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 failed 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-depen-

dent 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; *MyoD*; *Myf5*; *cAbl* kinase

EMBO reports (2011) 12, 164–171. doi:10.1038/embo.2010.195

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 muscle-specific 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.

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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 teleangectasia 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 *MLC1/3F-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

Fig 1 | Effect of genotoxic drugs on somitic cultures derived from *MyoD*-null or *Myf5*:(*Mrf4*) double-null embryos. PSM from E9.5 (20–24 somite stage) embryos were cultured for 4 days (Cossu et al, 1996) in the presence of MMC (3 µg/ml for 1 h), Eto (0.5 µM), MMS (75 µM) or solvent alone. (A) Double immunofluorescence with monoclonal MyHC antibody (MF20; red) and rabbit polyclonal *MyoD* or *Myf5* antibodies (green) of control (upper panels) or MMC-treated (lower panels) cultures from *Myo*-null (left panels) or *Myf5*:(*Mrf4*) double-null (right panels) embryos. (B) Western blot of total protein extracts of somitic cultures from *MyoD*-null and *Myf5*:(*Mrf4*) double-null embryos reacted with MyHC, *Myf5*, *MyoD* and phospho-ATM antibodies. β-Tubulin antibody was used to normalize the amount of protein loaded. (C) Quantitative analysis of the number of differentiated (grey bars) and committed, non-differentiated (black bars) cells in cultures from *MyoD*-null or *Myf5*:(*Mrf4*) double-null embryos treated with genotoxic drugs. Data are the average ± s.d. of five separate experiments, each performed in triplicate. β-Tub, β-tubulin; CTR, control; E, embryonic day; Eto, etoposide; MMC, mitomycin C; MMS, methylmethanesulphonate; MyHC, myosin heavy chain; p-ATM, phospho-ataxia teleangectasia mutated; PSM, presomitic paraxial mesoderm.

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

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 teleangiectasia; 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.

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 led to tyrosine phosphorylation of MyoD (supplementary Fig S2 online), as previously described (Puri *et al*, 2002). MMC caused a reduction of X-Gal staining in somites of *MLC1/3F-nLacZ* wild-type embryos, compared with controls (Fig 2F). Myotome was truncated

in its ventral domain (that is mainly dependent on MyoD), and this alteration was confirmed by histological analyses of the same embryos (Fig 2G). The myotome truncation is more visible in the higher magnification and in additional sections taken at different cranio-caudal levels (supplementary Fig S3A,B online). This suggests that *in vivo* Myf5-dependent myogenesis is also resistant to DNA-damage-activated signalling. To demonstrate this, we treated *MyoD*-null or *Myf5:(Mrf4)* double-null inter-crossed

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 YDDX 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 *Myf5* 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 Ser19 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 ires-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

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 μ M) 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 μ g/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 $\pm$ 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.

showed almost complete resistance to DNA-damage-dependent inhibition of MyoD-activated myogenesis, which was reversed by reintroducing a constitutive nuclear Abl— Δ nes, which lacks the nuclear export signal—but not a constitutive cytoplasmic Abl mutant— Δ nls, 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*^{+/-} (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*:(*Mrf4*) double-null//*MyoD*^{Cre}-*Abl*^{flx/+} embryos, by crossing *Myf5*:(*Mrf4*) double-null//*MyoD*^{Cre} mice with *Myf5*:(*Mrf4*) double-null//*Abl*^{flx/flx} mice (see supplementary information online for the generation of the *Abl*^{flx} allele in mice) to selectively eliminate one floxed allele

Fig 4 | Effect of constitutive nuclear cAbl kinase (FKBP-cAbl-Nuk) on *MyoD* or *Myf5:(Mrf4)* driven myogenesis. (A) Western blot analysis (*MyoD* or *Myf5*, and their tyrosine phosphorylation) of 10T1/2 fibroblasts transduced with lentiviral vectors expressing FLAG-tagged FKBP-cAbl-Nuk and then transduced with adenovectors expressing *MyoD* or *Myf5*, either WT or mutated (Y30F and S19P). Total protein extracts were immunoprecipitated with *Myf5* or *MyoD* antibodies. (B) Immunofluorescence analysis (MyHC (red) and GFP (green) antibodies) of PSM cultures infected with FLAG-tagged, FKBP-cAbl-Nuk-GFP (lenti-cAbl) or empty lentivector (CTR). (C) immunofluorescence analysis (MyHC (red) and *MyoD* (green) antibodies) of *Myf5:(Mrf4)* double-null//*Abl*^{flx/+} mice, crossed with *Myf5:(Mrf4)* double-null//*MyoD*^{Cre} mice, to delete one *Abl* allele in *MyoD*-expressing cells during embryogenesis. Cultures were treated with MMC and after 3 days processed for immunofluorescence. CTR, control; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; IP, immunoprecipitation; MMC, mitomycin C; MyHC, myosin heavy chain; PSM, presomitic paraxial mesoderm; p-Tyr, phospho-tyrosine; Tot. extr., total extract; WB, western blot; WT, wild type.

of *Abl* gene in *MyoD*-expressing cells. After treatment with MMC, skeletal myogenesis could be observed in the PSM of *Myf5:(Mrf4)* double-null//*MyoD*^{Cre}-*Abl*^{flx/+} heterozygous embryos ($n=3$; Fig 4C, right panel), but it was inhibited in the *Myf5:(Mrf4)* double-null//*MyoD*^{Cre} 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 developmental mechanism 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 consensus 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. Cossu, 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 *Amphyoxus*, *Ascidiae*, *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*:(*Mrf4*) double-mutant embryos at E9.5, as described previously (Cossu *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 cAbl lentiviral vectors, we excised cAbl inserts from pC4M-FV2(m)cAbl-WT and pC4M-FV2(m)cAbl-Nuk by *Bam*HI/*Eco*R1 double digestion. cAbl-WT and cAbl-Nuk inserts were then filled in with Klenow enzyme. cAbl- δ NLS was excised from the MSCV-cAbl- δ NLS vector by *Not*I 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 *Bam*HI, filled with Klenow and dephosphorylated with CIAP enzyme. The cAbl 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- δ NLS 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- δ NLS 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 (Cossu *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 Tyr 146 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*^{flx/flx} mice. Two homologous PCR products (A-B and Y-Z) were amplified from BAC clone-containing mouse *Abl* genomic DNA and inserted into pSK⁺ vector. A measure of 500 ng of vector linearized by *Pst*II was electroporated into recombination-competent cells, DY380, harbouring the BAC clone to generate pSK⁺ 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 *lox*p-floxed *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 *lox*p site and two FRT sites, using the homology arms G-H and I-J in DY380 cells. The *Abl*^{flx}-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 μ F, 200 Ω . The construction of the *Abl*^{flx} allele is summarized in supplementary Fig S7 online, and the derivation of *Abl*^{flx/flx} 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>).

ACKNOWLEDGEMENTS

We thank David Goldhamer for the gift of the MyoD-cre mice. P.L.P. is an associate scientist of Telethon Dulbecco Institute and of Sanford Children's Health Center at Burnham, and was partly supported by Associazione Italiana Ricerca sul Cancro (AIRC) and National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS; RO1AR052779). This work was also supported by grants from the European Community ('Optistem' and 'Angioscaff'), European Research Council (ERC), Fondo Istituzionale Ricerca di Base (FIRB), Telethon, Duchenne Parent Project, Italian Ministries of Research and Health, Compagnia San Paolo di Torino and AIRC. C.P. was partly supported by a Wright Foundation Award and a Beginning Grant-in-Aid from the American Heart Association.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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