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Acetylation is important for MyoD function in adult mice

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Acetylation is a post-translational modification that influences the activity of numerous proteins *in vitro*. Among them, the myogenic transcription factor MyoD shows an increased transcriptional activity *in vitro* when acetylated on two lysines (K): lysines 99 and 102. Here, we have investigated the biological relevance of this acetylation *in vivo*. Using specific antibodies, we show that endogenous MyoD is acetylated on lysines 99 and 102 in myoblasts. Moreover, we show the functional importance of acetylation in live animals by using a mutant of MyoD in which lysines 99 and 102 were replaced by arginines (R). Knock-in embryos homozygous for the *MyoD*^{R99,102} allele expressed slightly reduced levels of MyoD but developed normally. However, the knock-in homozygous adult mice showed a phenotype that was almost identical to that of MyoD-knockout animals, including delayed muscle regeneration *in vivo* and an increased number of myoblasts but with reduced differentiation potential *in vitro*. Together, these results show the importance of MyoD acetylation for adult myogenesis.

Keywords: MyoD; acetylation; knock-in; muscle; regeneration

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INTRODUCTION

Acetylation of lysines (K) in histones and non-histone proteins by histone acetyltransferases (HATs)—GCN5 (Brownell *et al*, 1996), PCAF (Yang *et al*, 1996) or CREB-binding protein (CBP)/p300 (Ogryzko *et al*, 1996)—is an essential process in transcriptional activation (Bayle & Crabtree, 1997). Non-histone protein acetylation can influence the stability or subcellular localization of proteins and activities such as DNA binding or transcriptional repression/activation (Soutoglou *et al*, 2000). Acetylation generally modifies the interaction between proteins and other components (Gu & Roeder, 1997; Kiernan *et al*, 1999; Martinez-Balbas *et al*, 2000; Polesskaya *et al*, 2000, 2001). The functional importance of non-histone protein acetylation has been extensively explored *in vitro*, but comparable *in vivo* data are lacking.

The myogenic transcription factor MyoD is a substrate for HATs *in vitro* (Sartorelli *et al*, 1999; Polesskaya *et al*, 2000). MyoD is a transcription factor of the basic helix–loop–helix (bHLH) family (Lassar *et al*, 1989) that converts non-muscle cells to myoblasts; it functions by transactivating muscle-specific promoters (Weintraub *et al*, 1991). MyoD is not essential for muscle formation during development in mice, and knockout embryos develop normally owing to compensation by overexpression of other members of the same family, such as myogenic factor 5 (Myf-5; Rudnicki *et al*, 1992). MyoD-null myoblasts, however, have a reduced differentiation potential *in vitro* (Sabourin *et al*, 1999). Moreover, adult MyoD-null mice show impaired skeletal muscle regeneration, indicating that MyoD is specifically required for this function (Megeney *et al*, 1996). *In vitro*, MyoD is acetylated by two HATs: CBP/p300 and PCAF (Sartorelli *et al*, 1999; Polesskaya *et al*, 2000; Polesskaya & Harel-Bellan, 2001). Acetylation occurs on lysines 99 and 102, located at the vicinity of the DNA-binding/dimerization domain—the bHLH domain. There is evidence indicating that acetylation of MyoD increases its affinity for the HAT CBP/p300 (Polesskaya *et al*, 2001). Acetylation of recombinant MyoD before its introduction into cultured myoblasts results in increased transcriptional activity, as monitored by using a reporter assay (Polesskaya *et al*, 2000). Conversely, mutation of lysines 99, 102 and 104 strongly reduces the myogenic activity of MyoD (Sartorelli *et al*, 1999; Polesskaya *et al*, 2000). Endogenous MyoD is acetylated in live cells (Sartorelli *et al*, 1999; Polesskaya *et al*, 2000), but the residues that are acetylated *in vivo* have not been characterized. We now show, for the first time, that acetylation of MyoD is important for skeletal muscle regeneration *in vivo* and terminal differentiation *in vitro*.

RESULTS AND DISCUSSION

MyoD is acetylated on lysines 99 and 102 in live cells

Endogenous MyoD was previously shown to be acetylated on uncharacterized lysines in myoblast cell lines (Sartorelli *et al*, 1999; Polesskaya *et al*, 2000). *In vitro*, recombinant MyoD is acetylated by HATs on lysines 99 and 102, but so far the lysines acetylated *in vivo* have not been identified. To address this issue, we raised a polyclonal antibody that specifically recognizes

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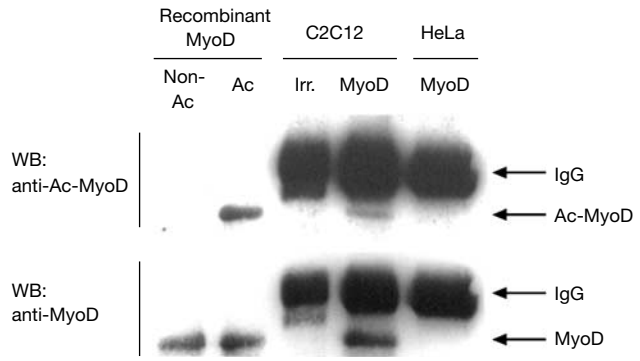

Fig 1 | MyoD is acetylated on lysines 99 and 102 in myoblasts. Proteins were extracted from trichostatin A-treated cells, using either myoblasts (C2C12) or, as a negative control, non-muscle cells (HeLa). Extracts were immunoprecipitated using MyoD antibody (MyoD) or irrelevant antibodies (Irr.) and then analysed by western blot using purified anti-acetyl-K99,102-MyoD serum (WB: anti-Ac-MyoD) or anti-non-acetylated-MyoD (WB: anti-MyoD) to monitor the total level of MyoD. Recombinant MyoD, either mock acetylated (Non-Ac) or acetylated (Ac) *in vitro*, was run on the same gel. K, lysine.

MyoD acetylated on K99 and K102 but does not bind to non-acetylated MyoD (Fig 1) or to an irrelevant protein acetylated *in vitro* (supplementary information S1 online). This antibody was used to detect acetylated MyoD in extracts from myogenic cells (C2C12) or from the non-muscle cell line HeLa used as a negative control. Equivalent amounts of recombinant MyoD, non-acetylated or acetylated *in vitro,* were used as internal controls. The acetyl-MyoD antibody detected a protein that migrates at the position of MyoD in C2C12 extracts but which is absent from HeLa extracts. Together, these results show that MyoD is acetylated on lysines 99 and 102 in C2C12 cells.

MyoD^{R99,102} has reduced myogenic activity

We next addressed the functional importance of acetylation of MyoD by replacing lysines 99 and 102 by arginines (R). This lysine mutation does not affect the nuclear localization of MyoD, its DNA-binding capacity or its basic interaction with other proteins such as E12 and CBP (Polesskaya *et al*, 2000, 2001). The effect of the double mutation was evaluated using an *in vitro* myogenic assay. Totipotent mouse embryonic stem (ES) cells were chosen as recipients because a low-level expression of wild-type MyoD has been shown to convert 100% of the population to myogenesis (Dinsmore *et al*, 1996; see also Fig 2C). Stably transfected ES cells expressing similar amounts of MyoD, either wild type or mutant (Fig 2A), were placed under differentiation conditions. Expression of the early marker myogenin was monitored by reverse transcription–quantitative PCR (RT–Q-PCR; Fig 2B) and that of the late markers myosin heavy chain (MHC) and muscle creatine kinase (MCK) by immunofluorescence (Fig 2C) and western blot (Fig 2D). Expression of all differentiation markers was both delayed and reduced in ES-MyoD^{R99,102} cells as compared with their wild-type counterparts. These results confirm previously published data obtained with a triple mutant (Polesskaya *et al*, 2000), and show that mutation of lysines 99 and 102 is sufficient to significantly reduce MyoD activity.

Establishing *MyoD*^{R99,102} knock-in mice

ES cells heterozygous for the *MyoD*^{R99,102} allele were created as described in the supplementary information S2 online. Mice heterozygous for the *MyoD*^{R99,102} allele were intercrossed, and the resulting newborn mice were genotyped by analysing MyoD intron I, the mutant allele of which is predicted to have an increased size. The proportion of homozygous mice was as expected, and this analysis did not show any embryonic or perinatal lethality associated with the mutant *MyoD*^{R99,102} allele. Homozygous or heterozygous mice carrying this allele developed into apparently normal adults, with normal weight gain (data not shown), reminiscent of *MyoD*-null mice, which likewise develop normally.

MyoD^{R99,102} homozygous embryos

The level of MyoD in embryos was evaluated by RT–Q-PCR. Results (Fig 3A) showed some individual variability between animals and suggested that, in homozygous mutants, MyoD expression was slightly reduced overall. RNAs produced from the wild-type and mutant alleles, in heterozygous embryos, were discriminated by an RT–PCR–restriction fragment length polymorphism (RFLP) assay in which PCR amplification products were subsequently digested with *Pst*I (a *Pst*I site is created by the *MyoD*^{R99,102} mutation; supplementary information S2 online). Specificity of the RT–PCR step is shown in Fig 3B (upper panel). Quantification of digestion products showed equal amounts of the two messenger RNA species, indicating equal expression of the two alleles and eliminating the possibility that *MyoD*^{R99,102} is impaired in its expression capacity (Fig 3B, middle and lower panels). Reduced levels of MyoD might thus reflect the existence of an autoregulatory loop for MyoD gene transcription: the non-acetylatable form of the protein is less efficient than the wild-type protein in transactivating target promoters; this is probably true for its own promoter as well.

Other muscle markers were analysed in homozygous embryos by RT–Q-PCR (Fig 3C). Myf-5 seemed to be slightly overexpressed in mutants but did not seem to reach the high levels observed in knockout mice; moreover, the difference was not statistically significant. MRF4 (data not shown) and myogenin (Fig 3C) expression was normal or close to normal.

Phenotypic analyses of *MyoD*^{R99,102} homozygous adults

To assess the functional impact of *MyoD*^{R99,102} mutation *in vivo*, we monitored the regeneration capacity of adult mice after injection of cardiotoxin. Embryonic MHC (eMHC) acted as a marker of regeneration: in adults, eMHC is re-expressed only in the regenerating muscle. The results indicated that eMHC expression was significantly delayed (3 days) in mutant mice (maximal expression between days 5 and 10) compared with wild-type mice (days 2–7; Fig 4A,B). By contrast, no significant difference was observed with regard to MyoD expression, showing that the defect in MyoD expression observed in embryos does not persist throughout the whole lifespan, and indicating a normal activation of myoblasts on the regeneration site. These data eliminate the possibility that decreased regeneration in adult *MyoD*^{R99,102} mice is due to reduced MyoD levels. Cross-sections of the muscles were analysed on day 2 by immunofluorescence for expression of eMHC (Fig 4C): eMHC-expressing cells were either differentiating individual myoblasts or cells fused to form

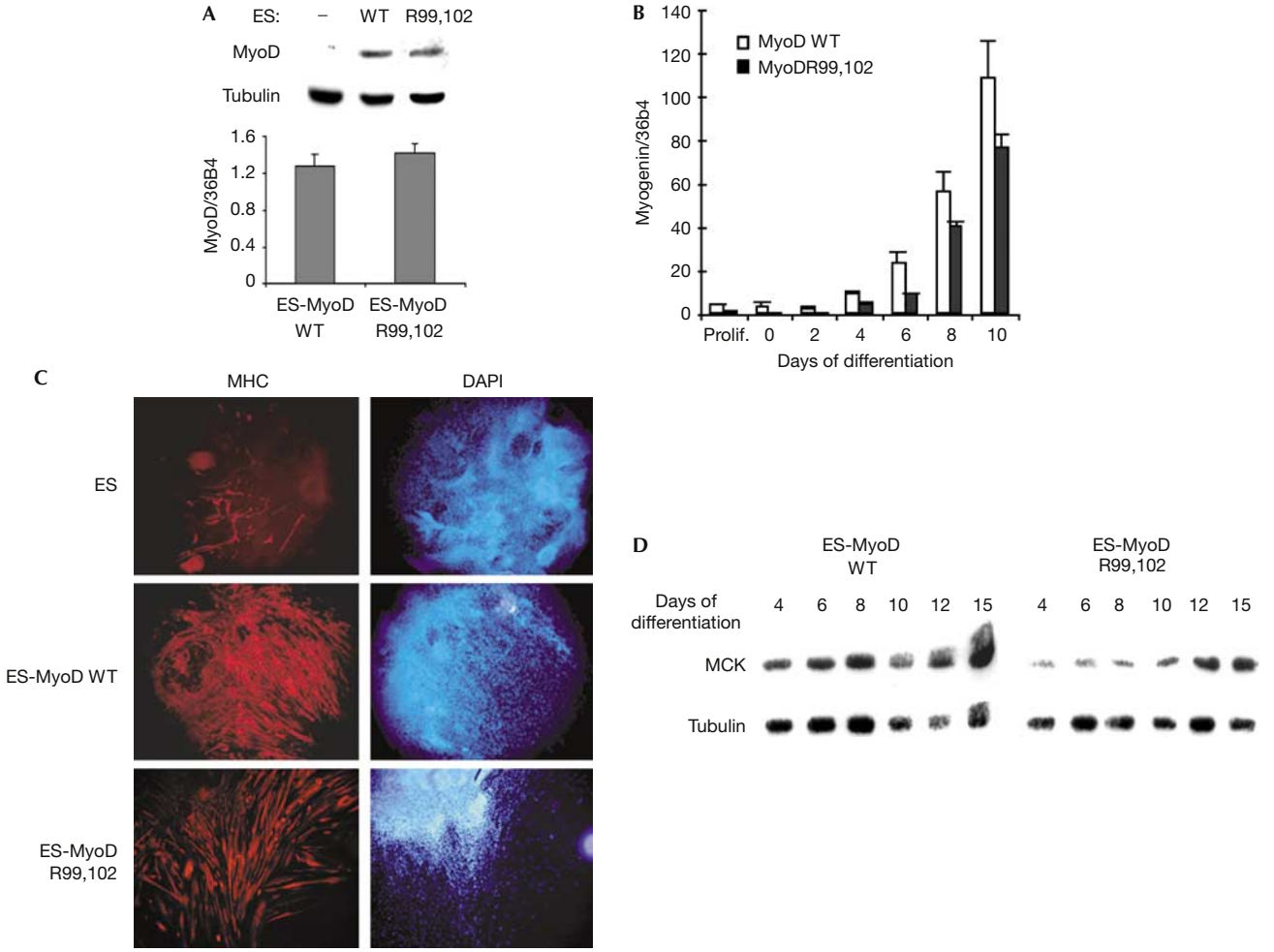

Fig 2 | MyoD^{R99,102} has reduced myogenic activity. (**A**) MyoD expression in stably transfected embryonic stem (ES) cells. ES cells were stably transfected with plasmids expressing wild-type (WT) MyoD or MyoD^{R99,102} (R99,102) and analysed by western blot (upper panel) or by reverse transcription–quantitative PCR (RT–Q-PCR; lower panel). RT–Q-PCR results were normalized using the housekeeping messenger RNA 36B4; transcripts were quantified in triplicate; –, non-transfected. (**B**) Myogenin expression in converted ES cells. RNAs extracted from embryoid bodies expressing wild-type MyoD (MyoD WT) or MyoD^{R99,102} (MyoD R99,102) were analysed in triplicate by RT–Q-PCR and normalized on 36B4 mRNA as in (**A**). (**C**) Myogenic conversion of ES cells by wild-type MyoD (ES-MyoD WT) or MyoD^{R99,102} (ES-MyoD R99,102): ES cells were stably transfected with pEMSV-MyoD or left untreated for the controls, and placed under differentiation conditions. Embryoid bodies were analysed by immunofluorescence for myosin heavy chain (MHC) expression, and nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). (**D**) Muscle creatine kinase (MCK) expression in converted ES cells. Proteins were extracted from embryoid bodies at different stages of differentiation and analysed by western blot. Tubulin is shown as a loading control. These experiments were carried out twice with similar results. R, arginine.

new fibres and showing central nuclei; muscles from *MyoD*^{R99,102} mice showed a reduced number of fibres scoring positive for eMHC (Fig 4C,D). However, on day 15, no difference could be detected between the wild-type and mutant mice, and abnormalities such as fibrosis or fat infiltration were not observed (supplementary information S3 online). Moreover, the average size of mutant fibres was similar to that of wild-type fibres (data not shown). Together, these results indicate that regeneration was not abolished but was significantly delayed in these 7-week-old mutant mice.

MyoD^{R99,102} mice had normal numbers of satellite cells, as assessed by paired-box protein 7 (Pax7) staining (data not shown) and previously observed for MyoD-knockout mice (Megeney *et al*, 1996). Although the yield of satellite-cell-derived myoblasts that can be obtained from skeletal muscle of MyoD-knockout mice is higher than that of wild-type controls, MyoD-knockout cells differentiate less efficiently than their wild-type counterparts. We found that, in *MyoD*^{R99,102} homozygous mice, the number of myoblasts derived from the muscles of hind leg, foreleg and diaphragm was two- to threefold higher than that of the wild-type controls (Fig 5A). However, when placed under differentiation conditions, myoblasts derived from the hind leg of *MyoD*^{R99,102} mice showed a strongly perturbed expression of early (myogenin) and late (MCK, MHC) differentiation markers (Fig 5B). In particular, in contrast to what happens in wild-type myoblasts, MyoD^{R99,102} levels decreased as soon as differentiation was

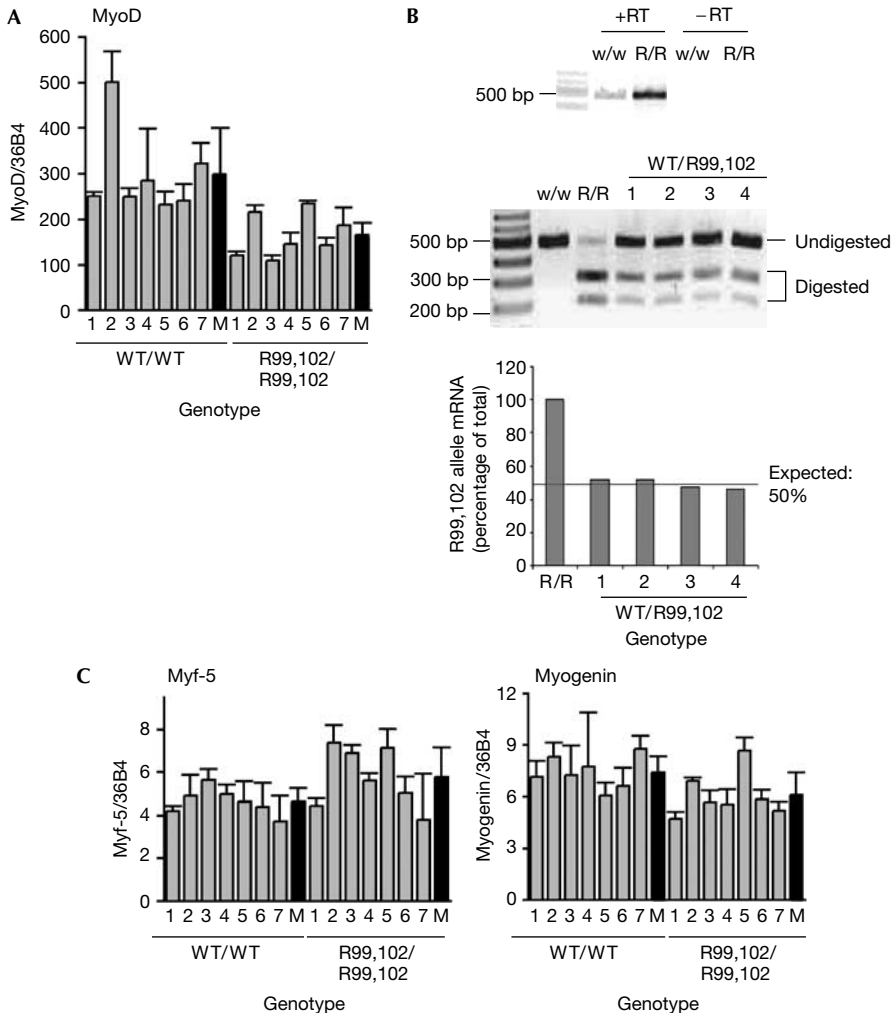

Fig 3 | Expression of myogenic factors in embryos at embryonic day 14. (**A**) MyoD expression is reduced in MyoD^{R99,102} homozygous embryos (R99,102/R99,102). MyoD RNA was extracted from seven individual embryonic day (E) 14 embryos for each genotype, and MyoD was detected by reverse transcription–quantitative PCR (RT–Q-PCR); results are normalized with reference to 36B4 messenger RNA; M, mean values; WT, wild type; $P<0.01$. (**B**) MyoD^{R99,102} and WT MyoD are equally expressed in heterozygous embryos. RNAs from four wild-type (w/w), heterozygous (WT/R99,102) or homozygous MyoD^{R99,102} (R/R) E14 embryos were analysed by RT–PCR–restriction fragment length polymorphism; the upper panel shows that amplification is dependent on RT; the centre panel shows the patterns obtained after digestion with *Pst*I (note that it is incomplete); the lower panel shows quantification of the gel using the Image Quant software; the proportion of MyoD^{R99,102} allele was evaluated by comparison with embryos homozygous for the MyoD^{R99,102} allele (R/R). (**C**) Expression of myogenic factors. RNAs from individual E14 embryos were extracted and myogenic factors analysed in triplicate by RT–Q-PCR; results were normalized using 36B4 RNA. $P>0.05$, Student's *t*-test. R, arginine.

triggered. In addition, the level of myogenin, which was higher in proliferating *MyoD*^{R99,102} myoblasts than in wild-type myoblasts, likewise decreased on differentiation, in contrast to the induction observed in normal cells. The levels of MHC and MCK were strongly decreased and only a few myotubes were formed, most likely as a consequence of these perturbations (Fig 5C). Moreover, with *MyoD*^{R99,102} myoblasts, the absolute number of cells decreased after several days under differentiation conditions, suggesting a high level of cell death in this population.

Neither wild-type nor *MyoD*^{R99,102} myoblasts expressed high amounts of Myf-5. In both cell types, however, Myf-5 expression increased with the number of passages. This phenomenon did not restore the normal expression of muscle markers in differentiating *MyoD*^{R99,102} myoblasts (Fig 5D), suggesting that Myf-5 is not able to fully compensate for MyoD modification in these cells. However, Myf-5 did seem to affect the differentiation process, as its downregulation by two distinct short interfering RNA (siRNA) oligonucleotides further reduced differentiation efficiency (Fig 5D; supplementary information S4 online). Interestingly, differentiating myoblasts derived from the diaphragm of *MyoD*^{R99,102} mice also showed a marked defect in fusion, indicating the functional importance of MyoD acetylation in distinct skeletal muscles *in vivo* (supplementary information S5 online).

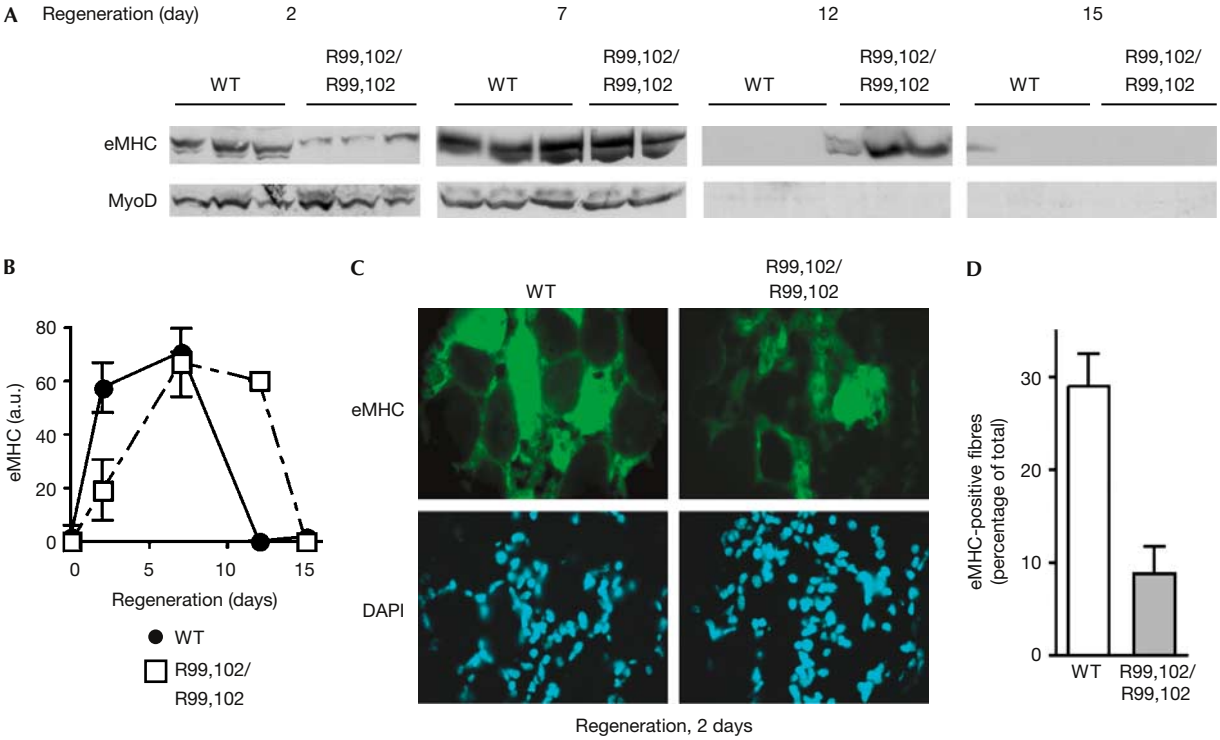

Fig 4 | Delayed regeneration in mice homozygous for the MyoD^{R99,102} allele. (**A**) Tibialis anterior muscles of wild-type (WT) or *MyoD*^{R99,102} mice (R99,102/R99,102; the results of three individual mice, two for the 7-day time point in *MyoD*^{R99,102} mice owing to the accidental death of one animal) were injected with cardiotoxin, and regeneration was monitored at the indicated time points for a period of 15 days; embryonic myosin heavy chain (eMHC) and MyoD were detected by western blot. (**B**) Quantification of the western blots shown in (**A**) using Image Quant software. a.u., arbitrary units. (**C**,**D**) Immunohistochemical staining of cross-sections of regenerating tibialis anterior muscle with eMHC antibodies 2 days after cardiotoxin treatment. (**D**) Quantification of the results shown in (**C**). eMHC-positive fibres were counted on 15 cross-sections of three individual regenerating tibialis anterior muscles from WT and MyoD^{R99,102} mice. DAPI, 4,6-diamidino-2-phenylindole.

Together, our data support a model in which acetylation of MyoD serves as an important mechanism for enhancing its transcriptional activity both *in vivo* and *in vitro*. Regeneration of adult skeletal muscle is delayed in the absence of MyoD acetylation on lysines 99 and 102. Satellite-cell-derived primary myoblasts from the limbs and diaphragm of *MyoD*^{R99,102} mice are more abundant, but their differentiation is significantly reduced. Thus, MyoD acetylation is important for repression of proliferation, for myoblast fusion, and for muscle marker expression. However, myogenesis is not completely abolished by the introduction of non-acetylatable MyoD, which is consistent with previous *in vitro* studies in which acetylation of MyoD was shown to increase its transcriptional activity considerably, but was not indispensable for its basic function (Sartorelli *et al*, 1999; Polesskaya *et al*, 2000). Our data indicate the importance of non-histone protein acetylation in live mice.

METHODS

Plasmids. A detailed description of plasmid construction is given in the supplementary information online.

Cell cultures. C2C12 and HeLa cells were maintained under standard conditions. The AT1 ES cell line was provided by Dr Muriel Vernet (CEA, Grenoble, France). ES cells were cultured on a feeder layer of inactivated primary mouse embryonic fibroblasts in standard medium supplemented with 100 μM β-mercaptoethanol (Sigma, St Quentin Fallavier, France) and 500 IU/ml leukaemia inhibitory factor (Esgro, Euromedex, Mundolsheim, France). ES-MyoD cell lines were established by electroporating ES cells with 15–60 μg of linearized wild-type pEMSV-MyoD or pEMSV-MyoD^{R99,102} together with 2 μg of linearized pGK-Neo and culturing under conditions of selection (geneticin 100–250 μg/ml) for 8 days. Embryoid bodies were generated according to Rohwedel *et al* (1994).

Primary myoblasts were obtained from the dissected muscle tissue, as described previously (Megeney *et al*, 1996), and cultured in Ham's media supplemented with 20% FCS and 2.5 ng/ml basic fibroblast growth factor (Invitrogen, Cergy-Pontoise, France) on collagen-coated dishes. Differentiation was induced by transferring to Dulbecco's modified Eagle medium supplemented with 5% horse serum (Invitrogen).

RNA interference assays on primary myoblasts were carried out using Hiperfect (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol.

PCR and Southern blotting. All primers are described in the supplementary information online. DNA from ES cells, mouse tails or the yolk sac of embryos at embryonic day (E) 14 was prepared using standard procedures. Genomic PCRs were performed using Dynazyme Ext DNA polymerase (Finnzymes,

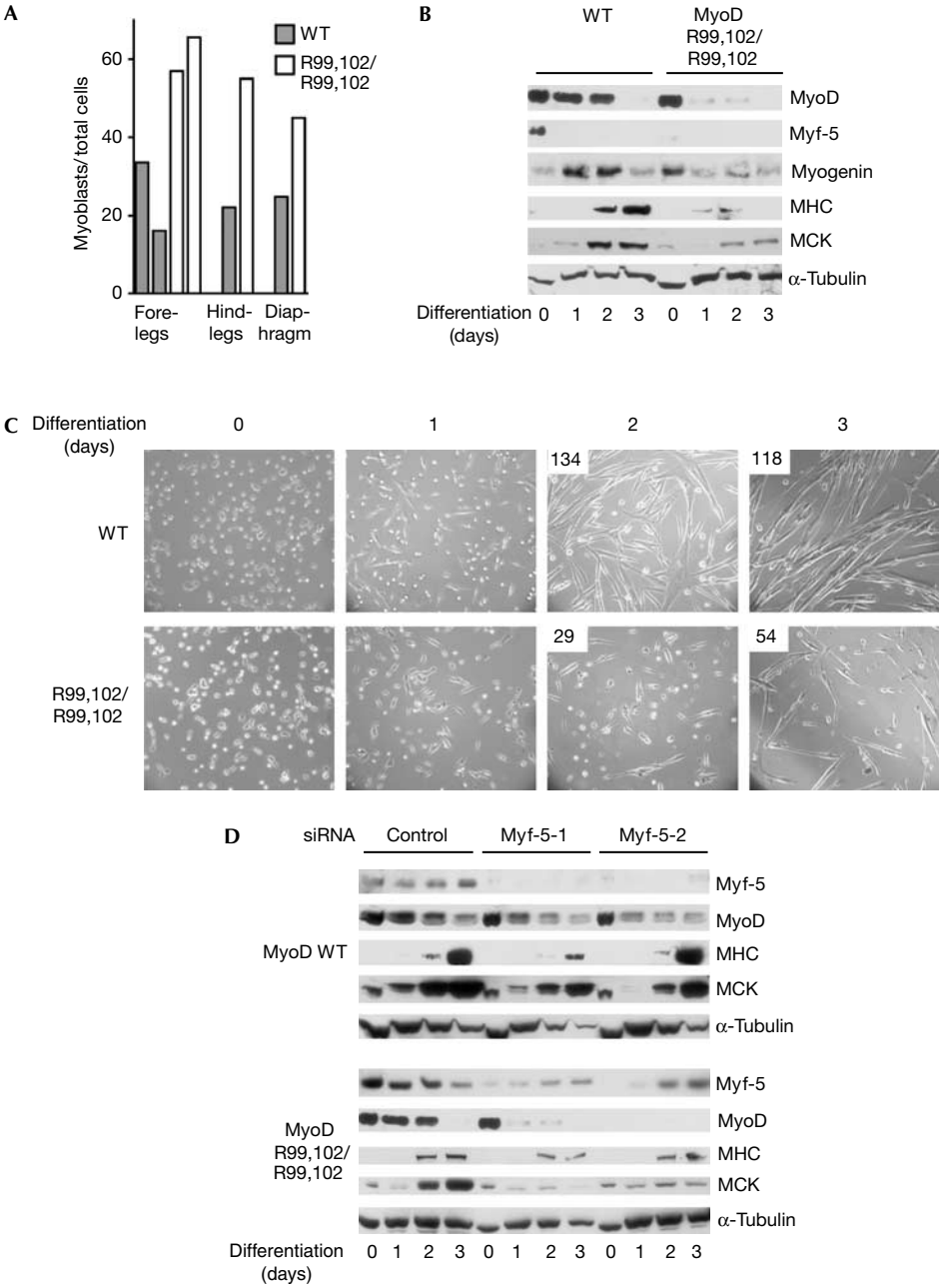

Fig 5 | Increased proliferation and delayed differentiation of adult primary myoblasts derived from MyoD^{R99,102} mice. (**A**) Primary myoblasts were isolated from the indicated skeletal muscle of wild-type (WT) and MyoD^{R99,102} (R99,102/R99,102) mice. After 5 days in culture, cells were fixed and stained for MyoD and desmin. The percentage of MyoD/desmin-positive cells in the total number of nuclei from individual mice is presented. (**B**,**C**) Impaired differentiation of passage-3 myoblasts, from the muscles of the hind leg of WT and MyoD^{R99,102} mice, placed under differentiation conditions and monitored for 72 h. (**B**) Western blot; (**C**) phase contrast; upper left corner: number of myotubes per field. (**D**) Downregulation of Myf-5 further decreases differentiation. Passage-5 myoblasts from the muscles of the hind leg of WT and MyoD^{R99,102} mice were depleted of Myf-5 by RNA interference, placed under differentiating conditions and analysed by western blot as in (**B**); the results obtained with two distinct Myf-5 short interfering RNAs (siRNAs) or a control scrambled siRNA are shown. (Phase contrast of the same samples is given in supplementary information S4 online.) MCK, muscle creatine kinase; MHC, myosin heavy chain; R, Arg, arginine; α-Tubulin, used as a loading control.

Ozyme, St Quentin en Yvelines, France) according to the manufacturer's recommendations, with the addition of 1% dimethylsulphoxide.

Southern blot analyses were carried out using standard procedures, and genomic DNA (5–10 mg) was digested with 15 U of restriction enzymes overnight. Probe A corresponds

to the *Nco*I–*Xho*I fragment (1.1 kb) adjacent to the 5′ flanking region of the transgene. Probe B is a 1 kb fragment corresponding to the −600 to +450 region. Probe C corresponds to the *Bam*HI–*Eco*RI fragment (1.2 kb) located 700 bp downstream of the 3′ flanking region.

RT–PCR–RFLP, RT–Q-PCR and RNA interference. RNA was isolated from ES cells, embryoid bodies or E14 embryos using TRIzol® Reagent (Invitrogen) according to the manufacturer's recommendations and then treated with DNase RQ1 (Promega, Lyon, France).

For RT–PCR–RFLP, RT was performed using Superscript III (Invitrogen). PCR products were digested with *Pst*I (New England Biologicals, Ozyme, St Quentin en Yvelines, France).

For RT–Q-PCR, RT was performed as described above, followed by quantitative PCR on a LightCycler (Roche Diagnostics, Meylan, France).

SiRNA oligonucleotides were obtained from Qiagen. Myf-5 siRNA sequences are given in the supplementary information online.

Immunoprecipitation and western blotting. Immunoprecipitations were carried out on 10 mg of whole-cell lysates using standard procedures. For detection of acetylated MyoD in C2C12, cells were treated with a deacetylase inhibitor, trichostatin A (Sigma), at 50 nM for 15 h before lysis. The rabbit antibody against acetylated MyoD was raised using a mouse MyoD peptide containing acetylated lysines at positions 99 and 102 (AC-acK-AC-acK-RKTTNADRR) and coupled to diphtheria toxin. Total IgGs were purified from the serum on a protein-G–Sepharose column (Mab-trap kit, Amersham Biosciences, Orsay, France). Antibodies against acetyl lysine and non-acetylated MyoD were eliminated by adsorption onto non-acetylated recombinant MyoD, followed by an irrelevant peptide containing one acetylated lysine, both coupled to cyanogen–bromide-activated beads. Specificity was verified by western blot. Other antibodies used for western blot were specific for MyoD (C20, Santa Cruz, Heidelberg, Germany), α-tubulin (DM 1A, Sigma), MCK (gift from K. Kato, Aichi, Japan), eMHC (F1.652, Stanford University School of Medicine, Stanford, CA, USA), or horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Sigma).

Immunofluorescence. Embryoid bodies were analysed by immunofluorescence using standard procedures after fixation in 4% paraformaldehyde, permeabilization with 0.4% Triton X-100 and incubation in a blocking solution (5% FCS and 1% BSA). Antibodies were mouse MHC antibodies (MY-32, Sigma) and Cy3-coupled anti-mouse IgG secondary antibody.

Cardiotoxin-induced regeneration assay. Regeneration assays were carried out by injecting 10 μl of cardiotoxin into the tibialis anterior muscle of 7- to 8-week-old mice, as described previously (Polesskaya *et al*, 2003). Each tibialis anterior muscle was weighed before lysis. Tissue extracts were normalized by total protein content and further controlled by staining with Ponceau red (an example is shown in supplementary Fig S1 online).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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