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## RESEARCH ARTICLE

## STEM CELLS AND REGENERATION

# Antagonistic regulation of p57<sup>kip2</sup> by Hes/Hey downstream of Notch signaling and muscle regulatory factors regulates skeletal muscle growth arrest

Antoine Zalc<sup>1,2,3,\*</sup>, Shinichiro Hayashi<sup>1,2,3,\*</sup>, Frédéric Auradé<sup>1,2,3,†</sup>, Dominique Bröhl<sup>4,‡</sup>, Ted Chang<sup>1,2,3,‡</sup>, Despoina Mademtoglou<sup>1,2,3</sup>, Philippos Mourikis<sup>1,2,3</sup>, Zizhen Yao<sup>5,§</sup>, Yi Cao<sup>5,§</sup>, Carmen Birchmeier<sup>4</sup> and Frédéric Relaix<sup>1,2,3,¶</sup>

## ABSTRACT

A central question in development is to define how the equilibrium between cell proliferation and differentiation is temporally and spatially regulated during tissue formation. Here, we address how interactions between cyclin-dependent kinase inhibitors essential for myogenic growth arrest (p21<sup>cip1</sup> and p57<sup>kip2</sup>), the Notch pathway and myogenic regulatory factors (MRFs) orchestrate the proliferation, specification and differentiation of muscle progenitor cells. We first show that cell cycle exit and myogenic differentiation can be uncoupled. In addition, we establish that skeletal muscle progenitor cells require Notch signaling to maintain their cycling status. Using several mouse models combined with *ex vivo* studies, we demonstrate that Notch signaling is required to repress p21<sup>cip1</sup> and p57<sup>kip2</sup> expression in muscle progenitor cells. Finally, we identify a muscle-specific regulatory element of p57<sup>kip2</sup> directly activated by MRFs in myoblasts but repressed by the Notch targets Hes1/Hey1 in progenitor cells. We propose a molecular mechanism whereby information provided by Hes/Hey downstream of Notch as well as MRF activities are integrated at the level of the p57<sup>kip2</sup> enhancer to regulate the decision between progenitor cell maintenance and muscle differentiation.

**KEY WORDS:** Myogenesis, Cell cycle regulation, p57<sup>kip2</sup>, Cdkn1, Notch signaling, MRF

## INTRODUCTION

The formation of functional organs of an appropriate size is highly controlled during development. Organ transplantation and regeneration studies have revealed that organ size relies on both intrinsic and extrinsic mechanisms (reviewed by Cook and Tyers, 2007). Systemic factors, such as growth hormones and nutritional status, have been known for many years to regulate organ size, while more recently the role of the Hippo and insulin/TOR pathways has emerged (Tumaneng et al., 2012). Of note, increasing evidence links these pathways with stem cell self-renewal and differentiation (Cherrett et al., 2012). Nevertheless, how cell fate decisions and differentiation programs are coordinated with cell cycle progression and arrest remains poorly understood.

Skeletal muscle provides a suitable model for such studies because the molecular pathways regulating differentiation and growth arrest have been identified. Muscle formation relies on a proliferating population of progenitor cells that express and require the Paired homeobox transcription factors Pax3 and Pax7 (Buckingham and Relaix, 2007). These resident progenitors are maintained in the developing muscles, where they provide a source of cells for muscle growth during development and eventually generate the adult stem cells population, termed satellite cells (Gros et al., 2005; Kassam-Duchossoy et al., 2005; Lepper and Fan, 2010; Relaix et al., 2006). Initially, muscle progenitor cells are located in the somite where they give rise to the trunk musculature of the myotome (Ben-Yair and Kalcheim, 2005; Kassam-Duchossoy et al., 2005; Relaix et al., 2005) or migrate out of the somitic dermomyotome to form limb skeletal muscles (Birchmeier and Brohmann, 2000; Schienda et al., 2006). During limb embryonic myogenesis, Pax3/7<sup>+</sup> progenitor cells undergo consecutive steps of differentiation via sequential expression of bHLH myogenic regulatory factors [MRFs; Myf5, MyoD1 and myogenin (Myog)], and first form committed progenitor cells that express Pax3/7 and Myf5, which correspond to a transit amplifying population (Picard and Marcelle, 2013), followed by the generation of myoblasts that express Myf5 and MyoD1, culminating in the appearance of differentiating myoblasts marked by Myog (Fig. 1) (Murphy and Kardon, 2011). The Myog<sup>+</sup> cells then fuse to form multinucleated muscle fibers. In the absence of MyoD1, despite upregulated Myf5 expression, myogenic differentiation is delayed during early limb development, resulting in a transient absence of differentiating (Myog<sup>+</sup>) myoblasts and fibers prior to E14.5 (Kablar et al., 1998). When both Myf5 and MyoD1 are impaired, Pax3/7<sup>+</sup> cells do not enter the myogenic program and skeletal muscle formation is abolished at all sites of myogenesis (Rudnicki et al., 1993).

Building a tissue requires the coordination of cell cycle exit with differentiation. Despite the identification of key molecular regulators of myogenic specification and differentiation (Buckingham and Relaix, 2007), how cell cycle exit is synchronized with skeletal muscle differentiation is not well understood. Cell cycle exit in muscle cells is orchestrated by cyclin-dependent kinase inhibitors (CDKIs) belonging to the CIP/Kip family: p21<sup>cip1</sup> (Cdkn1a, p21<sup>waf1</sup>), p27<sup>kip1</sup> (Cdkn1b) and p57<sup>kip2</sup> (Cdkn1c), abbreviated here as p21, p27 and p57, respectively. These CDKIs can bind and inhibit all combinations of cyclin-CDK complexes (reviewed by Besson et al., 2008). Most notably, in the absence of both p21 and p57, skeletal muscle development is severely affected and fiber formation is impaired, with myogenic cells undergoing apoptosis. This points to an essential function of p21 and p57 in cell cycle arrest during myogenesis (Zhang et al., 1999). *In vitro*, MyoD1 has been suggested to be a direct regulator of p21, thus controlling cell cycle exit during

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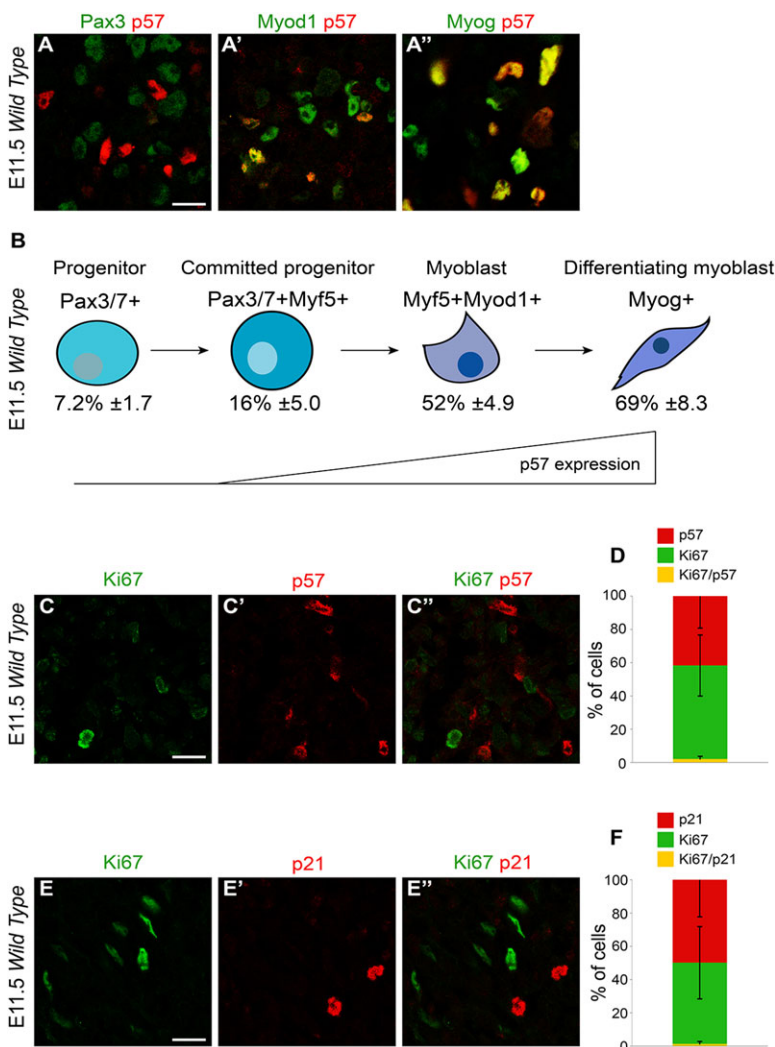
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**Fig. 1. Cell cycle exit occurs at the determination stage.**

(A–A'') Co-immunostaining for Pax3 (A), Myod1 (A') and myogenin (Myog, A'') in green, and p57 (A', A'') in red in E11.5 embryonic limb muscles. (B) Percentage of p57-expressing cells during forelimb myogenesis is given for each population. Progenitors and committed progenitors are mostly proliferating, whereas myoblasts and differentiating myoblasts are exiting the cell cycle. (C–C'') Co-immunostaining for Ki67 (C, C'', green) and p57 (C', C'', red) in E11.5 embryonic limb muscles. (D) Quantification of C–C''. (E–E'') Co-immunostaining for Ki67 (E, E'', green) and p21 (E', E'', red) in E11.5 embryonic limb muscles. (F) Quantification of E–E''. Ki67 is not expressed in cells expressing p21 or p57. For all experiments  $n=3$  embryos; error bars indicate s.d. Scale bars: 10  $\mu\text{m}$ .

adult muscle differentiation (Halevy et al., 1995). It has also been shown, both in mammalian cells (Reynaud et al., 2000) and in zebrafish (Osborn et al., 2010), that p57 interacts and stabilizes Myod1 to promote muscle differentiation, demonstrating a role for CDKIs beyond that in growth arrest. Analysis of *p21*; *p57* double-mutant mouse embryos suggested that cell cycle exit occurs in parallel to, but independently of, Myog-dependent terminal differentiation, while the lack of Mef2c expression in these mice suggested that late differentiation is defective (Zhang et al., 1999).

Previous studies have implicated the Notch signaling pathway as a key regulator of proliferation and differentiation of muscle progenitor cells (Buas and Kadesch, 2010; Mourikis and Tajbakhsh, 2014). This pathway is highly conserved during evolution and plays key roles during development, including the regulation of cell fate decisions, differentiation and homeostasis of progenitor cells in a wide variety of tissues (reviewed by Artavanis-Tsakonas and Muskavitch, 2010). Notch signaling requires direct interaction between a cell expressing at least one of the ligands [ $\delta$ -like 1 (Dll1) and 4 and jagged 1 and 2 in mammals] with a cell expressing one of the receptors (notch 1–4 in mammals). This interaction leads to a proteolytic cleavage of the receptor that releases the Notch intracellular domain, which translocates into the nucleus and interacts with the Rbpj transcription factor to induce downstream effectors, such as the Hes/Hey family of bHLH transcriptional repressors (reviewed by Borggreve and Liefke, 2012).

The role of Notch signaling in skeletal muscle development has been assessed in two mouse models: in a hypomorphic *Dll1* mutant (Schuster-Gossler et al., 2007) or in mice in which *Rbpj* expression was conditionally abrogated specifically in the myogenic lineage (Vasyutina et al., 2007). These *in vivo* models, along with studies performed in chick embryos, have demonstrated that Dll1-triggered canonical Notch signaling is required for the maintenance of muscle progenitor cells (Delfini et al., 2000; Hirsinger et al., 2001; Mourikis et al., 2012a; Schuster-Gossler et al., 2007; Vasyutina et al., 2007). Dll1 absence leads to early onset differentiation (Schuster-Gossler et al., 2007; Vasyutina et al., 2007), resulting in rapid exhaustion of the muscle progenitor cell pool and near complete absence of skeletal muscles at the fetal stage (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). This is in part mediated by the repression of Myod1 target genes through direct binding of Hey1 to their promoters (Bröhl et al., 2012; Buas et al., 2010). Interestingly, the role of Notch can be context dependent, since in the young somite of the chick embryo, *Dll1*<sup>+</sup> neural crest cells provide a transient stimulation of Notch activity that is important for the initiation of early myogenesis (Rios et al., 2011).

Here, we evaluated the *in vivo* expression of p57 and its link with muscle cell differentiation. Although cell cycle exit is normally synchronous with cell differentiation, we show that these events can be uncoupled. In fact, we found that during embryonic myogenesis p57-mediated cell cycle arrest occurs earlier than

previously recognized, namely in determined muscle cells. Moreover, we demonstrate that in the absence of terminal differentiation muscle progenitor cells aberrantly induce p57 expression, leading to growth arrest. We further show that this growth arrest is associated with a loss of Notch signaling. This is confirmed by conditional genetic ablation of *Rbpj* that leads to upregulation of p21 and p57 in muscle progenitors associated with increased growth arrest. We finally identify a muscle-specific p57 regulatory element and show that this enhancer is the target of both positive regulation by MRFs in myoblasts and negative regulation by Hes/Hey repressors downstream of Notch in progenitor cells. Our data therefore demonstrate that the regulation of cell cycle exit integrates both negative (via Hes/Hey downstream of Notch signaling) and positive (by MRFs) regulation at the same p57 regulatory element during muscle differentiation, and that Notch signaling acts upstream, but independently, of both differentiation and cell growth arrest.

## RESULTS

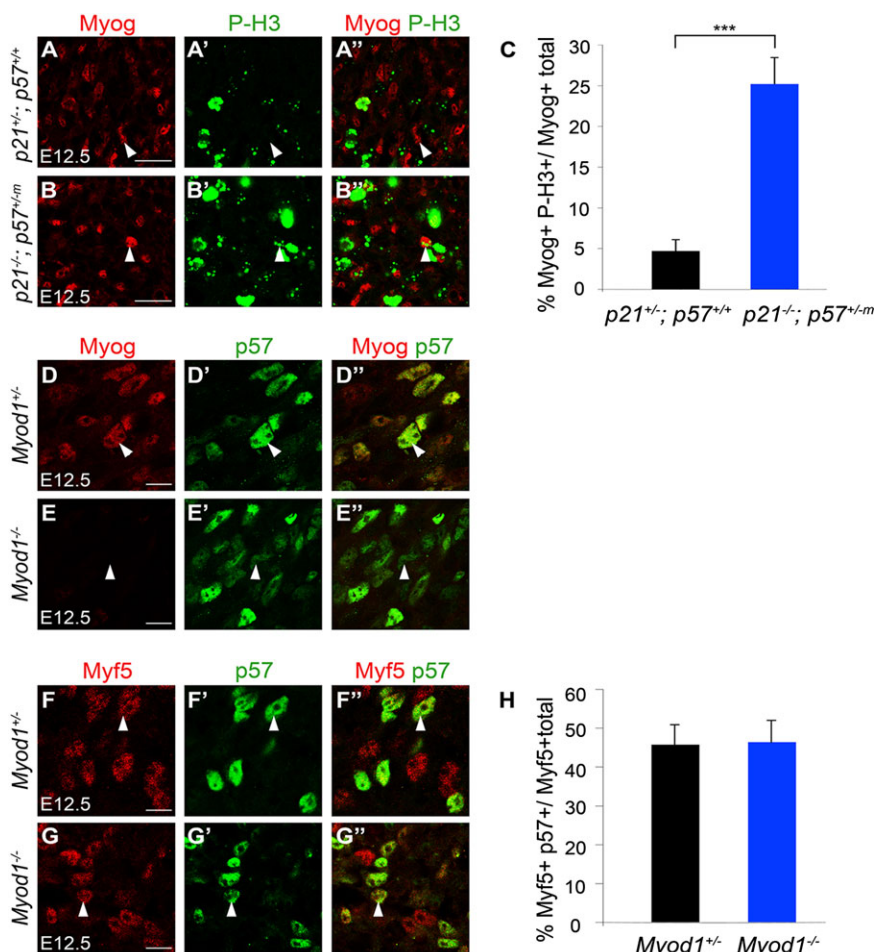
### Cell cycle exit and differentiation can be uncoupled during skeletal muscle development

We first assessed whether myogenic progenitors leave the cell cycle at specific steps of the MRF-mediated differentiation program, by comparing p57 expression with that of MRFs in E11.5 mouse limbs by immunofluorescence (Fig. 1A-A"). As expected, p57 expression was very low in Pax3/7<sup>+</sup> progenitors (7.2 ± 1.7%). By contrast, a proportion of the Pax3/7<sup>+</sup>/Myf5<sup>+</sup> committed progenitor cells did express p57 (16 ± 5%), and this proportion

increased significantly in Myf5<sup>+</sup>/Myod1<sup>+</sup> (52 ± 4.9%) and Myog<sup>+</sup> (69 ± 8.3%) populations (Fig. 1B). Similar results were obtained with p21 (data not shown). We verified that p21 and p57 are accurate markers of cell cycle exit of myogenic progenitors as their expression almost never co-localized with that of Ki67, a marker of cycling cells (Fig. 1C-F). Our data are consistent with the results of previous *in vivo* studies analyzing the proliferation of myogenic cells during development (Gros et al., 2005; Lagha et al., 2008; Relaix et al., 2005).

In order to test the existence of a link coupling cell cycle arrest with muscle differentiation, we first investigated whether muscle differentiation is affected when cell cycle exit is impaired. We examined whether the differentiation program proceeds normally in *p21*: p57 double-null embryos, in which growth arrest is abolished (Zhang et al., 1999). In limb muscles of control mice, 4.7 ± 1.4% of Myog-positive cells underwent proliferation as assessed by phospho-histone H3 (P-H3) (Fig. 2A-A", C). By contrast, *p21*<sup>-/-</sup>; *p57*<sup>+/-m</sup> double-mutant embryos displayed a marked increase in Myog<sup>+</sup>/P-H3<sup>+</sup> cells (25.2 ± 3.2%; Fig. 2B-C). Taken together, we conclude that p21- and p57-mediated cell cycle exit and MRF-mediated myogenic differentiation can occur independently of each other.

We then examined whether the uncoupling of proliferation and differentiation that we observed in the *p21*<sup>-/-</sup>; *p57*<sup>+/-m</sup> double-mutant embryos holds true in a complementary condition. Delayed myogenesis in *Myod1* mutant embryos provides a useful model for such analysis (Kablar et al., 1997). As expected, Myog and p57 co-localized in the forelimbs of control *Myod1*<sup>+/-</sup> mice at E12.5 (Fig. 2D-D"). By contrast, in the E12.5 *Myod1*<sup>-/-</sup> forelimbs, even



**Fig. 2. Cell cycle exit can be uncoupled from cell differentiation.** (A-B") Co-immunostaining for Myog (A, A", B, B", red) and P-H3 (A', A", B', B", green) in *p21*<sup>+/-</sup>; *p57*<sup>+/+</sup> (A-A") or *p21*<sup>-/-</sup>; *p57*<sup>+/-m</sup> (B-B") forelimbs at E12.5. Myog<sup>+</sup> cells (A) do not normally express P-H3 (A', A"), whereas in *p21*<sup>-/-</sup>; *p57*<sup>+/-m</sup> embryos Myog<sup>+</sup> cells aberrantly proliferate (B-B"). (C) Quantification of A", B". (D-G") Co-immunostaining for Myog (D, D", E, E", red), p57 (D', D", E', E", F', F", G', G", green) and Myf5 (F, F", G, G", red) in *Myod1*<sup>+/-</sup> (D-D", F-F") or *Myod1*<sup>-/-</sup> (E-E", G-G") embryonic limb muscles at E12.5. Myog<sup>+</sup> cells express p57 in *Myod1*<sup>+/-</sup> embryos (D-D", arrowheads). p57 is expressed in *Myod1*<sup>-/-</sup> embryos (E') despite the absence of Myog (E). Myf5 is co-expressed with p57 in both *Myod1*<sup>+/-</sup> (F-F", arrowheads) and *Myod1*<sup>-/-</sup> (G-G") embryos. (H) Quantification of F", G". For all experiments *n*=3 embryos for each genotype; error bars indicate s.d.; \*\*\**P*<0.001. Scale bars: 10 μm.



though Myog is not expressed, p57 is detected in the forming muscle masses (Fig. 2E-E''), where it labels nearly half of the Myf5<sup>+</sup> cells in both *Myod1*<sup>+/-</sup> (Fig. 2F-F'',H) and *Myod1*<sup>-/-</sup> (Fig. 2G-H) forelimb (45.6±5.1% versus 46.3±5.5%). These data suggest that cell cycle exit coincides with Myf5 expression in myoblasts and is unaffected when Myod1/Myog-mediated differentiation is impaired.

### In the absence of differentiated myoblasts, muscle progenitors precociously express p57 and exit the cell cycle

It has been previously shown that differentiating myoblasts are required for the survival of muscle progenitor cells throughout development (Kassar-Duchossoy et al., 2005). We examined in more detail the impact of differentiating myoblasts on the proliferation state of Pax3<sup>+</sup> cells by analyzing different allelic combinations of *Myod1*: *Myf5* double-null embryos to allow key steps during myogenic commitment to be separated. In the absence of Myod1<sup>+</sup> myoblasts but in the presence of Myf5<sup>+</sup> myoblasts in *Myod1*<sup>-/-</sup>; *Myf5*<sup>+/-</sup> mice (Rudnicki et al., 1993; Tajbakhsh et al., 1997) (supplementary material Fig. S1), the proliferation rate of Pax3<sup>+</sup> cells was comparable to that observed in control mice at E12.5 (23.6±3.9% versus 25.6±4.6%; Fig. 3A-B'',D). By contrast, in the double-mutant *Myod1*<sup>-/-</sup>; *Myf5*<sup>nlacZ/nlacZ</sup> forelimbs, which lack both committed progenitors and myoblasts (supplementary material Fig. S1), we observed a significant decrease in the proliferation of Pax3<sup>+</sup> cells (12.8±3.6% versus 25.6±4.6%; Fig. 3C-D). These data suggest that committed progenitors are required to maintain the proliferation of muscle progenitor cells, whereas differentiated myoblasts are dispensable.

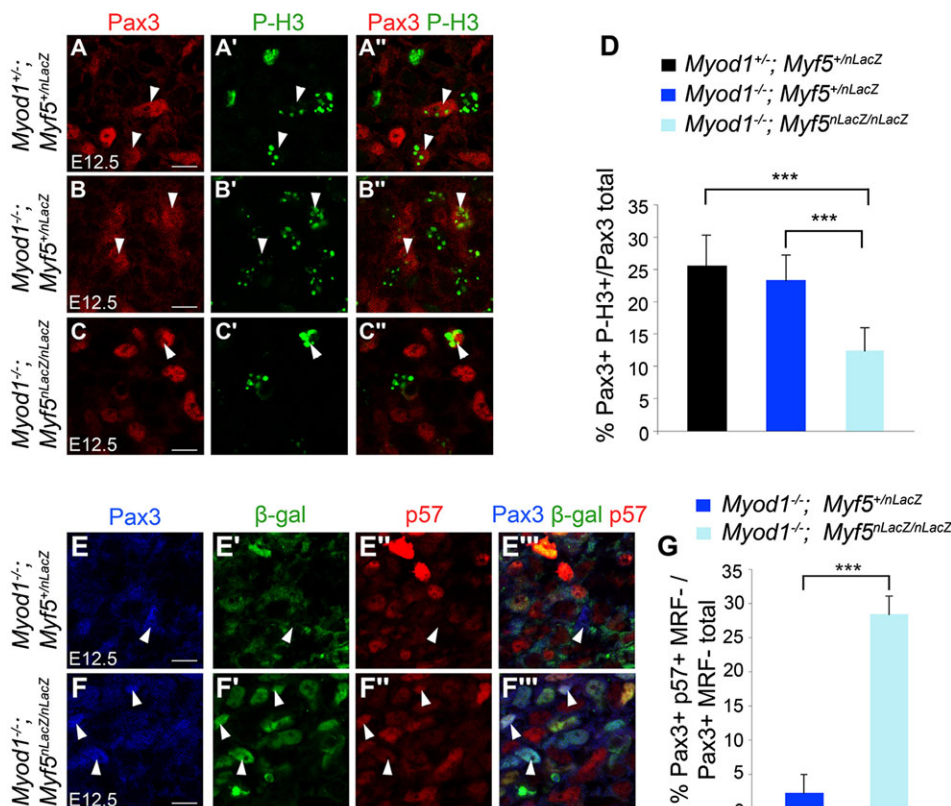
Consistent with the proliferation profile, the cell cycle inhibitor p57 was aberrantly expressed in Pax3<sup>+</sup>/MRF<sup>-</sup> progenitor cells of *Myod1*<sup>-/-</sup>; *Myf5*<sup>nlacZ/nlacZ</sup> embryos compared with control embryos (28.4±2.7% versus 2.3±2.7%; Fig. 3E-G). These data suggested that myoblasts are required to maintain cycling muscle progenitor cells by preventing p57 expression and cell cycle arrest.

### Impaired Notch signaling in *Myod1*; *Myf5* mutant embryos

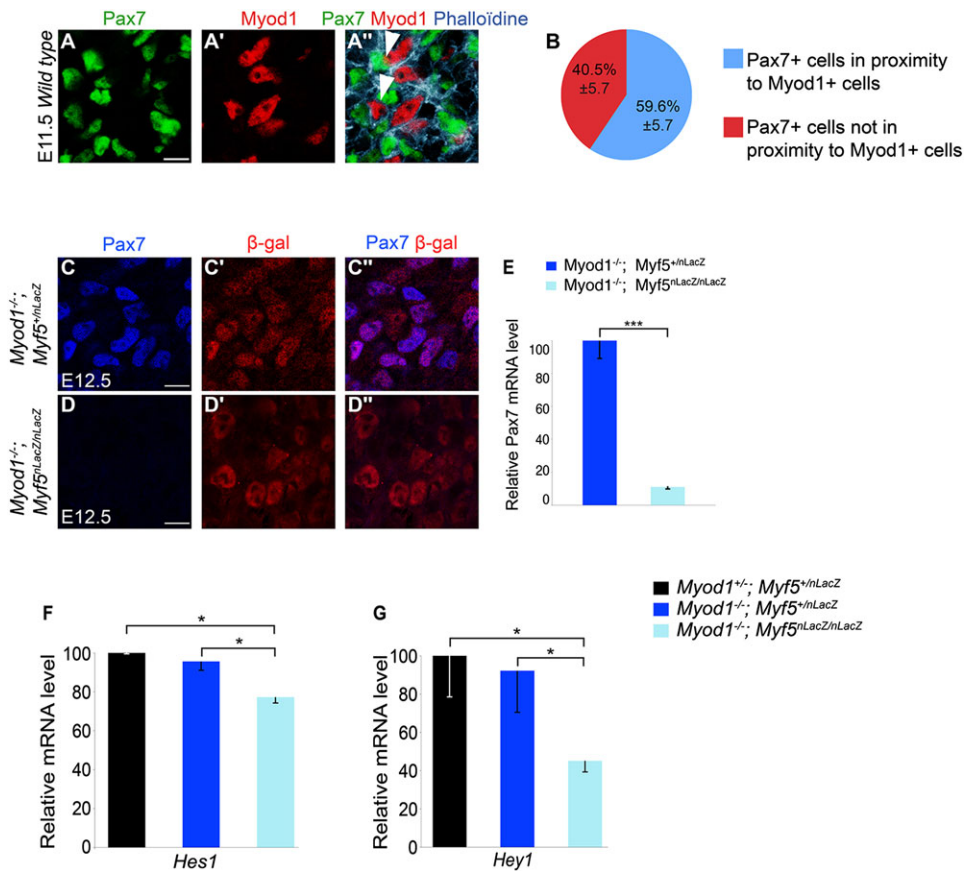
Our analysis of *Myod1*; *Myf5* mutant embryos reinforced the notion that functional interactions are taking place between myoblasts and muscle progenitor cells. A strong candidate pathway to mediate these interactions is Notch signaling. It has been previously shown that differentiating myogenic cells express Dll1 and possibly signal to the upstream population that expresses higher levels of Notch receptors (mainly notch 1, 2 and 3) (Delfini et al., 2000; Hirsinger et al., 2001; Mourikis et al., 2012b; Schuster-Gossler et al., 2007). This feedback mechanism of receptor/ligand regulation is supported by many independent *in vivo* studies. However, it has not been formally shown that such cell-cell interactions occur during development, a prerequisite for Notch signaling.

To demonstrate an interaction between myoblasts and muscle progenitor cells, we analyzed the cellular organization on sections of embryonic forelimb muscle masses by co-immunostaining, and found that the majority of Pax3<sup>+</sup> progenitor cells are in close proximity to Myod1<sup>+</sup> myoblasts (Fig. 4A-B). Our analysis therefore suggests that direct cell-cell signaling via Notch can occur between progenitors and myoblasts.

To further assess the significance of differentiating muscle cells in Notch activation, we measured endogenous pathway activity in E12.5 *Myod1*; *Myf5* double-mutant embryos that lack differentiated muscle due to the MRF deficiency. It was previously shown that Pax7 expression is lost when Notch signaling is abrogated in myogenic progenitor cells (Vasyutina et al., 2007). Consistent with impaired Notch activity, Pax7 protein was undetectable by immunofluorescence at E12.5 in *Myod1*<sup>-/-</sup>; *Myf5*<sup>nlacZ/nlacZ</sup> forelimbs (Fig. 4C-E), whereas it was expressed in *Myod1*<sup>-/-</sup>; *Myf5*<sup>+/-</sup> embryos (Fig. 4C-E). In addition, we found downregulation of the Notch target genes *Hes1* and *Hey1* in the forelimbs of *Myod1*<sup>-/-</sup>; *Myf5*<sup>nlacZ/nlacZ</sup> compared with *Myod1*<sup>+/-</sup>; *Myf5*<sup>+/-</sup> or with *Myod1*<sup>-/-</sup>; *Myf5*<sup>+/-</sup> at E12.5 (Fig. 4F,G).



**Fig. 3. Myoblasts control muscle progenitor cell proliferation by preventing cell cycle exit.** (A-C'') Co-immunostaining for Pax3 (red) and P-H3 (green) in *Myod1*<sup>+/-</sup>; *Myf5*<sup>+/nlacZ</sup> (A-A''), *Myod1*<sup>-/-</sup>; *Myf5*<sup>+/nlacZ</sup> (B-B'') and *Myod1*<sup>-/-</sup>; *Myf5*<sup>nlacZ/nlacZ</sup> (C-C'') embryos at E12.5. Arrowheads indicate Pax3<sup>+</sup> cells undergoing mitosis. (D) Quantification of A'', B'', C''. (E-F'') Co-immunostaining for Pax3 (blue),  $\beta$ -gal (green) and p57 (red) in *Myod1*<sup>-/-</sup>; *Myf5*<sup>+/nlacZ</sup> (E-E'') or *Myod1*<sup>-/-</sup>; *Myf5*<sup>nlacZ/nlacZ</sup> (F-F'') embryos at E12.5. *Myf5*<sup>-</sup>/ $\beta$ -gal<sup>-</sup> cells do not express p57 (arrowheads in E-E'') in *Myod1*<sup>-/-</sup>; *Myf5*<sup>+/nlacZ</sup> embryos, whereas in *Myod1*<sup>-/-</sup>; *Myf5*<sup>nlacZ/nlacZ</sup> embryos Pax3<sup>+</sup> cells are p57<sup>+</sup> (arrowheads in F-F''). (G) Quantification of E'', F''. For all experiments  $n=3$  embryos for each genotype; error bars indicate s.d.; \*\*\* $P<0.001$ . Scale bars: 10  $\mu$ m.



**Fig. 4. Close proximity of Pax7<sup>+</sup> and Myod1<sup>+</sup> cells, with decreased Pax7 and Hes1/Hey1 expression in muscle progenitor cells of the Myod1; Myf5 double mutant.** (A-A'') Co-immunostaining for Pax7 (green) and Myod1 (red), with phalloidin (cyan) to label actin to visualize cell membranes, in wild-type limb muscles at E11.5. (B) Percentage of Pax7<sup>+</sup> cells in proximity to Myod1<sup>+</sup> cells in limb muscle masses. (C-D'') Co-immunostaining for Pax7 (blue) and β-gal (red) in Myod1<sup>-/-</sup>; Myf5<sup>+/nLacZ</sup> (C-C'') and Myod1<sup>-/-</sup>; Myf5<sup>nLacZ/nLacZ</sup> (D-D'') embryos at E12.5. (E-G) qRT-PCR for Pax7 (E), Hes1 (F) and Hey1 (G) on E12.5 forelimbs of the genotypes indicated. For all experiments  $n=3$  embryos for each genotype; error bars indicate s.e.m.; \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . Scale bars: 10  $\mu\text{m}$ .

### Notch signaling prevents activation of p57 in muscle progenitor cells

Based on our results (Fig. 4) and previous reports (Georgia et al., 2006), we hypothesized that myoblasts control progenitor cell proliferation by activating the Notch/Hes1/Hey1 pathway, which would then repress p57 expression.

First, to establish whether Notch signaling participates directly in the coordinated control of cell cycle exit and differentiation, we used an *ex vivo* whole limb culture system (Zúñiga et al., 1999). We cultured E11.5 mouse forelimbs for 28 h, with or without 20  $\mu\text{M}$   $\gamma$ -secretase inhibitor DAPT, an inhibitor of Notch signaling. As expected, we saw decreased expression of the Notch target genes *Hes1* and *Hey1* after DAPT treatment (Fig. 5A). In addition, inhibition of Notch signaling led to reduced numbers of Pax7<sup>+</sup> cells ( $56.8 \pm 5.6\%$  in control versus  $27.7 \pm 7.0\%$  in DAPT-treated limb explants; Fig. 5B',C',D), whereas the Myod1<sup>+</sup> cell population was increased ( $62.7 \pm 9.0\%$  compared with  $32.6 \pm 5.3\%$  in control DMSO-treated explants; Fig. 5B'',C'',D), confirming previous reports (Schuster-Gossler et al., 2007; Vasyutina et al., 2007) and the robustness of our *ex vivo* model. Accordingly, we found decreased levels of Pax7 mRNA and increased levels of Myod1 mRNA in DAPT-treated samples (Fig. 5A). We next examined whether pharmacological inhibition of Notch signaling induces cell cycle arrest in cultured muscle progenitor cells. We found a 5-fold increase in p57 expression in Pax3<sup>+</sup>/MRF<sup>-</sup> cells in DAPT-treated limb explants compared with controls (Fig. 5E-G).

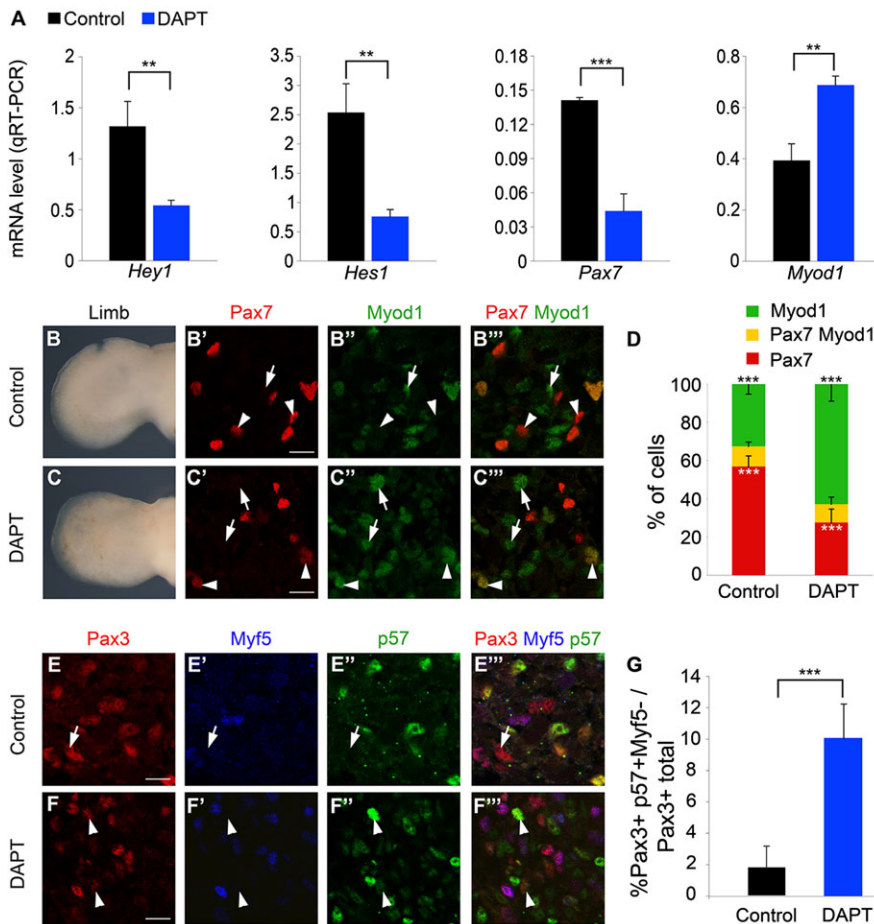
To confirm these results *in vivo*, we genetically abrogated Notch signaling in progenitor cells by conditionally deleting *Rbpj*. RbpJ is a DNA-binding transcription factor and the major effector of all four Notch receptors (Fortini and Artavanis-Tsakonas, 1994; Jarriault et al., 1995; Kopan and Ilagan, 2009; Schweisguth and Posakony, 1992).

We performed a conditional deletion of *Rbpj* in the Pax3 lineage by crossing *Rbpj*<sup>flox/flox</sup> mice (Han et al., 2002) with a *Pax3*<sup>Cre/+</sup> allele (Engleka et al., 2005). Ablation of *Rbpj* led to increased myogenic differentiation as previously reported (Vasyutina et al., 2007), with a severe loss of progenitor cells leading to tiny limb muscles at a fetal stage. Strikingly, both p57 and p21 were upregulated in the Pax3<sup>+</sup>/Myf5<sup>-</sup> muscle progenitor cells in the forelimbs of *Rbpj*<sup>flox/flox</sup>; *Pax3*<sup>Cre/+</sup> mice at E11.5, whereas Pax3 and these CDKIs were rarely co-expressed in such cells in control mice (Fig. 6A-D, see also Fig. 1). To demonstrate that expression of p21 and p57 is associated with growth arrest in these mutants, we analyzed the co-expression of Ki67 with either p57 or p21 in Pax3<sup>+</sup> muscle progenitors (Fig. 6E,F) in the forelimbs of *Rbpj*<sup>flox/flox</sup>; *Pax3*<sup>Cre/+</sup> mice at E11.5. We found a small but significant increase of Pax3<sup>+</sup> cells co-expressing p21 or p57 with Ki67 in the mutant embryos; nevertheless, the large majority of the Pax3<sup>+</sup>/p57<sup>+</sup> cells did not express Ki67, as predicted.

Altogether, these results demonstrate that in embryonic muscle progenitor cells Notch signaling antagonizes cell cycle exit by repressing p57 expression.

### A p57 muscle-specific enhancer is directly regulated by Notch signaling and MRFs

To gain insight into the molecular mechanisms of p57 regulation, we used data generated by a Myod1 ChIP sequencing experiment (Cao et al., 2010) to identify Myod1 binding sites in the vicinity of the p57 locus. A previous study had predicted that p57 muscle-specific regulatory elements are located between +35 and +225 kb from the p57 transcription start site (John et al., 2001). In keeping with this, a high density of Myod1 binding sites was found in a conserved region located +59 kb from p57. We isolated an evolutionarily conserved



**Fig. 5. The Notch pathway prevents activation of p57 in progenitor cells.** (A) qRT-PCR for *Hey1*, *Hes1*, *Pax7* and *Myod1* mRNA in control (DMSO-treated) and DAPT-treated *ex vivo* whole limb culture. (B, C) An E11.5 forelimb kept in culture for 28 h treated with DMSO (B) or 20  $\mu$ M DAPT (C). (B'-B''', C'-C''') Co-immunostaining for Pax7 (red) and Myod1 (green) in DMSO-treated (B'-B''') or 20  $\mu$ M DAPT-treated (C'-C''') explants from E11.5 limb muscles. Arrowheads indicate Pax7<sup>+</sup>/Myod1<sup>-</sup> cells in B'-B''' and Pax7<sup>+</sup>/Myod1<sup>+</sup> cells in C'-C'''; arrows indicate Myod1<sup>+</sup>/Pax7<sup>-</sup> cells. (D) Quantification of B''', C'''. (E-F''') Co-immunostaining for Pax3 (red), Myf5 (blue) and p57 (green) in DMSO-treated (E-E''') or 20  $\mu$ M DAPT-treated (F-F''') explants from E11.5 limb muscles. Arrow in E-E''' indicates a Pax3<sup>+</sup>/Myf5<sup>-</sup>/p57<sup>+</sup> cell. Arrowheads in F-F''' indicate Pax3<sup>+</sup>/Myf5<sup>-</sup>/p57<sup>+</sup> cells. (G) Quantification of E''', F'''. For all experiments  $n=3$ ; error bars indicate s.d.; \*\* $P<0.01$ , \*\*\* $P<0.001$ . Scale bars: 10  $\mu$ m.

686 bp fragment that contains 15 E-boxes, which are binding sites for MRFs, Hey1 and Hes1 (supplementary material Fig. S2).

We first validated this *p57* muscle regulatory element (*p57MRE*) as a functional enhancer *in vivo* by generating transgenic embryos carrying a *p57MRE-tk-nlacZ* construct. Following analysis of *lacZ* expression at E12, we detected robust reporter expression in all myogenic domains (Fig. 7A,A'), with an expression profile that matched that of Myod1. Interestingly, this element is skeletal muscle specific, since no other sites of p57 expression, such as parenchymal organs and intestine (Westbury et al., 2001), were observed. In order to characterize the myogenic cell type that expresses the p57 reporter, we performed immunohistochemical analyses on limb buds from these transgenic embryos.  $\beta$ -Gal<sup>+</sup> cells co-expressed p57 (Fig. 7B-B') and Myod1 (Fig. 7C-C') but not Pax7 (Fig. 7D-D'), defining the cellular specificity of the *p57MRE*.

We next hypothesized that this regulatory element integrates negative regulation by Hes/Hey proteins and positive regulation via direct activation by the MRFs. We performed ChIP experiments on E12.5 wild-type forelimbs and found that both Myod1 and Hes1 were bound *in vivo* to the *p57MRE* fragment (Fig. 8A). To ensure that our assay was specific, and given the lack of known positive controls for Hes1 in the myogenic lineage, we performed ChIP experiments in HEK293 cells transfected with either Hes1 or Myod1 and either wild-type *p57MRE* or containing mutations in the MRF and Hes binding sites (*p57MRE $\Delta$ E-Boxes*). Robust binding was observed for Hes1 (Fig. 8B) and Myod1 (Fig. 8C) on the *p57MRE* and this binding was abrogated on *p57MRE $\Delta$ E-Boxes* (Fig. 8B,C).

Finally, to further establish this interplay between positive and negative regulation, we tested the transcriptional activity of Myod1,

Hes1 and Hey1 on *p57MRE-tk-nlacZ* in transient transfection experiments in C2C12 muscle cells. Myod1 enhanced the activation of the *p57MRE* (Fig. 8D), but was not able to activate the *p57MRE $\Delta$ E-Boxes* element. Furthermore, Myod1 transcriptional activation was abolished when exposed to increasing concentrations of Hes1 or Hey1 (Fig. 8D), suggesting that both are able to repress the Myod1-dependent activation of *p57MRE*.

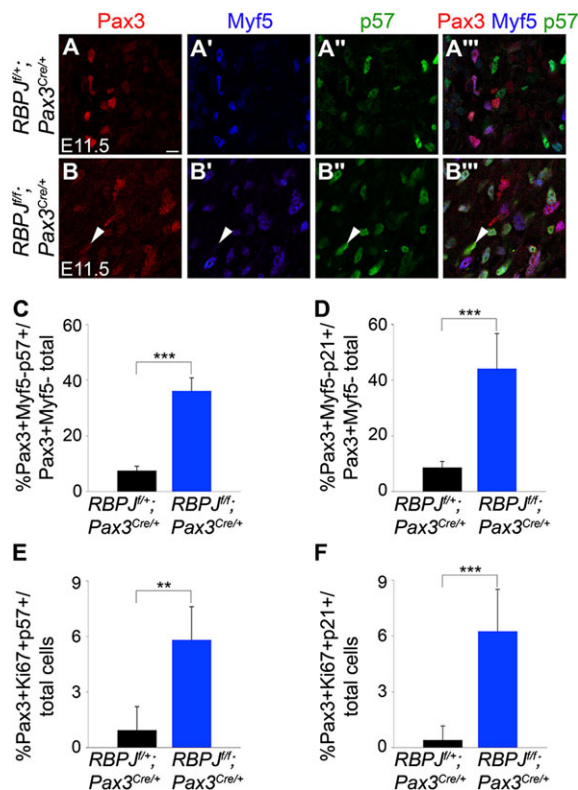
We propose a model in which the integration of Notch and MRF activities at the level of a muscle-specific enhancer of the key cell cycle arrest gene *p57* provides a means to control the equilibrium between progenitor pool amplification and the establishment of definitive functions of skeletal muscle (Fig. 8E).

## DISCUSSION

The generation of organs of a defined size requires a balance between proliferation and differentiation. This balance is ensured by regulated cell growth, which prevents prolonged proliferation or premature differentiation, both of which are deleterious for normal development.

During skeletal muscle development and postnatal regeneration, Notch signaling activity is crucial for sustaining stem/progenitor cell self-renewal and its downregulation is required to allow myogenic differentiation. Cell cycle exit was previously thought to be controlled by the differentiation program (Halevy et al., 1995). In this report we show that growth arrest is also negatively regulated by Notch signaling and demonstrate that these two events, despite appearing synchronous, can be uncoupled. In *Myod1*<sup>-/-</sup> forelimbs, myogenesis is paused between E11.5 and E14.5 (Kablar et al., 1998). Although Myf5 is unable to drive myogenesis and activate *Myog* at these stages,

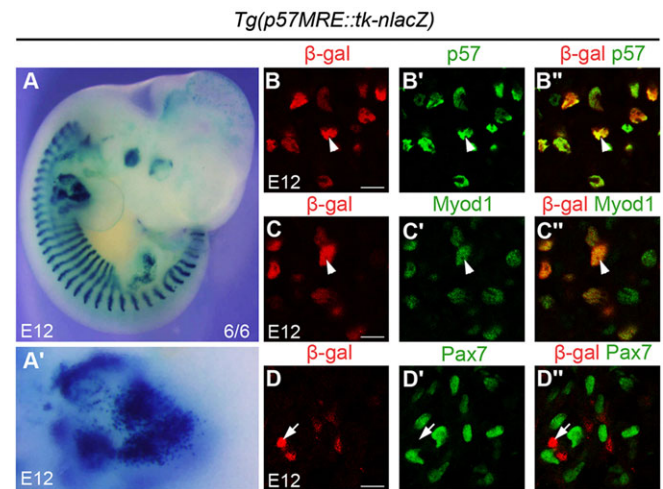




**Fig. 6. Conditional ablation of *Rbpj* leads to upregulation of p57 and p21 and to cell cycle arrest in muscle progenitor cells.** (A–B''') Co-immunostaining for Pax3 (red), Myf5 (blue) or p57 (green) in *Rbpj*<sup>flox/+</sup>; *Pax3*<sup>Cre/+</sup> (A–A''') or *Rbpj*<sup>flox/flox</sup>; *Pax3*<sup>Cre/+</sup> (B–B''') forelimbs at E11.5. Arrowhead indicates a Pax3<sup>+</sup>/Myf5<sup>+</sup>/p57<sup>+</sup> cell. Scale bars: 10  $\mu$ m. (C) Quantification of A''', B'''. (D) Quantification of co-immunostaining for Pax3, Myf5 or p21 in *Rbpj*<sup>flox/+</sup>; *Pax3*<sup>Cre/+</sup> or *Rbpj*<sup>flox/flox</sup>; *Pax3*<sup>Cre/+</sup> forelimbs at E11.5. (E) Quantification of co-immunostaining for Pax3, Ki67 or p57 in *Rbpj*<sup>flox/+</sup>; *Pax3*<sup>Cre/+</sup> or *Rbpj*<sup>flox/flox</sup>; *Pax3*<sup>Cre/+</sup> forelimbs at E11.5. (F) Quantification of co-immunostaining for Pax3, Ki67 or p21 in *Rbpj*<sup>flox/+</sup>; *Pax3*<sup>Cre/+</sup> or *Rbpj*<sup>flox/flox</sup>; *Pax3*<sup>Cre/+</sup> forelimbs at E11.5. For all experiments  $n=3$  embryos for each genotype; error bars indicate s.d., \*\* $P<0.01$ , \*\*\* $P<0.001$ .

we found that Myf5<sup>+</sup>/Pax3/7<sup>+</sup> cells expressed p57 at E12.5 and this did not prevent them from resuming differentiation at E14.5 (presumably when Mrf4 is activated). Given our finding that Myod1 directly binds and activates *p57* via the *p57MRE* sequence, we believe that Myf5 operates in the same way, thereby providing a functional uncoupling between MRF myogenic activity and growth arrest. Moreover, our study and those of others indicate that cell cycle exit occurs at the transition from committed progenitors to determined myoblasts (Fig. 1A). Consistently, we found that committed progenitor cells express Pax3/7 and Myf5, but neither p21 nor p57. This finding is consistent with the robust repressive activity exerted by Hes/Hey on MRF-mediated transactivation (Fig. 8D). The cycling status of committed progenitor cells is therefore of interest. A recent study showed that whereas the undifferentiated resident progenitor cells that express Pax7 represent a slow-cycling pool, the Pax3/7<sup>+</sup>/Myf5<sup>+</sup> committed progenitors correspond to a fast-cycling population (Picard and Marcelle, 2013). Our study did not address the subtle cell cycle regulation of these progenitor cell populations and future studies will be required to determine whether these changes in cell proliferation are linked to Myf5 or to other, as yet unidentified, factors.

The model of coordinated regulation that we propose, with a single *p57* element integrating positive (from the MRFs) and negative (from Hes/Hey) regulatory information suggests that the interplay



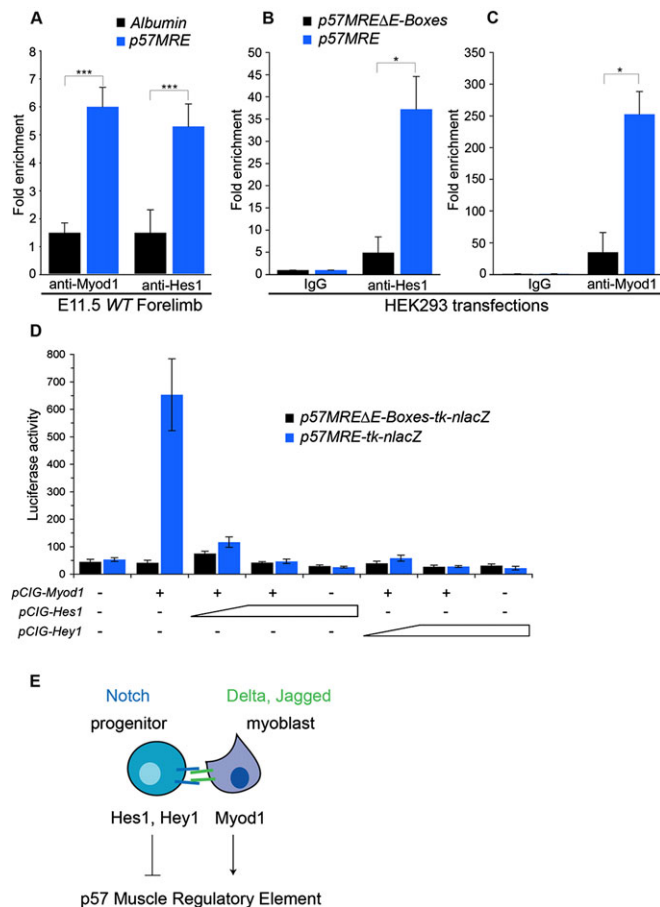
**Fig. 7. Expression of a *p57* muscle regulatory enhancer (MRE) in transgenic mice.** (A, A') X-Gal staining on a transgenic *p57MRE-tk-nlacZ* embryo. A' is a higher magnification of the forelimb region from A. (B–D''') Co-immunostaining for  $\beta$ -gal (B, C, D, B'', C'', D'', red), p57 (B', B'', green), Myod1 (C', C'', green) and Pax7 (D', D'', green). Arrowheads indicate  $\beta$ -gal<sup>+</sup>/p57<sup>+</sup> (B–B'') and  $\beta$ -gal<sup>+</sup>/Myod1<sup>+</sup> cells (C–C''); arrows indicate  $\beta$ -gal<sup>+</sup>/Pax7<sup>−</sup> cells. Scale bars: 10  $\mu$ m.

between Notch repression of *p57MRE* in Pax3/7 progenitors and its activation by MRFs in myoblasts is crucial for growth arrest. The molecular mechanisms regulating Notch signaling components during myogenesis are not fully characterized. It was reported that during *Xenopus* development Dll1 expression is regulated by Myod1 (Wittenberger et al., 1999) and that Myod1 expression is repressed by Hairy-1 (Umbhauer et al., 2001). It is unclear if these regulatory mechanisms also exist in amniotes, but our data are compatible with such a sequence of events. Resolving the precise molecular interplay between Pax gene expression, cell growth arrest, MRF regulation and the switch in Notch signaling will require additional investigations.

Notch signaling plays a key role in maintaining the homeostasis of muscle stem cells in the adult (Bjornson et al., 2012; Carlson et al., 2008; Fukada et al., 2011; Kitamoto and Hanaoka, 2010; Mourikis et al., 2012b) and in colonization of the satellite cell niche (Bröhl et al., 2012). In particular, Notch controls quiescence of muscle satellite cells (Bjornson et al., 2012; Mourikis et al., 2012b). This activity might be mediated by Hey1 and HeyL, which are required in the adult lineage for satellite cell homeostasis and skeletal muscle regeneration (Fukada et al., 2011). Conditional deletion of *Rbpj* in Pax7<sup>+</sup> satellite cells led to spontaneous differentiation without activation or division of the cells (Bjornson et al., 2012; Mourikis et al., 2012b). Strikingly, *Rbpj* ablation does not lead to an immediate and complete differentiation or growth arrest in the Pax3<sup>+</sup> population during embryonic development, leaving open the possibility that other pathways are involved. For instance, Notch activity on adult muscle stem cells is counteracted by TGF $\beta$  signaling (Carlson and Conboy, 2007). This is mediated through the activation of phosphorylated Smad3, which can directly bind and activate the *p15* (*Cdkn2b*), *p16* (*Cdkn2a*), *p21* and *p27* promoters (Carlson and Conboy, 2007) to favor muscle stem cell differentiation. Interestingly, during chicken myogenesis myostatin, which is a member of the TGF $\beta$  family, has also been implicated in the control of terminal differentiation through indirect activation of p21 (Manceau et al., 2008).

In addition to driving cell cycle exit during adult myogenesis, p57 has also been implicated in stabilization of Myod1 through direct association in C2C12 cells, resulting in enhanced myogenesis





**Fig. 8. Direct regulation of the *p57MRE* by *Myod1* and *Hes1/Hey1*.**

(A) Chromatin immunoprecipitation followed by qPCR on wild-type forelimbs at E11.5. *p57MRE* is enriched when precipitated with anti-Myod1 or anti-Hes1 antibodies compared with an albumin gene control. (B,C) Validation of antibody ChIP capacities on transfected HEK293 cells: enrichment with anti-Hes1 (B) or anti-Myod1 (C) is obtained with the *p57MRE* compared with the construct in which all putative E-boxes have been mutated (*p57MREΔE-Boxes*).  $n=3$ ; error bars indicate s.e.m.; \* $P<0.05$ , \*\*\* $P<0.001$ . (D) Transactivation assay on C2C12 cells with the expression plasmids and reporters indicated ( $n\geq 3$ ). (E) Schematic representation of the regulation of cell cycle exit during myogenesis. In muscle progenitors, Notch downstream effectors *Hes1* and *Hey1* repress the activation of *p57* to allow the amplification of the pool, while in the neighboring myoblasts that express the Notch ligands, *Myod1* directly activates *p57* expression.

(Reynaud et al., 2000). A similar mechanism has also been identified in zebrafish, in which *p57* cooperates with *Myod1* to drive the differentiation of several early zebrafish muscle fiber types (Osborn et al., 2010). It is not known if this positive-feedback loop also operates during early murine skeletal muscle formation. One could propose that, although the initiation of myogenic differentiation and growth arrest are independent, these events may synergize subsequently, for instance to enhance *Myod1* activity and reinforce terminal differentiation. In zebrafish, *p57* cooperates with *Myod1* to drive *myog* expression (Osborn et al., 2010); nevertheless, proliferating *Myod1*<sup>+</sup> and *Myog*<sup>+</sup> cells are detected in *p21*<sup>-/-</sup>; *p57*<sup>+/-</sup> mice (see Fig. 2A–C; our unpublished observations). Interestingly, expression of *Mef2c* is impaired in these mutant mice (Zhang et al., 1999), raising the possibility that *p57* may also be involved in terminal differentiation in murine myogenesis during development.

In our study, the expression of *p57* is firmly linked to an absence of cell cycle progression, since we observe no overlap between *p57*

(or *p21*) expression and Ki67 (Fig. 1C–F) under normal conditions. Strikingly, a small but significant proportion of the *Pax3*<sup>+/+</sup>/*p21*<sup>+/+</sup> or *Pax3*<sup>+/+</sup>/*p57*<sup>+/+</sup> cells are Ki67<sup>+</sup> in the *Pax3*<sup>Cre/+</sup>; *Rbpj*<sup>flax/flax</sup> mutant context. Although this might correspond to a transitory state due to the differentiation phenotype of these mutant embryos, one cannot exclude the possibility that Notch might also be involved in both cell cycle progression and cell cycle arrest via a complex regulatory loop.

*p57* expression has been reported previously in adult satellite cells (Fukada et al., 2007), but the precise timing of expression has yet to be characterized. The identification of *p57MRE* through a *Myod1* ChIP-seq screen performed in C2C12 cells raises the possibility that this element is reused in adult muscle cells *in vivo*. Owing to the perinatal death of *p57* mutant mice, the role of *p57* in postnatal myogenesis cannot be studied *in vivo*. *p21*-deficient mice display normal muscle development but impaired skeletal muscle regeneration (Hawke et al., 2003). Given the functional overlap between *p21* and *p57* during development, it would be interesting to evaluate the combined role of these two proteins in postnatal satellite cell homeostasis and skeletal muscle regeneration.

The recent identification of the role of *p57* in the maintenance of quiescent hematopoietic (Matsumoto et al., 2011), neural (Furutachi et al., 2013) and lung (Zacharek et al., 2011) stem cells indicates that *p57*, along with other CDKIs, is important for stem cell function. Whether such a regulatory mechanism for CDKI expression is redeployed in other systems remains to be investigated. For example, Notch has been implicated in maintaining progenitor cell proliferation in intestinal stem cells (Riccio et al., 2008), in adult neural stem cells (Imayoshi et al., 2010) and in Rathke's pouch progenitors of the pituitary (Monahan et al., 2009) and, indeed, one proposed mechanism is the repression of CDKIs by the product of the Notch target gene *Hes1* (Monahan et al., 2009; Riccio et al., 2008). Unfortunately, these studies did not define which cells provide the ligands. Nevertheless, our data and the role of Notch and *Hes1* in intestinal stem cells, neural stem cells and pituitary progenitor cells might suggest a general mechanism whereby the expansion of the progenitor cell population is regulated via modulation of CDKI genes. Such a regulatory mechanism could be used as a safeguard to prevent tumor formation by progenitor/stem cells, for instance when differentiation is impaired. It is also tempting to speculate that fine-tuning of this system could also be used for intrinsically regulating organ size.

## MATERIALS AND METHODS

### Mouse lines and harvest of embryos

*Myf5*<sup>+/-</sup>/*nlacZ*, *Myod1*<sup>+/-</sup>, *p21*<sup>+/-</sup>, *p57*<sup>+/-</sup>/*m* (*p57* is an imprinted gene; we indicate maternal origin of the allele by a superscript *m*), *Pax3*<sup>Cre/+</sup> and *Rbpj*<sup>flax/+</sup> lines have been described previously (Deng et al., 1995; Engleka et al., 2005; Han et al., 2002; Rudnicki et al., 1992; Yan et al., 1997). For explant and ChIP experiments, C57BL/6J embryos were used (Janvier). For timed pregnancies, the morning when a vaginal plug was found was defined as embryonic day (E) 0.5. All experiments were performed on three independent embryos for each genotype.

### Immunohistochemistry and X-Gal staining

Embryos and forelimbs were harvested and fixed for 2 h and for 20 min, respectively, in PBS/4% paraformaldehyde at 4°C. Cryoprotection was performed by equilibration in PBS/15% sucrose overnight at 4°C. Frozen sections were permeabilized in PBS/0.1% Triton X-100, blocked in PBS/2% bovine serum albumin for 1 h at room temperature, then immunolabeled with primary antibodies overnight at 4°C. For X-Gal staining, embryos were collected in PBS, fixed 20 min in PBS/4% paraformaldehyde at room temperature and incubated in X-Gal solution (Life Technologies) overnight at 37°C on a rotary shaker.

## Antibodies

The following antibodies were used: mouse anti- $\beta$ -galactosidase 1/500 (Promega, Z378), mouse anti-Myod1 5.8A 1/200 (DAKO, M3512), mouse anti-Myog F5D 1/200 (DSHB, F5D), mouse anti-p21 1/100 (BD Pharmingen, 556431), mouse anti-p57 1/100 (Santa Cruz, sc-56341), mouse anti-Pax7-c 1/100 (DSHB, Pax7-c), mouse anti-Pax3-c 1/100 (DSHB, Pax3-c), rabbit anti- $\beta$ -galactosidase 1/1000 (Life Technologies, A-11132), rabbit anti-Myod1 M318 1/100 (Santa Cruz, sc-760), rabbit anti-Myf5 C20 1/500 (Santa Cruz, sc-302), rabbit anti-p57 H91 1/100 (Santa Cruz, sc-8298), rabbit anti-phospho-histone 3 Ser10 1/1000 (Cell Signaling, 9701), goat anti-p57 M20 1/50 (Santa Cruz, sc-1039) and goat anti-Pax3 1/100 (Santa Cruz, sc-34916). Phalloidin (649 nm) 1/500 was from Life Technologies. Secondary antibodies were coupled to Alexa Fluor 488 1/250, 594 1/1000 (Life Technologies) or 649 1/250 (Jackson ImmunoResearch).

## Explant and cell culture

Forelimbs from E11.5 wild-type embryos were cultured in 12-well plates in BGJb medium (Life Technologies), without serum, with 200  $\mu$ g/ml ascorbic acid (Sigma) and 100  $\mu$ g/ml penicillin/streptomycin (Life Technologies). For Notch inhibition, forelimbs were immediately treated with 20  $\mu$ M N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Sigma) or DMSO carrier (Sigma) for 28 h. Treated and control forelimbs originating from the same embryo were compared in each experiment. C2C12 and HEK293 cells were cultured in proliferating medium comprising DMEM with 10% fetal bovine serum and 100  $\mu$ g/ml penicillin/streptomycin (Life Technologies).

## Plasmid construct for transgenesis

The *p57* muscle regulatory element (*p57MRE*) (chr7: 150,587,238–150,587,924) was isolated by PCR. For cloning convenience, *EagI* restriction sites were added to the forward and reverse primers used for amplification: forward, 5'-AAGCGGCCGACCCAGTTTGGCCAGTGTAG-3'; reverse, 5'-AACGGCCGCGAGTAAAGACACCCAGAA-3'. After *EagI* digestion, the 686 bp fragment was cloned, respecting its genomic orientation, into the *NotI* site of *ptknlacZ*(-) plasmid (Hadchouel et al., 2000) (*tk*, thymidine kinase). The *p57MRE-tk-nlacZ* fragment was released by *SacII/XhoI* digestion and gel purified using the Nucleobond plasmid purification kit (Macherey-Nagel) before injection into pronuclei.

## $\beta$ -galactosidase assay

*Hey1*, *Hes1* cDNAs [gifts from S. Tajbakhsh (Pasteur Institute, Paris, France) and R. Kageyama (Institute for Virus Research, Kyoto University, Japan), respectively] and *Myod1* cDNA were cloned into the pCIG plasmid (Megason and McMahon, 2002). C2C12 cells were transfected with a total of 1.2  $\mu$ g DNA using Lipofectamine LTX plus reagent (Life Technologies). Fixed concentrations of *p57MRE-tk-nlacZ* or *p57MRE $\Delta$ E-Boxes-tk-nlacZ* (0.6  $\mu$ g), or pCIG-Myod1 (0.15  $\mu$ g) were used. For pCIG-Hes1 and pCIG-Hey1, 0.15 or 0.3  $\mu$ g was used. Each sample was co-transfected with 0.1  $\mu$ g *tk*-Luciferase reporter for sample-to-sample normalization. Forty-eight hours after transfection, the cells were collected and the proteins were extracted and assayed for  $\beta$ -galactosidase activity ( $\beta$ -Gal assay Kit K1455-01, Life Technologies) and for luciferase activity (Luciferase assay system E1500, Promega) to normalize transfection variation. Measurements were made at least in triplicate and expressed as the mean (with s.e.m.) of the amount of  $\beta$ -galactosidase substrate (ONPG) hydrolyzed.

## Reverse transcription and quantitative PCR (qPCR)

Total RNA from embryo forelimbs was extracted using the RNeasy mini kit (Qiagen). 1  $\mu$ g RNA was used to generate cDNA using the Superscript II reverse transcriptase kit (Life Technologies). qPCR was performed using the Lightcycler 480 SYBR Green mix (Roche) and Lightcycler 480 II (Roche). RT-qPCR on FACS-isolated cells was performed using the Superscript III cell direct cDNA kit (Life Technologies). qPCR results are expressed as relative ratios of target cDNA to *Hprt*. The following oligonucleotides were used (5'-3'; forward and reverse): *Hes1*, ACACCGGACAAACCAAGAC and AATGCCGGGAGCTATCTTTC;

*Hey1*, CACCTGAAAATGCTGCACAC and ATGCTCAGATAACGGG-CAAC; *Myod1*, GGCTACGACACCGCCTACTA and GAGATGCGCT-CCACTATGCT; *Pax7*, AGGCCTTCGAGAGGACCCAC and CTGA-ACCAGACCTGGACGCG.

## Chromatin immunoprecipitation (ChIP)

Myod1 ChIP-seq has been described in detail (Cao et al., 2010). For qPCR ChIP experiments, forelimbs from E11.5 embryos were frozen in liquid nitrogen and processed for ChIP according to the manufacturer's protocol (Active motif). 150  $\mu$ g of chromatin was used for each experiment. 2 g of a rabbit anti-Myod1 M318 (Santa Cruz, sc-760) and 2 g of a goat anti-Hes1 (Santa Cruz, sc-13844) were used; 2 g of a rabbit anti- $\beta$ -galactosidase (Life Technologies, A-11132) or 2 g of a goat anti- $\beta$ -galactosidase (Santa Cruz, sc-19119) were used as the corresponding IgG negative control. The precipitated and input chromatin were analyzed by qPCR using *p57MRE* primers (forward, 5'-ATGTGCACACAG-CTCAGAGG-3'; reverse, 5'-GGAAGGATGGAGGGCTTTAC-3') with albumin primers as negative control (forward, 5'-GGGACGAGATGGT-ACTTTGTG-3'; reverse, 5'-GATCAGTCCAAACTTCTTTCTG-3').

For ChIP on transfected cells, HEK293 cells were transfected with a total of 7.5  $\mu$ g DNA using FuGENE6 (Promega). A mutant *p57MRE* sequence, *p57MRE $\Delta$ E-Boxes*, was synthesized (GeneART) in which all putative E-boxes were mutated according to Iso et al. (2003). Fixed concentrations of *p57MRE-tk-nlacZ* or *p57MRE $\Delta$ E-Boxes-tk-nlacZ* (4  $\mu$ g) were used together with either pCig-Myod1 or pCig-Hes1 (2  $\mu$ g). After 48 h, chromatin was extracted and processed as above; 100  $\mu$ g chromatin was used for each experiment. For ChIP, 2  $\mu$ g normal mouse (Santa Cruz) and goat (Santa Cruz) IgG were used for negative controls for the Myod1 and Hes1 antibodies mentioned above. Results are expressed as fold change compared with IgG control.

## Statistical test

Immunostainings were performed on at least three embryos of each genotype. Quantifications were performed using images of all muscle masses present in an embryo section (6–8 sections per slide, 2–3 frames per mass). All qPCR experiments were performed at least three times independently. Cell counting and qPCR results were analyzed by Mann–Whitney or Student's *t*-test. In Fig. 3D and Fig. 4F,G, quantifications were analyzed by ANOVA. In Fig. 5D, quantifications were analyzed by a chi-square test.

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## Competing interests

The authors declare no competing financial interests.

## Author contributions

A.Z. designed and performed experiments, analyzed data and wrote the paper. S.H. and F.A. designed and performed experiments, analyzed data and edited the manuscript. T.C., D.M. and P.M. designed and performed experiments, analyzed data. Z.Y. and Y.C. provided data on Myod1 ChIP-seq. D.B. provided *Rbpj* mutant embryos. C.B. analyzed data and edited the manuscript. F.R. oversaw the entire project, designed experiments, analyzed data and wrote the paper.

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### Supplementary material

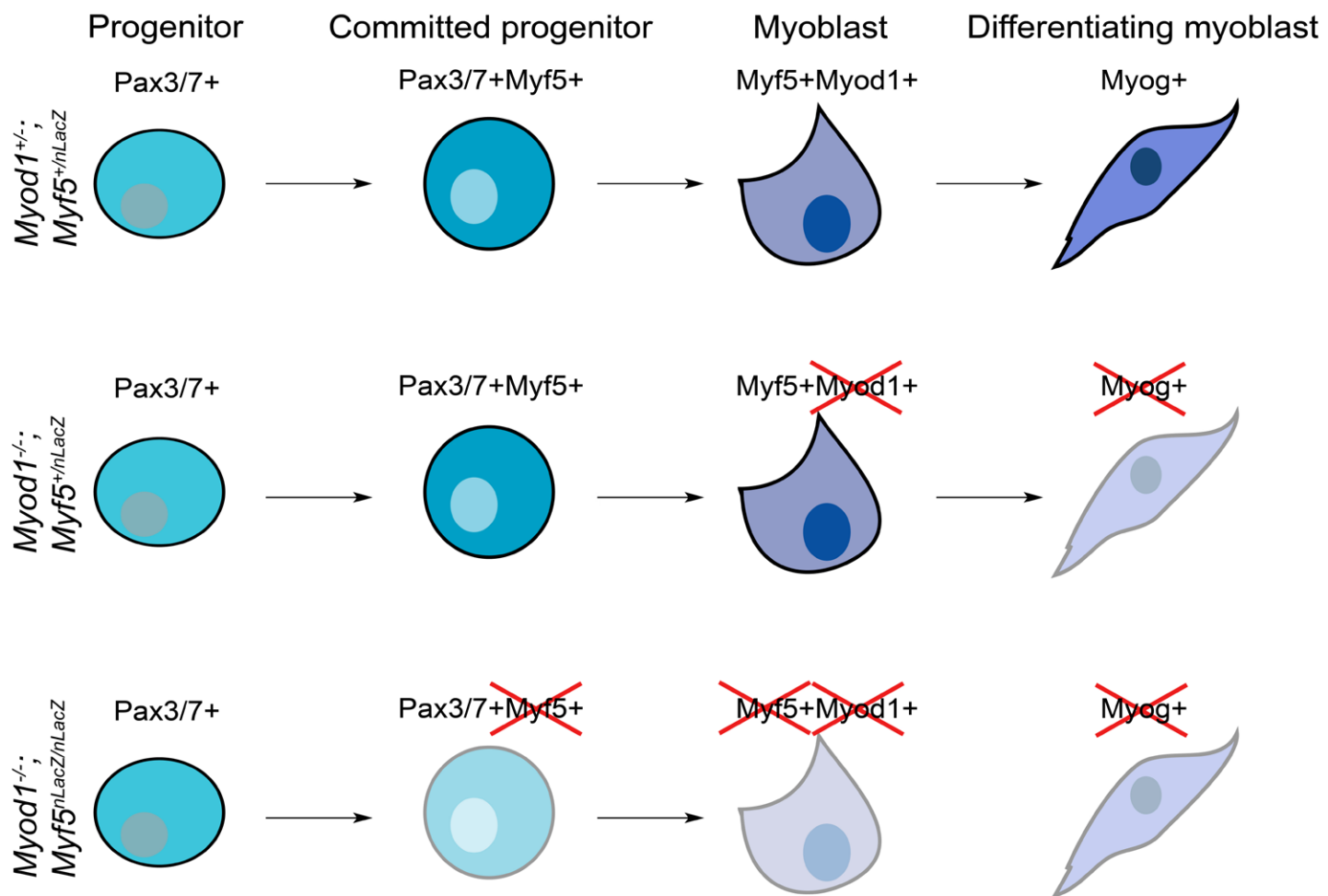
Supplementary material available online at  
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.110155/-/DC1

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Supplementary Figure 1. Stages of myogenic differentiation impairment using the different *Myod1: Myf5* mutant compounds.

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      .       .       .       .       .
1-  ACCCAGTTTGCCCAGTGTAGAGTGCCCAAAGCCCTCAGAAAAGACAAAGAAAGAGACCT
      .       .       .       .       .
61-  TTCAGACTAGAAAACAGAAGCAGGATACTTTGACATATGTCACCATGTGCCTGGGCCACT
      .       .       .       .       .
121-  TGGCTTGGCTACCCAAGCAAGGGCCCAAGAGAGTGCCCCCTTTGTCCCTTCTTCACAAAT
      .       .       .       .       .
181-  GCTGTGTATCTACTCCACACATGTGCACACAGCTCAGAGGCTCTTGCCTTTGGAATGCAG
      .       .       .       .       .
241-  TCCACCCACCTCCAGTTTTTCCCCTAAGCAGTTCAGCTGCTAAGCCCTGAACTCTCCAC
      .       .       .       .       .
301-  CTGCCGGGTCCTTAGCACAGCTGCCAGAGCTCCTGGACCCAGGCACAGCTGGATGACC
      .       .       .       .       .
361-  ACCCCTCCGAGCTGGCTAGGCCTGCTGTGAGCTGCCAGTAAAGCCCTCCATCCTTCCCAA
      .       .       .       .       .
421-  CAGGTGTCCCCACAACCTGCTGGGGGGGTGTGCTTAGCTAGGCAGCTATTCCACTGACCCAG
      .       .       .       .       .
481-  GCCTGCAGAATGATGTCACACTTACCACTGTGGAAGGAGGTAGCCCGAGGGATCTGACAG
      .       .       .       .       .
541-  ACTCCACGGAAGTTATTGGTTCTAGAGAAACGGCACACCAGTCATACTCAGCTCCAGCTG
      .       .       .       .       .
601-  GGTGTTGGGTCAGACCTATCCTTTTAAAGACCAACTGGACATTGGCCAGAGAAGTTTGGCCC
      .       .       .       .       .
661-  TGAAGGGTCTGGGGTGTCTTTACCTGG -687

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**Supplementary Figure 2. Genomic sequence of p57MRE enhancer cloned in p57MRE-tk-nlacZ reporter.**

Putative E-boxes are underlined with grey background. To generate *p57MREΔE-Boxes* mutant construct, every CANNTG motif has been replaced by CGNNAG.