA-3').

### Locomotion, muscle strength, and CK assay

Locomotive behavior was determined as previously described (Moll et al., 2001). In brief, mice were placed into a new cage and motor activity (walking, digging, and standing upright) was measured for 10 min. Grip strength was evaluated by placing the animals onto a vertical grid and measuring the time until they fell down. The cut-off time was 3 min. Blood for CK assays was collected from the tail vein. 2  $\mu$ l of serum was applied using the CK CK-NAC Liqui-UV kit (Rolf Greiner Biochemica). In all tests, at least three animals of each genotype were analyzed, and values were normalized to values obtained from WT animals.

### Histology, immunohistochemistry, and antibodies

Muscles were immersed in 7% gum tragacanth (Sigma-Aldrich) and rapidly frozen in liquid nitrogen-cooled isopentane ( $-150^{\circ}\text{C}$ ). 12- $\mu\text{m}$ -thick cross sections or longitudinal sections were cut and collected on SuperFrost Plus slides (Menzel-Glaser). In the case of longitudinal sections, the slides were pretreated with 3% aqueous EDTA. General histology was performed using HE (Merck). Masson's Trichrome staining (Luna, 1968) was used to visualize collagenous tissue. Membrane-bound and extracellular epitopes were visualized with Alexa Fluor 488-conjugated WGA (Invitrogen). Polyclonal rabbit anti-mouse laminin- $\alpha 5$  (Ab 405) and monoclonal rat anti-mouse laminin- $\alpha 1$  (Ab 198; Sorokin et al., 1992) were a gift from L. Sorokin (Lund University, Lund, Sweden). Polyclonal sheep anti-mouse  $\alpha$ -dystroglycan was a gift from S. Kröger (University of Mainz, Mainz, Germany). The remaining antibodies were produced in-house or obtained as follows: monoclonal mouse anti-rat dMyHC (Novocastra), monoclonal rat anti-mouse laminin- $\gamma 1$  chain (CHEMICON International, Inc.), polyclonal rabbit anti-chick (produced in-house; Gesemann et al., 1995), and anti-mouse agrin (produced in-house). Mouse monoclonal anti-myc antibody (9E10) was produced and purified from hybridoma cell line 9E10 and was biotinylated (D-Biotinoyl-E-aminocaproic acid-N-hydroxysuccinimide; Roche). Depending on the source of the primary antibody, appropriate Cy3-conjugated (Jackson ImmunoResearch Laboratories) Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) or TRITC-labeled streptavidin were used for visualization. DAPI was used to stain nuclei.

### Quantification of immunostainings

The muscle fiber size was quantified using the minimum distance of parallel tangents at opposing particle borders (minimal "Feret's diameter"), as previously described (Briguet et al., 2004). Pictures of WGA-stained cross sections were collected using a fluorescence microscope (DM5000B; Leica), a digital camera (F-View; Soft Imaging System), and analysis software (Soft Imaging System). Measurement of minimal Feret's diameter of notexin-treated muscle was done on cross sections stained for laminin- $\gamma 1$  and dMyHC. Normalization of the number of fibers in each fiber Feret class of 5  $\mu\text{m}$  was based on the total number of muscle fibers in each picture. Fibrosis was quantified by measuring the fibrotic area of WGA-stained muscle cross section and normalizing it to the entire area of the cross section. For quantification of immunostainings of m-mag, c-mag, c-Flag, laminin- $\alpha 5$ , or  $\alpha$ -dystroglycan, images were collected and analyzed by a confocal microscope (TCS-SP; Leica) and appropriate software. InSpeck Microscope Image Intensity Calibration kit (Invitrogen) was used to determine the linear range of the laser. Specific intensity was calculated for each image as the signal intensity of the muscle circumference minus that of an adjacent, unstained region (Turney et al., 1996). Five different pictures were taken using the same parameters on each section, and four different sections were used for each individual mouse. In all quantification experiments, at least three mice of each genotype were analyzed.

### Evaluation of full-length agrin expression

Transgenic c-mag and c-Flag were detected by the polyclonal rabbit anti-chick agrin (Gesemann et al., 1995). For comparison of the transgenic m-mag and the endogenous agrin, an antiserum recognizing the 95-kD, C-terminal half of mouse agrin was used. Chick and mouse agrin immunostainings were quantified separately, as described in the previous section. Under the premise that c- and m-mag ameliorate the disease phenotype to the same extent, the relative expression levels of both were set to 100%. This clearly shows that levels of endogenous agrin expressed in kidney (expression level, 113% of m-mag) are sufficient to at least produce the ameliorating effect of c-Flag (expression level, 81% of c-mag).

### Notexin-induced muscle damage

Tibialis anterior of 5-wk-old mice was injured by injection of 15–20  $\mu$ l notexin (50  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich), as previously described (Bentzinger

et al., 2005). Mice were killed 6, 14, or 28 d after injection, and muscles were isolated and processed as described in Quantification of immunostainings.

### Hydroxyproline assay

Fibrosis in triceps brachii muscles was measured by assaying for the exclusive collagen-specific modified amino acid hydroxyproline (Woessner, 1961; Edwards and O'Brien, 1980). Tendons were carefully removed before muscles were vacuum-speed dried and sent to Analytical Research Services (Bern, Switzerland) for amino acid analysis. There, each muscle was hydrolyzed under vacuum in 50  $\mu$ l of 6 N HCl for 22 h at  $115^{\circ}\text{C}$ . Hydrolysates were evaporated to dryness and resuspended in 0.1% trifluoroacetic acid. Aliquots were diluted 1:100 for determination of amino acids by a routine method (Cohen et al., 1986), including derivatization with phenylisothiocyanate, followed by HPLC, identifying, and quantifying the collagen-related amino acid hydroxyproline. Relative hydroxyproline amount was assessed in reference to the total amount of amino acids.

### Statistical analysis

To compare the different genotypes, p-values were calculated using the unpaired two-sample t-tests, assuming equal variances.

### Online supplemental material

Fig. S1 represents the regulation of expression by the inducible tetracycline-regulated tet-off expression system (Gossen and Bujard, 1992) and the breeding scheme to obtain  $dy^w/dy^w$  mice with a tight spatial and temporal regulation of mini-agrin expression. Fig. S2 shows the binding of the transgenic m-mag and the fusion protein AgPerl to laminin and  $\alpha$ -dystroglycan in both solid-phase and overlay binding assays. In Fig. S3, immunohistochemical staining of cross sections visualizes the regulation of different agrin-binding proteins, including laminin- $\alpha 5$ ,  $\alpha$ -dystroglycan, and laminin- $\alpha 1$  in  $dy^w/m$ -mag mice. The online version of this article is available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>.

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