Antagonistic regulation of p57kip2 by Hes/Hey downstream of Notch signaling and muscle regulatory factors regulates skeletal muscle growth arrest

Antoine Zalc1,2,3,*, Shinichiro Hayashi1,2,3,*, Frédéric Auradé1,2,3,*, Dominique Bröh1,4,*, Ted Chang1,2,3,†, Despoina Mademtzoglou1,2,3, Philippos Mourikis1,2,3, Zizhen Yao5,§, Yi Cao5,§, Carmen Birchmeier4 and Frédéric Relaix1,2,3,¶

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 (p21cip1 and p57kip2), 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 p21cip1 and p57kip2 expression in muscle progenitor cells. Finally, we identify a muscle-specific regulatory element of p57kip2 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 p57kip2 enhancer to regulate the decision between progenitor cell maintenance and muscle differentiation.

KEY WORDS: Myogenesis, Cell cycle regulation, p57kip2, 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; Kassar-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; Kassar-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+ progenitor cells undergo consecutive steps of differentiation via sequential expression of bHLH myogenic regulatory factors [MRFs; Myf5, MyoD and myogenin (Myog)], and first form committed progenitor cells that express Pnx3/7 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+ 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+4) myoblasts and fibers prior to E14.5 (Kablar et al., 1998). When both Myf5 and MyoD1 are impaired, Pnx3/7+ 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: p21cip1 (Cdkn1a, p21waf1), p27kip1 (Cdkn1b) and p57kip2 (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...
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 Borggrefe 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+ 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 by Hes/Hey repressors downstream of Notch in progenitor cells.

**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+ progenitors (7.2±1.7%). By contrast, a proportion of the Pax3/7+/Myf5+ committed progenitor cells did express p57 (16±5%), and this proportion increased significantly in Myf5+/Myod1+ (52±4.9%) and Myog+ (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−/− embryos, in which growth arrest is abolished (Zhang et al., 1999). In limb muscles of control mice, 4.7±1.4% of cycling cells (Fig. 2A–A″) expressed Myog and p57. Conversely, a proportion of the Myog+/P-H3+ MyoD1+/P-H3+ cells (25.2±3.2%; Fig. 2B–C) co-localized in the forelimbs of control embryos (Kablar et al., 1997). As expected, Myog and p57 expression almost never co-localized with that of Ki67, a marker of cycling cells. 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).

We then examined whether the uncoupling of proliferation and differentiation that we observed in the p21−/− p57−/− 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+/− mice at E12.5 (Fig. 2D–D″). By contrast, in the E12.5 Myod1−/− forelimbs, even...
though Myog is not expressed, p57 is detected in the forming muscle masses (Fig. 2E″), where it labels nearly half of the Myf5+ cells in both Myod1−/− (Fig. 2F″, H) and Myod1+/− (Fig. 2G–H) forelimbs (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+ 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+ myoblasts but in the presence of Myf5+ myoblasts in Myod1−/−; Myf5nlacZ/nlacZ mice (Rudnicki et al., 1993; Tajbakhsh et al., 1997) (supplementary material Fig. S1), the proliferation rate of Pax3+ cells was comparable to that observed in control mice at E12.5 (23.6±3.9% versus 25.6±4.6%; Fig. 3A-B″). By contrast, in the double-mutant Myod1−/−; Myf5nlacZ/nlacZ forelimbs, which lack both committed progenitors and myoblasts (supplementary material Fig. S1), we observed a significant decrease in the proliferation of Pax3+ 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+/MRF− progenitor cells of Myod1+/−; Myf5nlacZ/ZlacZ embryos compared with control embryos (28.4±2.7% versus 2.3±2.7%; Fig. 3E-G). These data suggested that committed progenitors 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 vitro 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 Pax7+ progenitor cells are in close proximity to Myod1+ 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 (Vasutina et al., 2007). Consistent with impaired Notch activity, Pax7 protein was undetectable by immunofluorescence at E12.5 in Myod1−/−; Myf5nlacZ/ZlacZ forelimbs (Fig. 4C-E), whereas it was expressed in Myod1−/−; Myf5nlacZ/nlacZ embryos (Fig. 4C-E). In addition, we found downregulation of the Notch target genes Hes1 and Hey1 in the forelimbs of Myod1−/−; Myf5nlacZ/ZlacZ compared with Myod1+/+; Myf5nlacZ/ZlacZ or with Myod1−/−; Myf5nlacZ/nlacZ at E12.5 (Fig. 4F,G).
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 µM γ-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+ cells (56.8±5.6% in control versus 27.7±7.0% in DAPT-treated limb explants; Fig. 5B), whereas the Myod1+ cell population was increased (62.7±9.0% compared with 32.6±5.3% in control DMSO-treated explants; Fig. 5B), confirming previous reports (Schuster-Gossler et al., 2007; Vasyutina et al., 2007) and the robustness of our ex vivo model system. 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+/MRF+ 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 Rbhp. Rbhp 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 Rbhp in the Pax3 lineage by crossing Rbhpflp/flp mice (Han et al., 2002) with a Pax3Cre/+ allele (Engleka et al., 2005). Ablation of Rbhp 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+/Myf5− muscle progenitor cells in the forelimbs of Rbhpflp/flp; Pax3Cre/+ 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+ cells in DAPT-treated limb explants; Fig. 6E–G). Overall, 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
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. β-Gal+ 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 the MRFs, Hey1 and Hes1 (supplementary material Fig. S2). We performed ChIP experiments on HEK293 cells transfected with either Hes1 or Myod1 and either wild-type p57 or containing mutations in the MRF and Hes binding sites (p57MREΔE-Boxes). Robust binding was observed for Hes1 (Fig. 8B) and Myod1 (Fig. 8C) on the p57MRE and this binding was abrogated on p57MREΔE-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 p57MREΔE-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−/− 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,
we found that Myf5+/Pax3/7 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 MRE-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+/Myf5+ 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 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 X. laevis 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). This activity might be mediated by Hey1 and HeyL, which are required in the adult lineage for satellite cell homeostasis and skeletal muscle regeneration (Bjornson et al., 2012; Carlson et al., 2008; Fukada et al., 2011; Kitamoto and Hanaoka, 2010; Mourikis et al., 2012b). Strikingly, Rbpj ablation does not lead to an immediate and complete differentiation or growth arrest in the Pax3+ 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 TGFβ 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 TGFβ 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...
Myod1 directly activates p57 expression. In our study, the expression of p57 is firmly linked to an absence of cell cycle progression, since we observe no overlap between p57 and p21 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/stem cell proliferation in intestinal stem cells (Riccio et al., 2008), 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>*/*lacZ*, *Myod1<sup>+/−</sup>*, *p21<sup>−/−</sup>*, *p57<sup>−/−</sup>* (p57 is an imprinted gene; we indicate maternal origin of the allele by a superscript m), Pax3<sup>Cre<sup>+</sup></sup> and Rbpjflox/flox 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.

(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>+/p21<sup>+</sup></sup> or Pax3<sup>−/p57<sup>+</sup></sup> cells are Ki67<sup>+</sup> in the Pax3<sup>Cre<sup>+</sup></sup>/Rbpjflox/flox 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.

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 antibodies compared with an albumin gene control. (D) Transactivation assay on C2C12 cells with the expression plasmids and with the construct in which all putative E-boxes have been mutated (p57MRE<sub>E-Boxes</sub>). ΔE-Boxes, n=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.

Notch
progenitor
Delta, Jagged
myoblast

p57 Muscle Regulatory Element

p57<sup>MRE</sup>ΔE-Boxes-tk-nlacZ
p57MRE-tk-nlacZ

p57<sup>−/−</sup>/p21<sup>+/−</sup>, p57<sup>−/−</sup>/p21<sup>−/−</sup>
Antibodies
The following antibodies were used: mouse anti-β-galactosidase 1/500 (Promega, Z378), mouse anti-Myod1 5.8A 1/200 (DAKO, M3512), mouse anti-Myo F5D 1/200 (DSHB, F5D), mouse anti-p21 1/100 (BD Pharmingen, 556431), mouse anti-p57 1/100 (Santa Cruz, sc-65341), mouse anti-Pax7-c 1/100 (DSHB, Pax7-c), mouse anti-Pax3-c 1/100 (DSHB, Pax3-c), rabbit anti-β-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 Signalling, 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 1/2,500, 594 1/1000 (Life Technologies) or 649 1/2,500 (Jackson ImmunoResearch).

Explant and cell culture
Forelimbs from E11.5 wild-type embryos were cultured in 12-well plates in BGGb medium (Life Technologies), without serum, with 200 µg/ml ascorbic acid (Sigma) and 100 µg/ml penicillin/streptomycin (Life Technologies). For Notch inhibition, forelimbs were immediately treated with 20 µM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Active motif). For Notch inhibition, forelimbs were immediately treated with 20 µM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Active motif). For Notch inhibition, forelimbs were immediately treated with 20 µM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Active motif). For Notch inhibition, forelimbs were immediately treated with 20 µM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Active motif). For Notch inhibition, forelimbs were immediately treated with 20 µM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Active motif).

Reverse transcription and quantitative PCR (qPCR)
Total RNA from embryo forelimbs was extracted using the RNeasy mini kit (Qiagen). 1 µg 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, ACACCCGACAAACCAAGAC and AATGCCGGAGCTATCCTT; Hey1, CACCTGAAAATGTCTGCACAC and ATGCTCAGATAAAGCAGGCAAC; Myod1, GGTCAGCAACGCCTACTA and GAGATGCTGCCT-CCACTGTCT; Pax7, AGGCGCTTGAGAGGACCACCC and CTGAMCCACGGTGAGCG.

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 µg 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-β-galactosidase (Life Technologies, A-11132) or 2 g of a goat anti-β-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'-ATGTCACACAG-CTCAGAG-3'; reverse, 5'-GGAAGGATGGAGGCCTTAC-3') with albumin primers as positive control (forward, 5'-GGGACGAGATGTG-ATTGTTG-3'; reverse, 5'-GACGATCCAAATCTTCTTCTG-3').

For ChiP on transfected cells, HEK293 cells were transfected with a total of 7.5 µg DNA using FuGENE6 (Promega). A mutant p57MRE sequence, p57MREΔE-Boxes, was synthesized (GeneART) in which all putative E-boxes were mutated according to Iso et al. (2003). Fixed concentrations of p57MRE-ΔE-Boxes or p57MREΔE-Boxes-ΔΔE-Boxes (4 µg) were used together with either pCig-MyoD1 or pCig-Hes1 (2 µg). After 48 h, chromatin was extracted and processed as above; 100 µg chromatin was used for each experiment. For ChiP, 2 µg 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 masse). 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.

Acknowledgements
We are grateful to Drs Sonia Alonso-Martin, Edgar Gomes, Revital Rattenbach, Vanessa Ribes and David Sassoon for their assistance with this work. We also acknowledge the animal care facilities at UPMC and CDTA, and Catherine Blanc and Bénédicte Hoareau from the Flow Cytometry Core CyPS. We are grateful to Drs Talpaw and Fukada for sharing unpublished data, Drs Tajbakhsh and Kageyama for Hef1 and Hes1 cDNAs and F. Langla Vives for transgenic services.

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 unpublished data, Drs Tajbakhsh and Kageyama for Hef1 and Hes1 cDNAs and F. Langla Vives for transgenic services.

Funding
This work is supported by funding to F.R. from Institut National de la Santé et de la Recherche Médicale (INSERM) Avenir Program, Association Française contre les Myopathies (AFM), Association Institut de Myologie (IAM), Labex REVIVE, the European Union Seventh Framework Program in the project ENDOSTEM [grant # 241440], Ligue Nationale Contre le Cancer (LNC), Association pour la Recherche contre le Cancer (ARC), Fondation pour la Recherche Médicale (FRM) [FDT20130928236], Institut National du Cancer.
unexpected heterogeneity between skeletal muscles in the mouse embryo. Development 127, 4455-4467.


