An evolutionarily acquired genotoxic response discriminates MyoD from Myf5, and differentially regulates hypaxial and epaxial myogenesis

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Despite having distinct expression patterns and phenotypes in mutant mice, the myogenic regulatory factors Myf5 and MyoD have been considered to be functionally equivalent. Here, we report that these factors have a different response to DNA damage, due to the presence in MyoD and absence in Myf5 of a consensus site for Abl-mediated tyrosine phosphorylation that inhibits MyoD activity in response to DNA damage. Genotoxins fail to repress skeletal myogenesis in MyoD-null embryos; reintroduction of wild-type MyoD, but not mutant Abl phosphorylation-resistant MyoD, restored the DNA-damage-dependent inhibition of muscle differentiation. Conversely, introduction of the Abl-responsive phosphorylation motif converts Myf5 into a DNA-damage-sensitive transcription factor. Gene-dosage-dependent reduction of Abl kinase activity in MyoD-expressing cells attenuated the DNA-damage-dependent inhibition of myogenesis. The presence of a DNA-damage-responsive phosphorylation motif in vertebrate, but not in invertebrate MyoD suggests an evolved response to environmental stress, originated from basic helix–loop–helix gene duplication in vertebrate myogenesis.

Keywords: DNA damage; skeletal myogenesis; Myo D; Myf5; cAbl kinase

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INTRODUCTION

Previous work suggests the existence of at least two programmes for embryonic myogenesis driven by MyoD and Myf5 (Cossu et al., 1996; Gensch et al., 2008; Haldar et al., 2008). Early (epaxial) myogenesis is dependent on Myf5 and/or Mrf4, which are induced by signals from the neural tube/notochord (NT/NC) complex. Subsequently, signals from the dorsal ectoderm activate MyoD in cells of the dorsolateral domain in developing somites leading to hypaxial myogenesis, which generates most of the skeletal muscles in the body wall and all muscles in the limbs, tongue and diaphragm (Cossu & Borello, 1999). Ablation of Myf5-expressing cells confirmed the existence of a Myf5-dependent and a Myf5-independent myogenic lineage (Gensch et al., 2008; Haldar et al., 2008), the latter requiring the activity of MyoD. This is not reflected by functional differences between Myf5 and MyoD proteins, as both activate myogenesis by redundant mechanisms through conserved functional domains (Gerber et al., 1997).

On exposure to genotoxins, myoblasts activate a ‘differentiation checkpoint’ that delays the transcription of musclespecific genes and coordinates DNA repair with the onset of the differentiation programme (Puri et al., 2002), thereby safeguarding the genomic integrity of terminally differentiated myotubes.
Phosphorylation of Tyr 30 in the amino-terminal region of MyoD by DNA-damage-activated nuclear Abl kinase mediates the transient block of MyoD-activated gene expression (Puri et al., 2002). The observation that the DNA-damage-sensitive phosphorylation site of MyoD is absent in the corresponding N-terminal region of Myf5 (see supplementary Fig S5A online) prompted us to investigate whether this sequence divergence results in a different response to genotoxic stress.

RESULTS AND DISCUSSION

Myf5-dependent myogenesis is insensitive to genotoxins

Explants of presomitic mesoderm (PSM) from embryos expressing the nuclear lacZ (nlacZ) gene under the control of the myosin light chain (MLC1/3F) regulatory region (Kelly et al., 1995) were cultured with either adjacent NT/NC—which activates Myf5—or dorsal ectoderm—which mainly activates MyoD—and exposed to the genotoxin mitomycin C (MMC), a DNA cross-linking agent that activates the nuclear Abl tyrosine kinase. Staining the cultures (n = 5) for lacZ expression showed that myogenesis was almost completely blocked by MMC in cultures of PSM with dorsal ectoderm (MyoD-dependent myogenesis), but only slightly affected in cultures of PSM with NT/NC (Myf5-dependent myogenesis; supplementary Fig S1 online). Thus, the majority of cells in the PSM avoided the DNA-damage-activated differentiation checkpoint when co-cultured with signals from NT/NC that activate Myf5.

To link this difference to Myf5 or MyoD, we exposed explants of presomitic tissues from embryos derived from either MyoD-null (n = 10) or Myf5:(Mrf4) double-null (n = 8) mice to several genotoxic agents. This approach eliminates the reciprocal activation between myogenic basic helix–loop–helix (bHLH) genes (Weintraub, 1993). Genotoxins did not affect myogenesis in PSM cultures (co-cultured with both NT/NC and dorsal ectoderm) derived from MyoD-null embryos (Fig 1A,B), in which Myf5 and/or Mrf4 drive the differentiation programme (Kassar-Duchossoy et al., 2004). By contrast, myogenesis was inhibited by genotoxic agents in cultures from Myf5:(Mrf4) double-null embryos, in which differentiation is driven by MyoD (Fig 1A,B). As genotoxins were used at equal concentrations and the same route of delivery and time of exposure were used, the DNA was damaged equally in PSM cultures from MyoD-null or Myf5:(Mrf4) double-null embryos. Furthermore, the DNA-damage-signalling machinery was not affected by the genetic ablation of MyoD or Myf5:(Mrf4), as shown by the auto-phosphorylation of the ataxia telangiectasia mutated protein (ATM; Bakkenist & Kastan, 2003), which was comparable in MyoD-null and Myf5:(Mrf4) double-null cells exposed to DNA damage (Fig 1B). The different responses of MyoD and Myf5 were also observed using different genotoxic agents: MMC, etoposide and methylmethanesulphonate (MMS), which are known to activate the nuclear Abl tyrosine kinase (Fig 1C).

To test the effect of MMC in vivo, we treated pregnant mice (wild type) expressing the MLCL1/3F-nlacZ reporter (n = 5), or MyoD (n = 4) and Myf5:(Mrf4) double-null mutant mice (n = 4) with two consecutive injections of MMC—2 mg/kg in 0.5 ml of PBS at embryonic day (E) 8.5 and 4 mg/kg in 0.5 ml of PBS at E9—and analysed at E10. Induction of phosphorylated-ATM (p-ATM) was observed in a few nuclei of untreated embryos (Fig 2A,C, red-stained nuclei), possibly reflecting the stochastic activation of the DNA-damage-signalling pathway in proliferating cells of developing embryos. By contrast, induction of p-ATM was observed in almost all the nuclei of embryos exposed to MMC, including the myotomal nuclei, which express high levels of the Myf5 protein (Fig 2B,D,E). This homogeneous response reflects the synchronous activation of DNA-damage signalling in the nuclei of embryos exposed to genotoxic stress. A similar activation of ATM phosphorylation was observed in embryos lacking one or two...
more myogenic factors—the MyoD-null and Myf5:(Mrf4) double-null mutants (data not shown). The activation of the DNA-damage response in MMC-treated embryos was also shown by the phosphorylation of Nijmegen breakage syndrome (p-Nbs1) and Myf5 antibody (green). (F) Whole-mount and (G) histology X-Gal staining of treated and control MLC1/3F–nLacZ embryos. (H) Whole-mount MyHC staining (with MF20 antibody) of MyoD-null or Myf5:(Mrf4) double-heterozygous pregnant mice treated as described in A. Note that MyHC+ myotomes in control WT and treated MyoD-null embryos, but not in MMC-treated Myf5:(Mrf4) double-null embryos. The heart is normally stained in all treated embryos. ATM, ataxia telangiectasia; CTR, control; E, embryonic day; MMC, mitomycin C; MLC, myosin light chain; MyHC, myosin heavy chain; nLacZ, nuclear lacZ; T, treated; WT, wild type; X-Gal; 5-bromo-4-chloro-3-indolyl-b-D-galactoside.

**Fig 2** Effect of mitomycin C on embryonic myogenesis in vivo. (A–E) MLC1/3F–nLacZ pregnant mice were treated with (B,D,E) two consecutive injections of MMC (2 mg/kg in 0.5 ml of PBS at E8.5 and, after a specified number of hours, 4 mg/kg in 0.5 ml of PBS at E9) or (A,C) PBS alone. Embryos were collected at E10, sectioned and stained with a phosphorylated ATM antibody, which identifies DNA-damaged nuclei (red) and Myf5 antibody (green). (F) Whole-mount and (G) histology X-Gal staining of treated and control MLC1/3F–nLacZ embryos. (H) Whole-mount MyHC staining (with MF20 antibody) of MyoD-null or Myf5:(Mrf4) double-heterozygous pregnant mice treated as described in A. Note that MyHC+ myotomes in control WT and treated MyoD-null embryos, but not in MMC-treated Myf5:(Mrf4) double-null embryos. The heart is normally stained in all treated embryos. ATM, ataxia telangiectasia; CTR, control; E, embryonic day; MMC, mitomycin C; MLC, myosin light chain; MyHC, myosin heavy chain; nLacZ, nuclear lacZ; T, treated; WT, wild type; X-Gal; 5-bromo-4-chloro-3-indolyl-b-D-galactoside.
heterozygous mice with MMC. Figure 2H shows the expression of myosin heavy chain (MyHC) in the myotome and in the heart of MyoD-null and Myf5:(Mrf4) double-null embryos either untreated or exposed to MMC. Each of the MMC-treated embryos expressed myosin in the heart, which was morphologically normal. However, although Myf5:(Mrf4) double-null embryos did not show MyHC expression in the myotome after exposure to MMC, the MyoD-null embryos did (Fig 2H, right panels). This suggests that MyoD-driven myogenesis is inhibited by genotoxins in vitro and in vivo, but Myf5:(Mrf4)-driven myogenesis is not.

We investigated this phenomenon in cultured muscle cells by downregulating MyoD expression with short hairpin RNA (shRNA) in myoblast cell lines exposed to genotoxic agents. In cells depleted of MyoD by shRNA interference, Myf5 becomes the most abundant bHLH factor (Sabourin et al., 1999). Independent clones of C2C12 and C2C7 myoblasts, in which MyoD was downregulated by at least 50% by retroviral-mediated delivery of specific shRNA, showed a threefold increase in levels of Myf5 and displayed a proportional resistance to DNA-damage-mediated inhibition of differentiation (supplementary Fig S4 online). In response to genotoxins, MyoD shRNA-infected myoblasts showed an increase in the formation of MyHC-positive myotubes (supplementary Fig S4B,D online) that expressed higher levels of myogenin and MCK than myoblasts infected with control shRNA (supplementary Fig S4C,D online) differences were found to be significant ($P<0.01$) by the analysis of variance test. These data support the conclusion that MyoD has a unique role, among the MRFs, in mediating DNA-damage-dependent inhibition of skeletal myogenesis, and that this effect is not restricted to the embryonic stage.

**Sensitivity to genotoxins depends on cAbl kinase**

Phosphorylation of Tyr 30 within the Abl consensus site YDDXX in the N-terminus of MyoD mediates the transient block of MyoD-activated gene expression in myoblasts exposed to DNA damage (Puri et al., 2002). This site is absent in the N-terminus of Myf5, although the phospho-acceptor tyrosine is present (supplementary Fig S5A online). Notably, this DNA-damage-responsive motif is also absent in the myogenic transcription factors myogenin and Mrf4 (Supplementary Fig S5A online). Moreover, when MyoD or Mrf5 was expressed in a heterologous cell type (U293 cells), tyrosine phosphorylation of MyoD, but not of Myf5, could be detected in response to genotoxic agents that activate Abl (Fig 3A). By replacing Ser 19 with a proline and thus generating an Abl consensus phosphorylation site (supplementary Fig S5A online), it was possible to achieve Myf5 tyrosine phosphorylation in response to DNA-damage (Fig 3A), indicating that absence of a DNA-damage-responsive sequence motif discriminates Myf5 from MyoD and suggests that these proteins might have non-redundant roles in the response to genotoxic insults.

We then evaluated the effect of genotoxins on myogenic conversion of fibroblasts induced by wild-type or mutant MyoD and Myf5 in which the Abl consensus site was either disrupted in MyoD (MyoD-Y30F) or created in Myf5 (Myf5-S19P). Ectopic expression of the wild-type MyoD converted 10T1/2 fibroblasts into myogenic cells with higher efficiency than wild-type Myf5 (30% of multinucleated MyHC-positive cells in MyoD-expressing fibroblasts compared with 5–10% MyHC-positive mononucleated cells in Myf5-expressing fibroblasts). Exposure to genotoxins reduced the number of MyHC-positive cells in wild-type MyoD-expressing cultures, but did not inhibit myogenic conversion in wild-type Myf5-expressing cells (Fig 3B,D). Introduction of the DNA-responsive phosphorylation motif in Myf5 induced sensitivity to DNA damage in cells expressing Myf5-S19P (Fig 3B,D), which became tyrosine phosphorylated in response to DNA-damage (Fig 3A). Furthermore, myogenic conversion induced by a Myf5-S19P/Y146F double mutant, in which an additional potential Abl consensus phosphorylation site at the carboxyl terminus (Tyr 146) was disrupted, was still sensitive to DNA damage (data not shown). Together, these results show that the integrity of the Abl consensus site within the N-terminal region of MyoD was required for genotoxins to inhibit myogenic conversion of 10T1/2 fibroblasts (Fig 3B,D).

Next, we analysed the effect of DNA damage on MyoD-null PSM explant cultures ($n=3$) in which wild-type MyoD or the MyoD-Y30F mutant were reintroduced by irs-GFP lentiviral transduction (Fig 3E). The explants were cultured without the neural tube, to prevent Myf5-dependent myogenesis that would have masked the effect of exogenous MyoD. Reconstitution with wild-type MyoD restored the response to MMC, leading to inhibition (about 80%) of myogenesis (Fig 3E, left panel). The residual myogenesis observed in embryos reconstituted with wild-type MyoD was probably due to cells that had committed to differentiation before the expression of lentiviral vector-driven MyoD. By contrast, embryos reconstituted with the MyoD-Y30F mutant activated myogenesis despite the exposure to MMC (Fig 3E, right panel), similar to the behaviour of the MyoD-null embryos.

**Nuclear cAbl phosphorylates MyoD but not Myf5**

Abl kinase shuttles between the nucleus and the cytoplasm in response to different stimuli, with only the nuclear fraction mediating the DNA-damage response (Wang, 2005). Therefore, we evaluated the effect of a nuclear-targeted and dimerizable FKBP-AblNuk (the dimerization of which is induced by the cell-permeable ligand AP20187; Vella et al., 2003). However, myoblasts expressing FKBP-AblNuk and exposed to AP20187 showed an apoptotic effect (data not shown). Nevertheless, in the absence of AP20187, FKBP-AblNuk had low but constitutive kinase activity that enabled survival, yet was sufficient to block differentiation. Wild-type FKBP-Abl, which showed predominant cytoplasmic localization, caused only a small reduction in the expression of myogenin (data not shown). Moreover, co-expression of FKBP-AblNuk and MyoD or Myf5 (wild-type and mutant) in 10T1/2 fibroblasts induced tyrosine phosphorylation of wild-type MyoD and Myf5-S19P, but not of MyoD-Y30F and wild-type Myf5 (Fig 4A). The functional consequence of these modifications was demonstrated by lentiviral delivery of FKBP-AblNuk to PSM cultures from MyoD-null or Myf5:(Mrf4) double-null embryos (Kassar-Duchossoy et al., 2004). Although MyoD-dependent myogenesis was inhibited by the expression of constitutive nuclear, active cAbl (FKBP-AblNuk), Myf5-dependent myogenesis was not affected by the same conditions (Fig 4B).

**cAbl kinase inhibits MyoD-dependent myogenesis**

Next, we investigated the Abl-dependent inhibition of MyoD-activated myogenesis in fibroblasts derived from Abl-deficient mice. Compared with wild type, fibroblasts from Abl-null mice
showed almost complete resistance to DNA-damage-dependent inhibition of MyoD-activated myogenesis, which was reversed by reintroducing a constitutive nuclear Abl—Δnes, which lacks the nuclear export signal—but not a constitutive cytoplasmic Abl mutant—Δnls, which lacks the nuclear localization signals (Puri et al., 2002; supplementary Fig S6 online). This is consistent with the ability of nuclear Abl tyrosine kinase to inhibit MyoD function through tyrosine phosphorylation (Puri et al., 2002). By contrast, cytoplasmic Abl is not activated by DNA damage and promotes, rather than inhibits, skeletal myogenesis (di Bari et al., 2006).

Reducing the enzymatic activity of Abl by deletion of one allele in fibroblasts derived from Abl<sup>+/−</sup> (heterozygous) mice was sufficient to attenuate the inhibition of MyoD-activated myogenesis by DNA damage (supplementary Fig S6 online).

To confirm the role of Abl as mediator of DNA damage-dependent inhibition of MyoD, we generated Myf5<sup>−/−</sup> (Mrf4) double-null/<sup>MyoD<sup>Cre−Ab<sup>flx+</sup>-</sup></sup> embryos, by crossing Myf5<sup>−/−</sup> (Mrf4) double-null/<sup>MyoD<sup>Cre+</sup></sup> mice with Myf5<sup>−/−</sup> (Mrf4) double-null/<sup>Ab<sup>flx</sup></sup> mice (see supplementary information online for the generation of the Ab<sup>flx</sup> allele in mice) to selectively eliminate one floxed allele

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**Fig 3** Effects of DNA damage on MyoD- and Myf5-mediated 'myogenic conversion' of fibroblasts. (A) Western blot analysis (for MyoD or Myf5, and their tyrosine phosphorylation) of 293T cells, transfected with either WT or mutated forms of HA-tagged MyoD or Myf5, treated or not with MMS (150 μM) and immunoprecipitated with HA antibodies. (B) Immunofluorescence with MyHC antibody (red) of 10T1/2 fibroblasts transfected with either the WT or mutated forms of MyoD or Myf5, treated or not with MMC (10 μg/ml) for 3 h and then shifted to DM for 3 days. Nuclei were stained with DAPI (blue). (C) Western blot analysis of protein extracts from the same cell cultures as in B. β-Tub was used to normalize the amount of loaded protein. (D) Quantitative analysis of the number of differentiated control (blue bars) and MMC-treated (red bars) cultures when similarly treated. Data are the average ± s.d. of five separate experiments, each conducted in triplicate. (E) PSM cultures (without the neural tube) from MyoD-null mice were transduced with lentiviral vectors expressing either WT or Y30F-mutant MyoD (all eGFP-tagged) treated or not treated (CTR) with MMC and, after 3 days, stained with MyHC and GFP antibodies. β-Tub, β-tubulin; CTR, control; DAPI, 4,6-diamidino-2-phenylindole; DM, differentiation medium; eGFP, enhanced green fluorescent protein; HA, haemagglutinin; IP, immunoprecipitation; MMC, mitomycin C; MMS, methylmethanesulphonate; MyHC, myosin heavy chain; PSM, presomitic paraxial mesoderm; p-Tyr, phosphotyrosine; WT, wild type.

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**Table 1**: Western blot analysis of protein extracts from the same cell cultures as in B. β-Tub was used to normalize the amount of loaded protein. (D) Quantitative analysis of the number of differentiated control (blue bars) and MMC-treated (red bars) cultures when similarly treated. Data are the average ± s.d. of five separate experiments, each conducted in triplicate.
of Abl gene in MyoD-expressing cells. After treatment with MMC, skeletal myogenesis could be observed in the PSM of Myf5:(Mrf4) double-null//MyoDCre:Ablflx/+ heterozygous embryos (n = 3; Fig 4C, right panel), but it was inhibited in the Myf5:(Mrf4) double-null//MyoDCre control embryos (n = 4; Fig 4C, left panel). This gene-dosage effect of Abl on skeletal myogenesis in response to DNA damage supports the crucial function of Abl full enzymatic activity in repressing MyoD function in myoblasts exposed to genotoxic stress.

Together, the data presented here show the first molecular difference between MyoD and Myf5, which were thought previously to be functionally indistinguishable. They are equivalent in their ability to open chromatin and activate transcription of downstream genes such as myogenin (Gerber et al., 1997).
However, we show here that Myf5 and MyoD differ in the manner by which their function is regulated by genotoxins, owing to the DNA-damage-responsive phosphorylation motif that enables MyoD, but not Myf5, to undergo transient inactivation in response to genotoxic insults. This suggests the existence of a developmentally sensitive checkpoint that diversifies epaxial and hypaxial myogenesis during somitogenesis.

The appearance of a lateral septum, which characterizes the increase in size and complexity of the vertebrate body, divides the skeletal muscles into a dorsal (epaxial) and a ventral (hypaxial) compartment (Romer & Parsons, 1978). The simultaneous duplication of the myogenic regulatory gene MyoD gave rise to a family of four genes (Atchley et al., 1994) the molecular diversity of which provided flexibility in the execution of the myogenic programme. Only MyoD acquired conserved sequences for regulatory enzymes, such as the Abl tyrosine kinase, which transmits genotoxic cues to the transcription machinery and reversibly blocks differentiation, thus protecting genome integrity in post-mitotic nuclei, in which the shutdown of global nucleotide excision repair and other pathways reduce the overall efficiency of DNA repair (Simonatto et al., 2007).

As the Myf5-dependent epaxial muscles are the first to develop during somitogenesis, their tolerance to the damage-activated differentiation checkpoint might have been selected during evolution, to ensure muscle formation despite the encounter of stress signals. Conversely, MyoD-dependent hypaxial myogenesis—which gives rise to the majority of skeletal muscles in the adult body of amniotes (which develop in a protected environment)—is responsive to the DNA-damage-activated differentiation checkpoint, possibly to protect the genomic integrity of cells that constitute the adult musculature to ensure long-term survival. Consequently, sequence diversification in functional homologues of myogenic bHLH proteins during evolution could be the result of a selective pressure to ignore or to respond to environmental stress and also to proliferation signals (A. Innocenzi, G. Messina and G. Cosso, unpublished data), although the latter remains to be investigated at the molecular level. Interestingly, the MyoD sequence in all analysed vertebrates shows the presence of the DNA-damage-responsive phosphorylation motif. However, this sequence is not present in any of the invertebrate MyoD analysed so far, in which only one myogenic factor is present. These include Amphioxus, Ascidia, Echinoderm and Caenorhabditis elegans (supplementary Fig S5B online), with the notable exception of Drosophila in which skeletal myogenesis is regulated by several structurally unrelated transcription factors and MyoD deletion does not compromise muscle formation, although the pattern is altered (Wei et al., 2007). Together, these data suggest that in invertebrates—in which myogenesis is driven by a single bHLH—the DNA-damage-activated differentiation checkpoint is ignored, possibly because of a selective pressure to acquire motility early in development, irrespective of genome integrity.

**METHODS**

**PSM** was dissected from wild-type or MyoD or Myf5; (Mrfr) double-mutant embryos at E9.5, as described previously (Cosso et al., 1996). Tissue explants were cultured in RPMI supplemented with 15% FCS and, when indicated, transduced (after 12 h) with lentiviral vectors. On day 3 (unless otherwise specified), cultures were processed for biochemical analysis or immunostaining.

Cell lines, culture conditions and generation of plasmids and viral vectors for cDNA and shRNA delivery (Puri & Sartorelli, 2000; Puri et al., 2002) are detailed in supplementary material online. Transient transfections were performed using the FUGENE reagent.

To engineer Abl lentiviral vectors, we excised cAbl inserts from pCMV-FV2(m)cAbl-WT and pCMV-FV2(m)cAbl-Nuk by BamHI/EcoR1 double digestion. cAbl-WT and cAbl-Nuk inserts were then filled in with Klenow enzyme. cAbl-8NLS was excised from the MSCV-cAbl-8NLS vector by NotI digestion and was also filled in with Klenow. The pHR-CMV-IREs-GFP lentiviral transfer vector was provided by Dr Noriyuki Kasahara (UCLA School of Medicine, Los Angeles, CA, USA). The transfer vector was digested with BamHI, filled with Klenow and dephosphorylated with CIAP enzyme. The Abl variants were then inserted in the linearized pHR-CMV-IREs-GFP lentiviral transfer vector. The resulting constructs pHR-CMV-cAbl-WT, pHR-CMV-cAbl-Nuk and pHR-CMV-cAbl-8NLS were verified by restriction digestion and sequence analysis. Lentiviral vectors were produced by transient co-transfection of 293T cells with pHR-CMV-cAbl-WT, pHR-CMV-cAbl-Nuk or pHR-CMV-cAbl-8NLS with the packaging plasmid pCMV-R8.71, and the plasmid pMD.G encoding the VSV-G envelope. Viral titres were determined by assessing the expression of GFP in transduced 293T cells by fluorescence-activated cell sorter analysis.

Immunofluorescence, immunoprecipitation and western blot analyses were performed as described previously (Cosso et al., 1996; Puri et al., 2002).

**MyoD mutant Y30F** was described previously (Puri et al., 2002). The cAbl consensus site was reconstituted in Myf5 by replacing Ser19 with proline (Myf5 S19P). The double mutant with a reconstituted N-terminal but disrupted C-terminal cAbl consensus sites was generated by replacing the Tyr146 with phenylalanine in the Myf5 S19P backbone. Quick Change II Site Directed Mutagenesis Kit from Stratagene was used to obtain mutants. Myogenic Conversion Assay was performed in 10T1/2 transfected with MyoD or Myf5 wild-type and mutants, as described previously (Puri & Sartorelli, 2000).

**Construction of Abl<sup>Flox</sup> mice.** Two homologous PCR products (A-B and Y-Z) were amplified from BAC clone-containing mouse Abl genomic DNA and inserted into the pSK<sup>þ</sup> vector. A measure of 500 ng of vector linearized by PstI was electroporated into recombination-proficient cells (EL350) to select recombinants on a selective medium. The plasmid was then recombined with the p5K<sup>þ</sup> vector to generate p5K<sup>þ</sup> vector with a 10.6-kb fragment of Abl genomic DNA (exons 4–7). A measure of 10 ng of the construct was then co-electroporated with 100 ng of lpsf-flxed Neo cassette flanked by two homology arms C-D and E-F into DY380 cells to select recombinants on a kanamycin plate. The recombinant plasmid was electroporated into EL350 cells to grow on Luria broth in the presence of 0.1% arabinose, which induces Cre expression, to excise the Neo cassette. The plasmid was then recombined with the Neo-targeting vector derived from pl451, which contains single lpsf site and two FRT sites, using the homology arms G-H and I-J in DY380 cells. The Abl<sup>Flox</sup>-targeting recombinant was selected on a kanamycin plate and exons of the vector were verified by DNA sequencing. The electroporation condition was the same for the whole procedure: 1.8 kV, 25 μF, 200Ω. The construction of the Abl<sup>Flox</sup> allele is summarized in supplementary Fig S7 online, and the derivation of Abl<sup>Flox</sup> mice from the appropriately targeted embryonic stem cells was achieved using standard techniques.
Supplementary information is available at EMBO reports online (http://www.emboreports.org).

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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