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The histone- and PRMT5-associated protein COPR5 is required for myogenic differentiation

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Myogenic differentiation requires the coordination between permanent cell cycle withdrawal, mediated by members of the cyclin-dependent kinase inhibitor (CKI) family, and activation of a cascade of myogenic transcription factors, particularly MYOGENIN (MYOG). Recently, it has been reported that the Protein aRginine Methyl Transferase PRMT5 modulates the early phase of induction of *MYOG* expression. Here, we show that the histone- and PRMT5-associated protein COPR5 (cooperator of PRMT5) is required for myogenic differentiation. C2C12 cells, in which *COPR5* had been silenced, could not irreversibly exit the cell cycle and differentiate into muscle cells. This phenotype might be explained by the finding that, in cells in which *COPR5* was downregulated, *p21* and *MYOG* induction was strongly reduced and PRMT5 recruitment to the promoters of these genes was also altered. Moreover, we suggest that COPR5 interaction with the Runt-related transcription factor 1 (RUNX1)–core binding factor-β (CBFβ) complex contributes to targeting the COPR5–PRMT5 complex to these promoters. Finally, we present evidence that COPR5 depletion delayed the *in vivo* regeneration of cardiotoxin-injured mouse skeletal muscles. Altogether, these data extend the role of COPR5 from an adaptor protein required for nuclear functions of PRMT5 to an essential coordinator of myogenic differentiation.

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Coordination between permanent cell cycle exit and initiation of terminal differentiation is critical during tissue development, a process that requires a controlled balance between cell proliferation, apoptosis and differentiation. The regulated expression of the cyclin-dependent kinase inhibitors (CKIs) $p21^{Cip1/Waf1}$ (p21), $p27^{Kip1}$ (p27) and $p57^{Kip2}$ (p57) has a key role in promoting and maintaining cell cycle arrest in quiescent cells and during differentiation of many cell types.[1] Importantly, only quiescent cells, and not terminally differentiating cells, retain the ability to reverse the proliferation block induced by p21. Moreover, quiescent cells are less likely to differentiate than proliferating cells, indicating that they employ active mechanisms to prevent the adoption of a non-dividing status (i.e., differentiation) associated with permanent cell cycle withdrawal.[2] Skeletal muscle formation is characterized by the fusion of myoblasts into myotubes and increased synthesis of muscle contractile proteins. Differentiating myoblasts strongly accumulate CKIs[3–6] and the differentiation programme is initiated by the ordered expression of several bHLH transcription factors of the myogenic regulatory factor family.[7] Among them, MYOD1, which is expressed in proliferating, undifferentiated committed myoblasts, and MYOGENIN (MYOG), which is expressed early during the differentiation programme, are essential for inducing muscle cell differentiation.[7] In the C2C12 myoblast cell line, MYOD1-dependent differentiation involves induction of MYOG while cells are still proliferating, followed by p21-dependent exit from the cell cycle.[3,5] Coupling the onset of differentiation with cell cycle withdrawal involves the direct activation by MYOD1 of genes involved in the regulation of the cell cycle, such as *p21*, *RB* and *CYCLIN D3* (*CCND3*).[3,4]

A growing body of evidence suggests that members of the Protein aRginine Methyl Transferases (PRMTs) family participate actively in the regulation of cell proliferation. Indeed, they modulate chromatin dynamics, transcriptional regulation, RNA metabolism, signal transduction and cell cycle checkpoint controls through methylation of histone and non-histone proteins.[8,9] Particularly, PRMT5 is essential for cell proliferation and *PRMT5* deficiency triggers cell cycle arrest in G1.[10] Conversely, stable expression of PRMT5 stimulates cell proliferation and transforms NIH3T3 cells, which can then grow in an anchorage-independent manner.[11] Moreover, PRMT5 is an important cytosolic factor, as evidenced by a recent study that implicates PRMT5 in the maintenance of ES cell pluripotency.[12] However, PRMT5 associates also with nuclear complexes to mediate mainly transcriptional repression,[13–17] although a recent work has reported that PRMT5 facilitates the activation of the *MYOG* promoter during MYOD-induced muscle differentiation.[18]

We previously reported that the adaptor protein COPR5 (cooperator of PRMT5) strongly binds to PRMT5 and histone H4, and that its silencing in proliferating U2OS cells reduces PRMT5 recruitment to the promoter of *CCNE1*, a key regulator of proliferation.[19] We now investigated whether

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Abbreviations: CKI, cyclin-dependent kinase inhibitor; *MYOG*, MYOGENIN; *PRMT5*, protein arginine methyl transferase 5; *COPR5*, cooperator of PRMT5; *RUNX1*, Runt-related transcription factor 1; CBFβ, core binding factor-β
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COPR5 could play a role in muscle cell differentiation by modulating the recruitment of PRMT5 to the promoter of genes involved in the coordination between cell cycle exit, such as p21, and differentiation, for instance, MYOG. We show that C2C12 cells in which *COPR5* is downregulated exit the cell cycle in a state which is not permissive for differentiation due to imbalanced CKI and MYOG expression. *COPR5* silencing impaired the recruitment of PRMT5 to the *p21* and *MYOG* promoters early during differentiation. Finally, we observe that *in vivo* downregulation of *COPR5* delayed skeletal muscle regeneration in mice following cardiotoxin (CTX)-induced injury. Collectively, these data suggest that COPR5 is a coordinator of PRMT5 activity targeted to a subset of key differentiation genes.

Results

***COPR5* silencing interferes with myogenic differentiation.** To investigate the role of the chromatin- and PRMT5-associated protein COPR5 during myogenic differentiation, C2C12 myoblasts, which can be induced to differentiate into muscle cells *ex vivo*,[20] were transduced with retroviral vectors that express *COPR5* shRNA (sh*COPR5*) or as control *Luciferase* shRNA (sh*LUC*). As expected, upon shift to differentiation medium (DM), control sh*LUC* cells stopped proliferating, aligned and fused to form multinuclear myotubes (Figure 1a, upper panels). In contrast, sh*COPR5* cells failed to form differentiated myotubes (Figure 1a, lower panels). *COPR5* downregulation in sh*COPR5* cells could be confirmed by quantitative RT-PCR (RT-qPCR) analysis (Figure 1b), but not by western blotting because the used antibody did not react with the mouse protein. Similar results were obtained with two other *COPR5* shRNAs, as well as with an shRNA directed against *PRMT5* (Supplementary Figures S1A and S1B). The failure of sh*COPR5* cells to differentiate was also confirmed by RT-PCR and immunofluorescence studies that showed the very low expression of the myogenic marker Myosin (*MHC*) in comparison with those detected in sh*LUC* cells soon after shifting to DM (Figure 1b, Supplementary Figures S1A and S1C). Similar results were obtained by silencing *COPR5* expression in primary human myoblasts (Figures 1c–e). Importantly, although we observed that ectopic COPR5 expression did not promote obviously by itself the differentiation phenotype in C2C12 cells (Supplementary Figure S1D), myotube formation was restored in sh*COPR5* C2C12 cells upon transduction with viral particles encoding human COPR5, the expression of which is not affected by the mouse anti-*COPR5* shRNAs (Figure 1f). Collectively, these data indicate that COPR5 is required for myogenic differentiation of C2C12 cells.

C2C12 cells in which *COPR5* has been downregulated fail to irreversibly exit the cell cycle. Differentiating myoblasts must arrest in the G1 phase of the cell cycle to terminally differentiate.[21] Thus, to address whether the differentiation defect observed in sh*COPR5* C2C12 cells could be due to a failure to irreversibly exit the cell cycle, we compared growth curves, cell cycle profiles and reversibility of cell cycle arrest in control sh*LUC* and sh*COPR5* C2C12 cells. In growth medium (GM), both cell populations behaved similarly (Figures 2a and b). Similarly, no differences were observed when cells were cultured in DM (Figures 2a and b), or in the presence of methylcellulose (+ MeC) to promote G1 or G0 arrest (Supplementary Figure S2). Conversely, when cells were first growth arrested in DM and then switched back to GM (DM→GM), a significant fraction of sh*COPR5* cells started to proliferate again and entered S phase, whereas most of control sh*LUC* cells remained arrested, as indicated by the growth curves and FACS analysis of BrdU-labelled cells (Figures 2c and d). These results show that sh*COPR5* cells can exit the cell cycle when transferred to DM, but not in a permanent way, suggesting that COPR5 controls indirectly or directly a subset of genes required for establishing the G1 arrest compatible with induction of myogenic conversion.

The expression of a subset of myogenic inducers and cell cycle regulators is altered in C2C12 cells, in which *COPR5* has been downregulated. To understand the mechanisms underlying the defects observed in sh*COPR5* C2C12 cells induced to differentiate, we first examined the expression of the cell cycle regulators involved in the tight control of the permanent G1 arrest required for differentiation.[21] As expected, quantification of the protein levels of the CKIs p21, p27 and p57 as well as of RB and CCND3 showed a significant increase in control sh*LUC* cells after switching to DM (Figure 3a). Conversely, in sh*COPR5* cells, expression of p21 and p27 was significantly lower, whereas the level of RB, CCND3 and p57 was only slightly affected (Figure 3a). Similar analysis of the main myogenic inducers MYOD1 and MYOG showed that both protein expression levels increased significantly during differentiation of sh*LUC* cells (Figure 3b). Conversely, in sh*COPR5* cells, MYOG induction was strongly reduced, while MYOD1 level was largely unaffected (Figure 3b). Moreover, the mRNA level of both the myogenic and cell cycle-regulated genes affected by COPR5 knockdown showed that it followed the protein variation level (Figure 3c). Interestingly, expression of *PRMT4* and *PRMT5,* the two PRMTs involved in C2C12 differentiation,[22] was not altered by *COPR5* downregulation (Figure 3c). By contrast, mRNA level of *p8*, which encodes an HMG protein involved in the mechanisms that restrict myogenic differentiation to the G1 phase,[23] was reduced, while those of *HES1* and *HES6*, which are involved in the control of cell cycle exit and myogenic differentiation, respectively,[24,25] remained unaltered (Supplementary Figure S3A). Finally, *COPR5* expression in sh*LUC* cells increased during muscle differentiation, reinforcing the hypothesis of a role of COPR5 in this process.

Altogether, these results indicate that the differentiation defect observed in sh*COPR5* cells is associated with reduced expression of p21, p27 and p8 (but not of RB, CCND3 and p57) and of MYOG (but not MYOD1), thus suggesting that only a subset of the differentiation programme is modulated by COPR5.

Early recruitment of PRMT5 to the *p21* and *MYOG* promoters requires COPR5, which can associate with the RUNX1 complex. We next investigated how COPR5

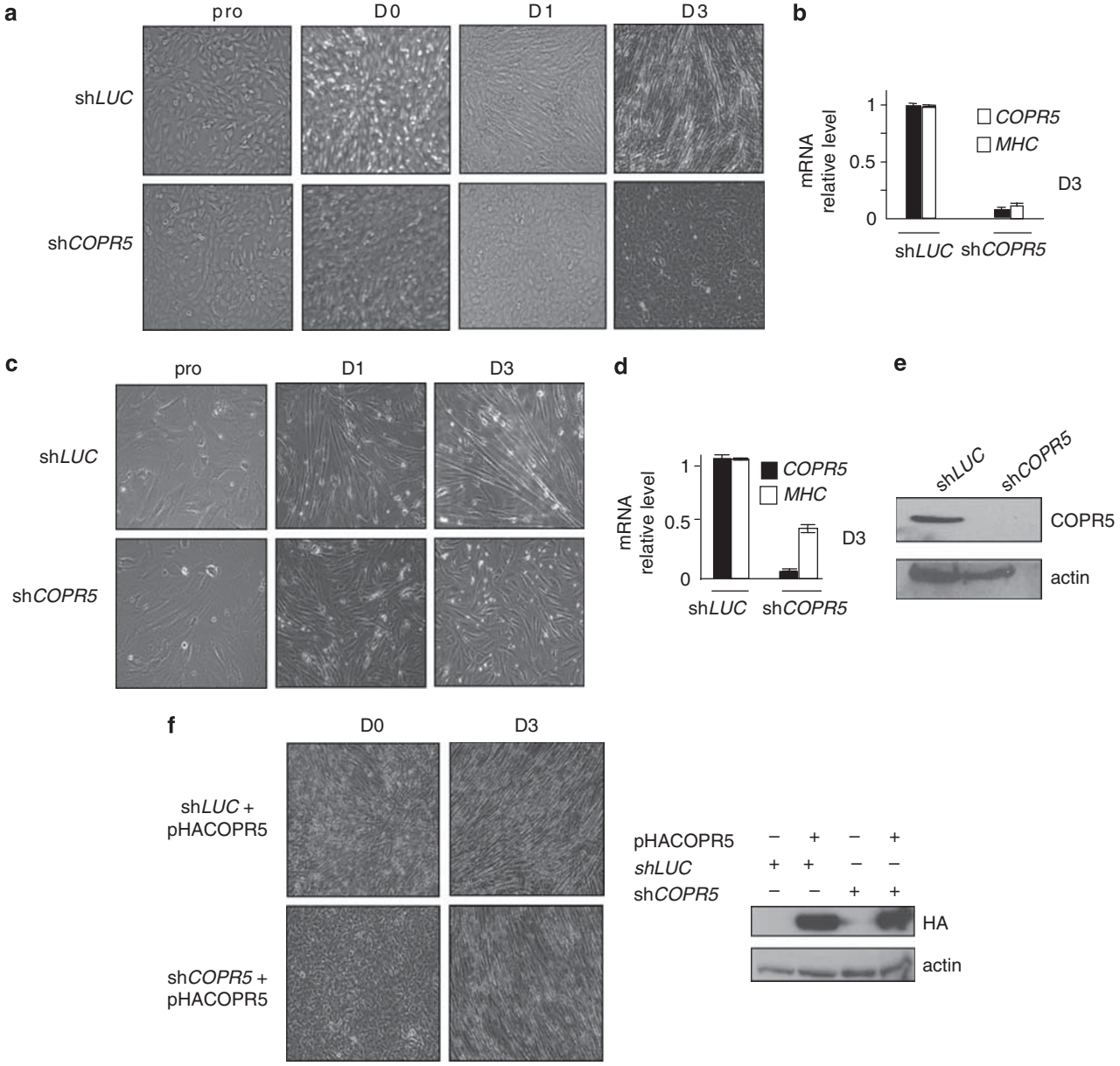

Figure 1 *COPR5* downregulation with shRNA interferes with myogenic differentiation. (**a**) Phase-contrast micrographs of proliferating (pro), confluent (day 0; D0) and differentiating (D1 and D3) C2C12 cells that express control (sh*LUC*) or *COPR5* (sh*COPR5*) shRNAs. (**b**) Expression analysis of *COPR5* and myosin heavy chain (*MHC*) was performed by RT-qPCR. Results were normalized to S26 RNA and values are expressed as the fold change compared with control cells. Values are the means ± SD of three independent experiments. (**c**) Phase-contrast micrographs of proliferating (pro) and differentiating (D1 and D3) human skeletal myoblasts transduced either with *LUC*- or *COPR5*-shRNA encoding viral particles. (**d**) Expression of *MHC* mRNA in primary human skeletal myoblasts transduced as in **a** and induced to differentiate (is shown at D3). The effect of *COPR5* downregulation on *MHC1* expression was monitored by RT-qPCR and results were normalized to *RPLP0* expression; analysis was performed as in **b**. (**e**) Protein extracts from *LUC* or *COPR5* shRNA-transduced human primary myoblasts were analysed by western blotting using an anti-COPR5 antibody. (**f**) Human HA-tagged COPR5 was expressed in mouse C2C12 cells in which endogenous *COPR5* was silenced. The phenotype in differentiating conditions is presented and visualized by the formation of myotubes (left panels) at D3 after induction of differentiation. Detection of HA–COPR5 by western blotting confirmed its resistance to the effects of shRNAs specific for mouse *COPR5* (right panels)

controls the expression of p21 and MYOG. As COPR5 is a histone- and PRMT5-associated protein,[19] we first tested whether it was present in the chromatin environment of the genes encoding these factors. To address this question, we performed chromatin immunoprecipitations (ChIPs) using an anti-HA antibody on C2C12 cells that had been stably transfected with a plasmid expressing HA-tagged COPR5. Consistent with the effect of *COPR5* silencing on p21, MYOG and p8 expression, transient recruitment of HA–COPR5 to the *p21*, *MYOG* and *p8*, but not to the *p57* and *MYOD1*, promoters was detected at day 1 (D1) after switching to DM (Figure 4a and Supplementary Figure S3B). Interestingly, reCHIP experiment using the anti-HA and anti-PRMT5 antibodies as first and second antibody, respectively,

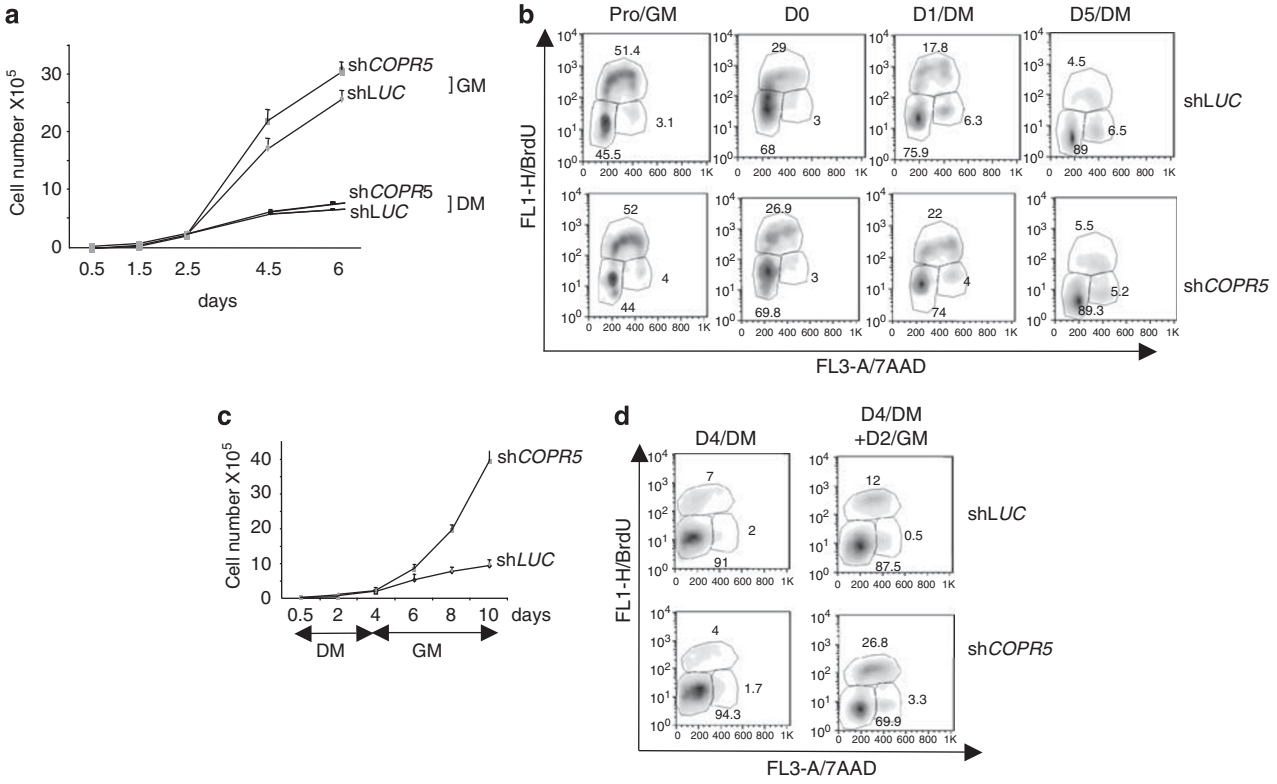

Figure 2 COPR5-silenced cells exit the cell cycle in a refractory state for differentiation. (**a**) Proliferation curves of selected C2C12 myoblast populations transduced with *LUC* or *COPR5* shRNA and maintained in growth medium (GM) or differentiation medium (DM) until day 6. Values represent the means ± SD of two independent experiments made in triplicates. (**b**) BrdU incorporation in proliferating (GM) or differentiating (DM) sh*LUC* or sh*COPR5* C2C12 cells analysed by FACS. The percentage of cells in the different phases of the cell cycle is indicated. (**c**) Proliferation curves as in **a** of selected C2C12 populations cultivated in DM for 4 days and then switched to GM. (**d**) The same experiment as in **b** but with cell culture conditions described in **c** is presented

showed on the *p21* promoter, but not the *MYOG* promoter, that COPR5 and PRMT5 were detected as part of a same complex (Supplementary Figure S4). As PRMT5 regulates the early phase of *MYOG* activation,[18] we then assessed whether *COPR5* downregulation could hinder the recruitment of PRMT5 to the *MYOG*, but also *p21* and *p8* promoters. ChIPs using differentiating sh*LUC* and sh*COPR5* C2C12 cells showed the presence of PRMT5 at the *p21* as well as *MYOG* and *p8* promoters in sh*LUC* cells at D1 after the switch to DM, whereas the recruitment of PRMT5 to these promoters was strongly reduced in sh*COPR5* cells (Figure 4b and Supplementary Figure S3C). Consequently to this observation we analysed whether the detection level of two PRMT5-mediated histone methylation marks (H3R8me2s and H4R3me2s) was affected as well. Strikingly, we failed to detect the presence of the H3R8me2s mark on both the *MYOG* and *p21* promoters on chromatin from C2C12 cells infected with a CTL shRNA (LUC), while the presence of the H4R3me2s mark was detected (Supplementary Figure S5). Interestingly, the detection of the latter mark decreased at D1 and D3 on the *MYOG* promoter only in *COPR5* shRNA-treated cells, probably consequently to the decreased recruitment of PRMT5 observed at D1 on this promoter. However, this mark could not be considered as a full readout of PRMT5 activity (see D0 with the *MYOG* promoter and results with the *p21* promoter).

A very slight increase, if any, in the detection of the H3R8me2s mark was observed in *COPR5* shRNA-treated cells at D3. We next tested whether *COPR5* silencing could affect the pool of PRMT5 associated with chromatin during differentiation. A fractionation assay showed that a small amount of PRMT5 was associated with the chromatin-enriched fractions (P3) in control sh*LUC* cells during differentiation. Conversely, the PRMT5 level in the P3 fraction of sh*COPR5* cells was strongly reduced, while it remained unchanged in the soluble cytosolic (S1) and nuclear (S2) fractions (Figure 4c). This effect is specific for PRMT5 as no alteration in the cellular distribution of another PRMT family member, PRMT1, was observed. Moreover, those of the chromatin remodeler BRG1 and MEKK1, used as controls of the S1 and P3 fractions, respectively, did not vary in sh*LUC* and sh*COPR5* cells (Figure 4c). Altogether, these results indicate that *COPR5* downregulation affects the activation of genes that play a key role during muscle cell differentiation by hindering the recruitment of PRMT5 to their promoters. We then asked whether COPR5 or the COPR5–PRMT5 complex could bind to specific factors present on these promoters. As both the *p21* and *MYOG* promoters are characterized by the presence of adjacent Runt-related transcription factor 1 (RUNX1) and MYOD1 functional DNA binding sites[26] and because MYOD1 is known to interact with PRMT5,[22] we assessed whether the

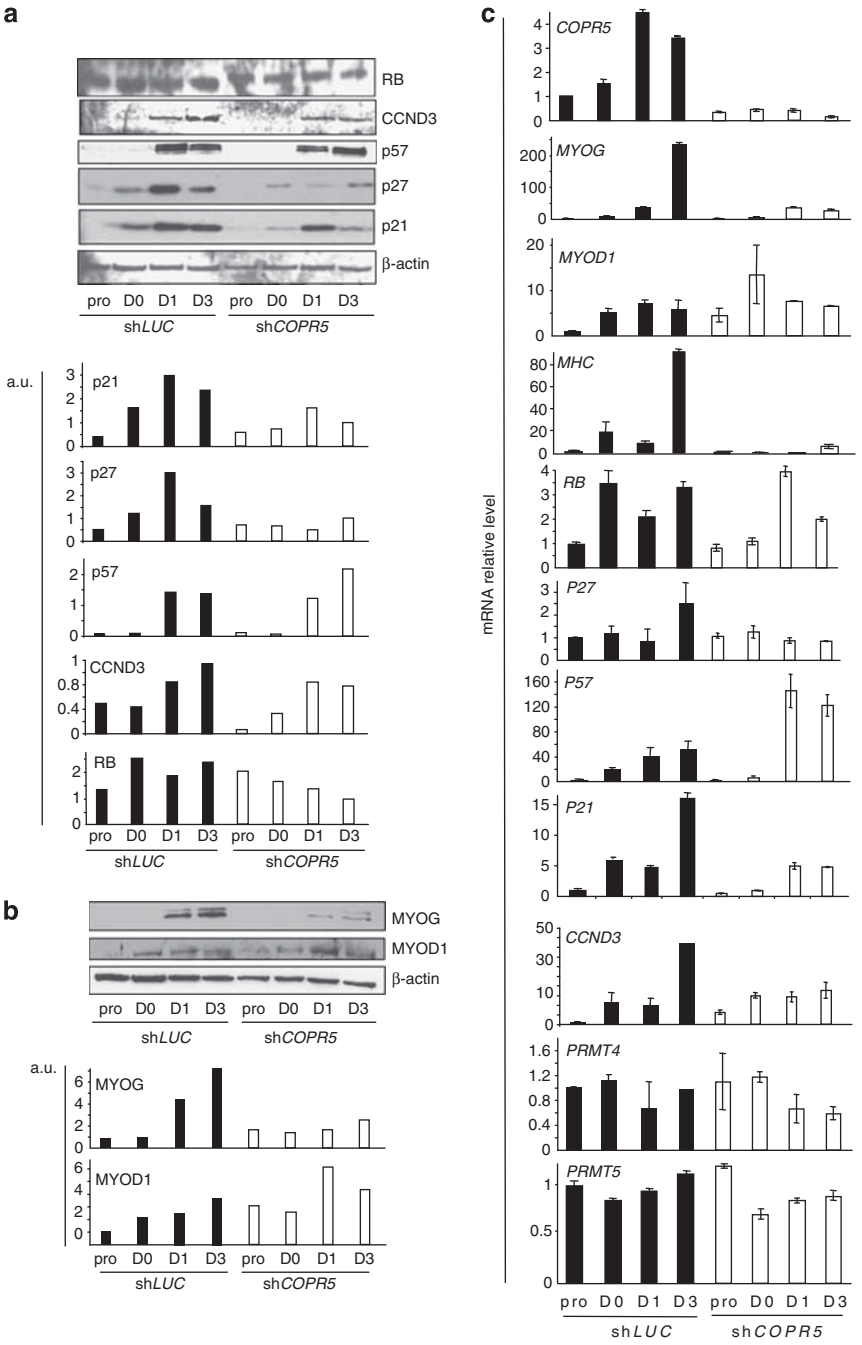

Figure 3 The expression of some cell cycle regulators and myogenic inducers are altered in COPR5-depleted cells. (**a**) Protein extracts from *LUC* or *COPR5* shRNA-transduced C2C12 cells were recovered at different time points (pro, D0, D1 and D3 of differentiation) and analysed by western blotting using antibodies against different cell cycle regulators involved in myogenic differentiation (upper panel). Quantification of the western blot is shown in lower panel. (**b**) The same analysis as in **a** was performed to analyse the protein level of different myogenic markers or inducers, as indicated. (**c**) The expression profile of different cell cycle regulators was assessed by RT-qPCR with RNA from sh*LUC* and sh*COPR5* C2C12 cells at different differentiation time points, as indicated. Results were normalized to *S26* RNA and values are expressed as the fold change compared with control cells (means ± SD of three independent experiments)

COPR5–PRMT5 complex could interact with RUNX1 and its partner protein core binding factor-β (CBFβ), which cooperate with MYOD1 in regulating target genes in myoblasts.[26] GST pull-down experiments using GST–COPR5 (GC) and protein extracts from U2OS cells transfected with HA–RUNX1 or Flag–CBFβ confirmed that COPR5 interacts with PRMT5, as expected,[19] as well as with CBFβ and RUNX1 (Figure 4d). Moreover, when using a GST–COPR5 deletion mutant (ΔC4), which does not interact with PRMT5,[19] only the interaction with CBFβ was observed (Figure 4d). The capacity of COPR5 to associate with CBFβ was confirmed by ChIP experiments (Figure 4e), suggesting that COPR5 could be targeted via the RUNX1 complex to promoters. Conversely, no RUNX1 DNA binding site was

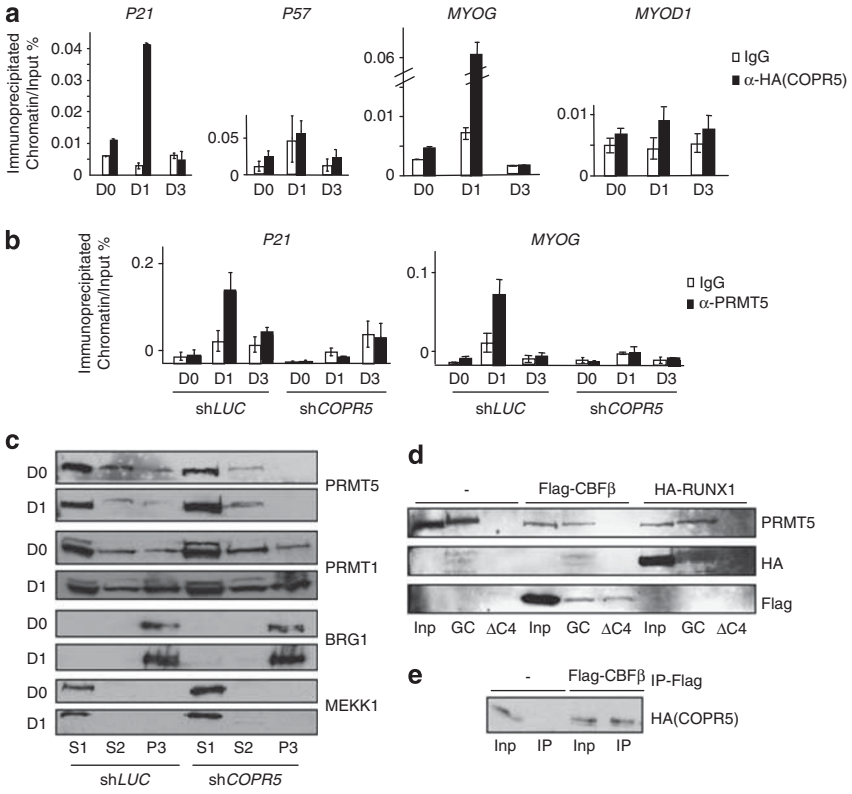

Figure 4 Early recruitment of PRMT5 to the *MYOG* and *p21* promoters requires COPR5, which binds to the RUNX1 complex. (**a**) Chromatin immunoprecipitation was performed using C2C12 cells that express HA–COPR5 and were grown in different culture conditions, as indicated. The recruitment of COPR5 to the *p21*, *p57*, *MYOG* and *MYOD1* promoters was analysed. Relative values are expressed as the percentage of immunoprecipitated chromatin relative to the input and represent the means ± SD of three independent experiments. (**b**) Chromatin immunoprecipitation was performed using *shLUC* and sh*COPR5* C2C12 cells in different culture conditions, as indicated. The recruitment of PRMT5 was performed on the *p21* and *MYOG* gene promoters. Results were analysed as in **a**. (**c**) Western blot analysis using anti-PRMT1 and -PRMT5 antibodies revealed a specific alteration in the repartition of PRMT5, compared with PRMT1, in a fractionation assay of sh*COPR5* C2C12 cells performed at D0 and D1. S1: cytoplasmic soluble, S2: nuclear soluble and P3: chromatin fraction. The anti-MEKK1 and -BRG1 antibodies were used as control of the S1 cytosolic and P3 chromatin fractions, respectively. (**d**) GST pull-down assay was performed after incubation of full-length GST–COPR5 (GC) or a mutant GST–COPR5 in which the C-terminal part had been deleted (ΔC4) with protein extracts from U2OS cells that had been transfected with HA-tagged RUNX1 or Flag-tagged CBFβ. (−) Corresponds to extracts of non-transfected cells. Anti-HA, -Flag and -PRMT5 antibodies were used. (**e**) Immunoprecipitation of COPR5 was validated in C2C12 cells that express HA–COPR5 and that had been transfected with Flag-CBFβ. (−) Corresponds to extracts from non-transfected cells. Inp: input (10% of total extract); IP: immunoprecipitation with anti-Flag antibodies

found in the *p27* promoter, although COPR5 binds to this promoter and recruits PRMT5 at a later stage of differentiation (data not shown), suggesting that alternative mechanisms are involved in COPR5 regulation of *p27* transcriptional activity.

Collectively, these results indicate that COPR5 regulates recruitment of PRMT5 to the promoters of *p21* and *MYOG*, a subset of genes that play key roles during muscle cell differentiation.

***COPR5* downregulation delays *in vivo* muscle regeneration.** To provide evidence of the role of COPR5 in myogenic differentiation *in vivo*, the two *tibialis anterior* muscles of mice were damaged by injection of CTX to reactivate satellite cells, which are considered the adult stem cells responsible for post-natal growth, regeneration and repair of skeletal muscle. Two days later, *COPR5* or control *LUC* shRNA (contralateral muscle) plasmid DNA was electroporated and then mice were killed at different time points to follow muscle regeneration. At D2 after electroporation, muscle tissue sections showed increased cellularity attributable to both proliferation of satellite cells and recruitment of inflammatory cells to damaged fibres (Figure 5a; haematoxylin–eosin staining). At this stage no obvious difference could be detected between control sh*LUC* and sh*COPR5* muscles. In contrast, at D5, high level of necrosis was only observed in muscles in which *COPR5* was silenced in comparison with control muscles (Figure 5a). At D7.5, regeneration had largely taken place in control muscles and many newly regenerated fibres were present with central nuclei, a known hallmark of recent muscle regeneration (Figure 5a). Such fibres were detected also in muscles in which *COPR5* was downregulated, but to a lesser extent than in controls. In accordance with results in C2C12 cells, the mRNA levels of *MYOG* and *p21* were reduced significantly in COPR5-depleted muscles compared with control but transiently (Figure 5b), probably due to a milder efficiency of the knockdown *in vivo* in the muscle compared with an *in vitro*-infected cell population. Consistently, immunohistochemical analysis using an anti-MYOG antibody on muscle sections performed at D5 showed less myofibres stained compared with control (Supplementary

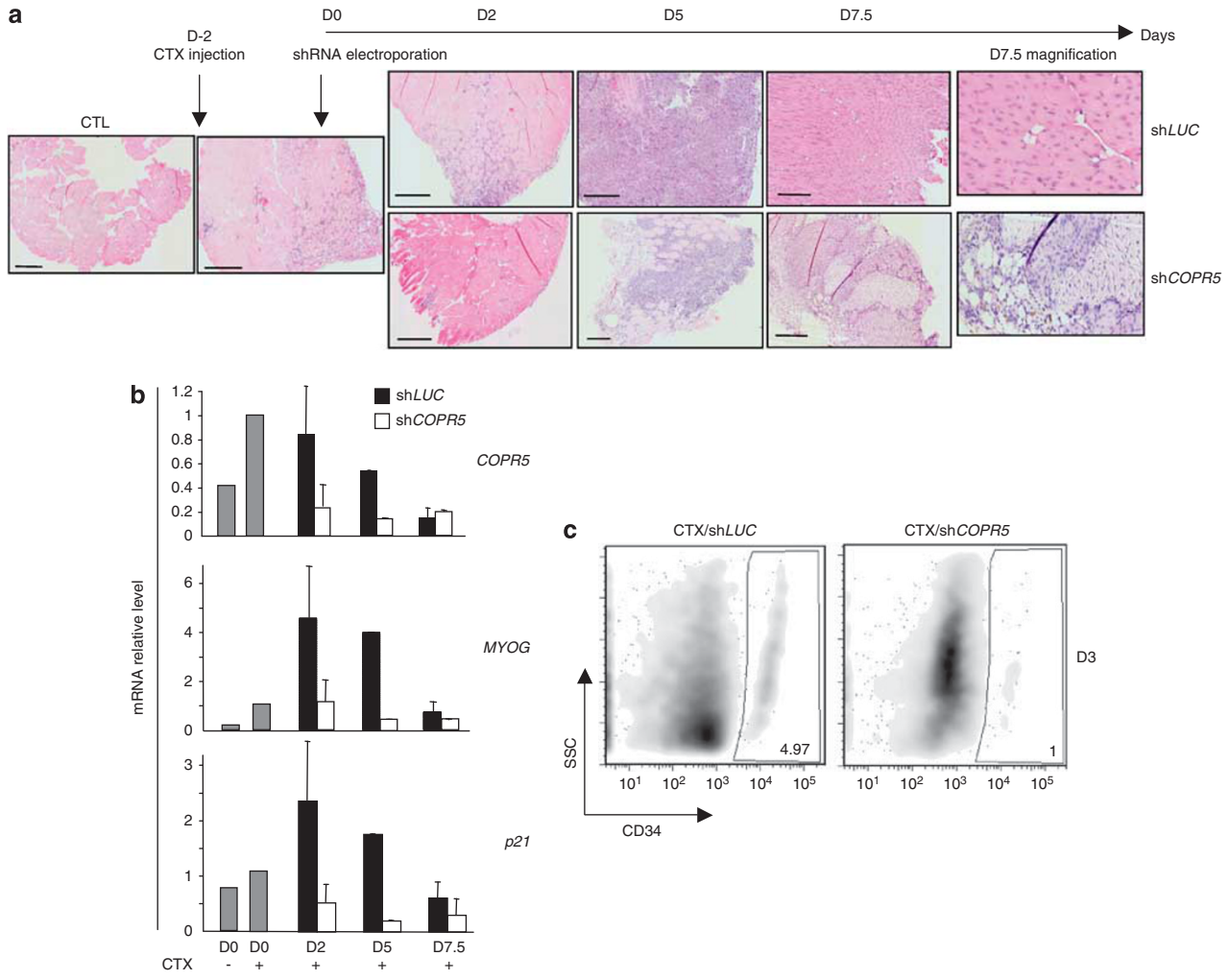

Figure 5 *COPR5* silencing slows down muscle regeneration *in vivo*. (**a**) Haematoxylin–eosin (HE) staining of paraffin-embedded muscle sections from *tibialis anterior* (TA) *muscles* of mice in which muscle necrosis was induced by cardiotoxin (CTX) injection, followed 2 days later by electroporation of *LUC* or *COPR5* shRNA-encoding plasmids. Bars: 200 μm. (**b**) mRNA level of *COPR5*, *p21* and *MYOG* genes was analysed by RT-qPCR after RNA extraction at D5 from muscle that had been treated as in **a**. Results were normalized and expressed as in Figure 3. Error bars of the means correspond to two independent experiments, except at D5. (**c**) Cells from muscles were injured and infected with ecotropic Moloney-based retroviral particles encoding either *LUC* or *COPR5* shRNAs, recovered at D3 and analysed by flow cytometry to quantify the CD34 + population (i.e., satellite cells). *x*-axis: CD34; *y*-axis: SSC (side scatter)

Figure 6). To assess whether this phenotype was due to a reduction in the pool of satellite cells activated following CTX-induced muscle injury, muscles were injected with retroviral particles expressing *LUC* or *COPR5* shRNAs that could only transduce activated and proliferating cells. An enriched satellite cell/isolated myoblast preparation was recovered from the *tibialis anterior* muscle at D3 after injury and the effect of *COPR5* downregulation on satellite cells was monitored by quantifying the number of CD34-positive (a satellite cell marker) cells that normally remains steady during homeostasis and injury-induced regeneration.[27] The number of CD34-positive cells in *COPR5* shRNA-transduced muscles was lower than in control muscles, indicating that the pool of satellite cells to generate committed myogenic progenitors was reduced following downregulation of *COPR5*. In accordance with the delayed regeneration observed in CTX-treated muscles, these findings strongly suggest that COPR5 plays a role during muscle regeneration, probably at the level of the satellite cell amplification and/or differentiation programme.

Discussion

Here we show that the PRMT5 adaptor COPR5 plays a role in myogenic differentiation of cultured C2C12 cells and modulated the transcriptional regulation of genes that are important for the control of cell cycle exit (*p27* and *p21*) and muscle differentiation (*MYOG)*. Increased levels of p27 and p21 have been correlated with the irreversible growth arrest required for differentiation.[3,4] Indeed, the level of p27 expression is critical for initiating growth arrest in differentiating myoblasts and in maintaining this arrest in terminally arrested mature myotubes, while in the presence of differentiation signals, p21 and p57 enhance cell survival.[28] C2C12 cells, in which *COPR5* was downregulated, could exit the cell cycle when switched to DM (Figure 2), suggesting that the residual levels

of the CKIs p21 and p27, and or a compensatory increase of p57 expression, are sufficient to trigger cell survival and this initial growth arrest. Consistent with this, while p21 and p27 expression was strongly reduced, p57 expression was slightly increased in differentiated *COPR5*-silenced cells (Figure 3). A similar MYOD1-mediated upregulation of p57 was previously reported in cells lacking *p21*.[29] Noteworthy, commitment to terminal differentiation is confined to the G1 phase of the cell cycle in differentiating myoblasts.[21] Strikingly, we also observed a decreased mRNA level of the G1-induced *p8* gene, which encodes an HMG protein involved in the mechanism to restrict myogenic differentiation to the G1 phase.[23] These findings together with our previous report that the COPR5–PRMT5 complex represses the *CCNE1* gene in U2OS cells[13] suggest that this complex is involved in coordinating CKI and p8 activation, while repressing CCNE1 expression as required for a stable cell cycle exit competent for differentiation. Consistent with this, we found that HA–COPR5 is recruited to the *p21*, *MYOG* and *p8* promoters (Figure 4a, Supplementary Figure S3). Moreover, the reduction of PRMT5 recruitment to these promoters in C2C12 cells, in which *COPR5* had been silenced (Figure 4b, Supplementary Figure S3), positions COPR5 centrally within the transcriptional regulatory mechanism that coordinates cell cycle exit and terminal muscle differentiation, and future studies will aim at identifying additional components and the upstream signalling that control this novel network. Our reCHIP data suggest that the accessibility of PRMT5 within COPR5-containing complex is different on the *p21* and *MYOG* promoters and might reflect a difference in the BRG1-mediated chromatin remodelling regulation at these promoters. In accordance with this, previous work supports that BRG1 is required for the induction of all muscle-specific gene expression by MyoD, thus including *MYOG*, whereas induction of the cell cycle regulators, p21, Rb, and cyclin D3 occurred independently of SWI/SNF function.[30] Our data also showed that COPR5–PRMT5 complex can interact with the transcription factor RUNX1–CBFβ complex, suggesting how COPR5 could target PRMT5 activity to a subset of genes, including *p21* and *MYOG*. Both the *p21* and *MYOG* promoters are characterized by the presence of adjacent RUNX1 and MYOD1 functional DNA binding sites. Interestingly, the interaction between the RUNX1 complex and MYOD1 participates in regulating the balance between proliferation and differentiation.[26] Moreover, the methylation of RUNX1 by PRMT1 regulates its interaction with SIN3A and modulates RUNX1-inducible gene expression.[31] Furthermore, crystal studies indicate that another GRG motif resembling those methylated by PRMT5 in other proteins is important for DNA binding.[32] Although we failed to methylate RUNX1 by COPR5–PRMT5 *in vitro* (data not shown), we do not exclude the possibility that such methylation could exist in a chromatin-dependent context to dissociate RUNX1 complex from MYOD1 and/or from DNA, allowing promoter activation.

Finally, consistent with a key role of COPR5 in myogenic conversion, its downregulation in CTX-injured muscles *in vivo* delayed muscle regeneration by reducing the pool of CD34-positive satellite cells (Figure 5). The presence of residual COPR5 indicated the *in vivo* depletion of COPR5 was milder than those obtained *in vitro*. Interestingly, muscle regeneration is also impaired in *p21−/−* mice,[33] thus stressing again the importance of COPR5 as a regulator of the expression of p21 and reinforcing the notion that PRMT5 is essential for myogenesis. Accordingly, beyond its impact on histone methylation, PRMT5 was shown recently to methylate Ash2L, a factor associated with PAX in a complex that directly controls entry into the myogenic programme of satellite cell-derived myoblasts.[34,35] Whether COPR5 downregulates directly the expression of satellite cell-driver genes such as *PAXs* or whether the phenotype due to *COPR5* silencing is an indirect consequence of the dysfunction of other myogenic regulators remains to be investigated.

This study clearly positions the COPR5–PRMT5 complex as a potential sensor and integrator of differentiation signals and a novel coordinator of cell cycle exit and differentiation. Noteworthy, the coupling of these two events is often perturbed during tumourigenesis and several types of tumours, including rhabdomyosarcoma, are characterized by an undifferentiated phenotype. Moreover, PRMT5 expression is deregulated and/or mislocalized in several types of cancer.[36,37] Therefore, it would be interesting to test whether alteration of COPR5 could contribute to the abnormal differentiation of tumours of mesenchymal origin.

Materials and Methods

Cell culture conditions. The C2C12 cell line was cultured in Dulbecco's modified Eagle medium supplemented with 15% fetal calf serum using standard conditions. To induce myogenic differentiation, GM was replaced with DM containing 2% serum (FetalClone III PerBio, Brebières, France). Primary human skeletal muscle myoblasts were cultured similar to C2C12 cells in the presence of Ultroser G (Gibco, St Aubin, France). Generation of retroviral particles and infection have already been described.[19]

RNA isolation, cDNA synthesis and RT-qPCR amplification. RNA isolation, reverse transcription and qPCR were performed as described.[38]

ChIP. ChIP was carried out essentially as described[13] using anti-H4R3me2s (Abcam, Cambridge, UK), PRMT5 (Euromedex, Mundosheim, France) and anti-HA (Sigma-Aldrich, St Quentin Fallavier, France) antibodies. H3R8me2s antibody was a gift from S Sif.

The sequences of the oligonucleotides used for shRNA, PCR and CHIP experiments are listed in Supplementary Information.

Western blot analysis. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Whatman, Maidstone, Kent, UK), and probed with anti-PRMT1, PRMT5, BRG1, MYOG, MYOD1, RB, p21 or p27 antibodies (Santa Cruz, Heidelberg, Germany), as indicated or an anti-HA antibody (12CA5, Sigma-Aldrich). Membranes were then incubated with the appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were detected by chemiluminescence. Quantification was performed using AIDA software (Dalian Software, Dalian, China).

Flow cytometry. For cell cycle analysis, experiments and analysis were performed as described.[38] Briefly, cells were incubated with 300 μM BrdU (Sigma-Aldrich) for 2 h, fixed in 70% ethanol solution and permeabilized with 0.2% Triton X-100 for 10 min. Then, cells were treated with 0.2 N HCl before incubation with a mouse anti-BrdU antibody (1 : 30 diluted in PBS with 0.2% Tween 20 and 1% BSA; BD Biosciences, Le Pont de Claix, France) at room temperature for 1 h followed by 1-h incubation with an FITC-conjugated secondary antibody (1 : 300; BD Biosciences). DNA was then counterstained with 7-amino-actinomycin D (1 : 50; Sigma-Aldrich) in the presence of RNase overnight. Cell cycle profiles were analysed by flow cytometry (FACScan; BD Biosciences) using the CellQuest software (BD Biosciences). For CD34 expression, 10^6 cells were labelled with a biotinylated anti-CD34 antibody (marker) in 100 μl PBS with 0.3% BSA on ice for 45 min, then washed and incubated with a streptavidin-Texas Red-conjugated secondary antibody for 45 min.

Cardiotoxin muscle injury. *Tibialis anterior* of mice (type of mice) were injected with 10 μM CTX (Calbiochem, Nottingham, UK) and processed 2 days later for electroporation, as described.[39] At the indicated time points, mice were killed, *tibialis anterior* muscles harvested and fixed in 4% formaldehyde overnight before to be paraffin-embedded for further analysis. For enriched satellite cell/myoblast preparations, isolation was performed as previously described,[22] after muscles were treated as above with CTX and co-injected with ecotropic Moloney-retroviral particles that encoded sh*LUC* or sh*COPR5*.

Mice and animal care. Animal experiments were approved by and performed in accordance with the guidelines of the Ethics Committee of the Languedoc-Roussillon region (France).

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)