Prmt5 is a regulator of muscle stem cell expansion in adult mice

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Skeletal muscle stem cells (MuSC), also called satellite cells, are indispensable for maintenance and regeneration of adult skeletal muscles. Yet, a comprehensive picture of the regulatory events controlling the fate of MuSC is missing. Here, we determine the proteome of MuSC to design a loss-of-function screen, and identify 120 genes important for MuSC function including the arginine methyltransferase Prmt5. MuSC-specific inactivation of Prmt5 in adult mice prevents expansion of MuSC, abolishes long-term MuSC maintenance and abrogates skeletal muscle regeneration. Interestingly, Prmt5 is dispensable for proliferation and differentiation of Pax7⁺ myogenic progenitor cells during mouse embryonic development, indicating significant differences between embryonic and adult myogenesis. Mechanistic studies reveal that Prmt5 controls proliferation of adult MuSC by direct epigenetic silencing of the cell cycle inhibitor p21. We reason that Prmt5 generates a poised state that keeps MuSC in a standby mode, thus allowing rapid MuSC amplification under disease conditions.

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Organ-specific adult stem cells enable continuous regeneration of various tissues throughout adult life. Most adult stem cells are assumed to undergo constant turnover to ascertain self-renewal and tissue homeostasis, although dormant adult stem cells have been described in the hematopoietic system, which remain in a quiescent state for most of their lifetime and divide only rarely under severe stress conditions. Adult skeletal muscle stem cells (MuSCs) are represented by a specialized subset of myofibre-associated cells called satellite cells and own a remarkable regenerative potential, which enables them to continuously replace myofibres by undergoing repeated rounds of activation and expansion under persisting disease conditions. Satellite cells originate during early embryonic development from a population of proliferating cells of the paraxial mesoderm. While most cells activate myogenic genes and form skeletal muscle fibres, some remain undifferentiated, adopt a satellite cell position in postnatal muscle and acquire a quiescent state. After injury or excessive exercise, Pax7+ cells exit quiescence, proliferate and differentiate to generate new myofibres or fuse with existing myofibres, thereby fully restoring damaged skeletal muscle tissues.

Several recent studies demonstrated that ablation of Pax7+ MuSCs prevents muscle repair under pathological conditions and during ageing (review in ref. 10). However, the molecular mechanisms that control satellite cell functions during skeletal muscle regeneration are only partially understood, although several factors directing the fate of MuSC have been identified (reviewed in refs 2,11).

To identify new regulators of MuSC activation, self-renewal, expansion and differentiation, we establish a systematic screening approach taking advantage of mass spectrometry-based protein profiling of FACS (fluorescence-activated cell sorting)-sorted MuSC6,7,12,13 combined with short hairpin RNA (shRNA)-mediated knockdown of MuSC-specific genes. Several genes are identified that strongly affect activities of MuSC in vitro, of which the histone arginine methyltransferase Prmt5 is chosen for in-depth functional analysis. We find that Prmt5 is essential for adult MuSC proliferation and muscle regeneration by restricting p21 expression via direct epigenetic silencing, thereby allowing rapid expansion of MuSC. Since the lack of Prmt5 does not affect embryonic myogenesis, we postulate that prenatal muscle development and adult muscle regeneration use distinct genetic and epigenetic mechanisms for the control of muscle progenitor cell expansion.

Results
Identification of novel regulators controlling MuSC homeostasis.
To determine the proteome of MuSCs, we isolated GFP-labelled stem cells (SCGF) from skeletal muscles of Pax7CreERT2/ZEG mice6,14 via FACS (Supplementary Fig. 1a), which all expressed Pax7 protein and readily differentiated into myocytes (Supplementary Fig. 1b,c). Protein extracts of freshly isolated MuSC were subjected to mass spectrometry analysis (n = 3) resulting in the identification of 135,341 peptides in all samples combined corresponding to 5,031 proteins in MuSC with at least one unique peptide (Supplementary Data 1). Notably, we detected numerous proteins known to be highly expressed in MuSCs including CD34, integrin α7, caveolin-1, Numb and β1-integrin, but not haematopoietic or endothelial cell markers such as CD45 or CD31 (refs 12,13,15). Comparison with proteome data sets obtained from myofibres, Pax7- mononuclear cells and the MuSCPG fraction (satellite cells after percoll gradient and before FACS sorting) allowed us to identify 441 proteins that are exclusively present in MuSC but not in differentiated myofibres (Fig. 1a and Supplementary Data 2). Mass spectrometry data of randomly selected proteins (Wdr61, ABCC4, Lxn, Mstn1, P2RX4 and Prmt5) were validated by immunofluorescence staining of freshly isolated myofibres (Fig. 1b).

To analyse the function of MuSC-specific proteins, we generated a custom-arrayed lentiviral shRNA library (400 genes, one shRNA per well, on average five different shRNAs per gene; Fig. 1c and Supplementary Data 2). FACS-purified Pax7CreERT2/Green MuSC6,12 were transduced with shRNA expressing lentiviruses and analysed by high-throughput fluorescent microscopy 96 h post transduction for the ratio of Pax7+ versus total 4,6-diamidino-2-phenylindole (DAPI)+ cells (Fig. 1d), providing a read-out for genes affecting self-renewal, proliferation and differentiation of MuSC. shRNAs targeting Pax7 and Nf1 were included as quality controls (n = 4 wells for each plate). After selecting for genes, which yielded a strong shift of the ratio of Pax7+ versus total DAPI+ cells after knockdown, we ended up with 30 genes inducing and 90 genes decreasing Pax7/DAPI- cell ratios after knockdown (Fig. 1e, Supplementary Fig. 1d and Supplementary Data 3).

Prmt5 is required for muscle regeneration.
Next, we initiated a thorough analysis of the function of an exemplary candidate, the arginine methyltransferase Prmt5, that mediates H3R8 symmetric dimethylation (H3R8me2s; ref. 16). Prmt5 was recently implicated in the regulation of proliferation of embryonic stem cells17,18 and neural progenitor cells (NPCs) during brain development19, but its function in adult stem cells has remained elusive. Inactivation of Prmt5 using Prmt5loxP/loxP (Supplementary Fig. 2a) and Pax7CreERT2 mice6,7 (= MuSC-specific Prmt5 knockout mice, Prmt5sKO) (Supplementary Fig. 2b) efficiently depleted Prmt5 mRNA in adult MuSC (Ctrl: 1.00 ± 0.339, n = 3; Prmt5sKO: 0.033 ± 0.020, n = 4; Supplementary Fig. 2c). Interestingly, tamoxifen (TAM)-treated Prmt5sKO mice remained viable and displayed no obvious phenotype under physiological conditions 21 days after treatment compared with control animals (Ctrl = Pax7CreERT2/ Prmt5+/loxP; Supplementary Fig. 2d). No significant change of body weight (Ctrl 23.60 ± 3.18; Prmt5sKO 22.67 ± 2.52, each n = 3) and no morphological alterations of skeletal muscle tissue were apparent (Supplementary Fig. 2e f). Likewise, the number of satellite cells on sections of tibialis anterior (TA) muscle (Ctrl 20.33 ± 2.08; Prmt5sKO 20.33 ± 1.53, each n = 3) and freshly isolated flexor digitorum brevis (FDB) myofibres (Ctrl 30.00 ± 3.61; Prmt5sKO 30.00 ± 2.00, each n = 3) did not differ between control and mutant littermates (Supplementary Fig. 2g,h). To investigate whether Prmt5-deficient satellite cells contribute to muscle regeneration, TA muscles of TAM-treated Prmt5sKO and control littermates were injected with cardiotoxin (CTX; Fig. 2a and Supplementary Fig. 2i). Strikingly, muscle regeneration was completely abolished in Prmt5sKO mice at all investigated time points (7 and 14 days, and 4 months after injury). The virtually complete lack of regenerated muscle fibres (Fig. 2b and Supplementary Fig. 2j) was accompanied by a massive increase of fibrosis (Fig. 2c and Supplementary Fig. 2k).

To analyse whether Prmt5 affects long-term satellite cell maintenance, we determined the number of MuSC 4 months after the initial TAM treatment. Importantly, we detected a significant decline of Pax7+ MuSC numbers both on cryosections from TA muscles (Fig. 2d; Ctrl 15.00 ± 2.19; Prmt5sKO 5.17 ± 2.32, each n = 6) and on freshly isolated myofibres from FDB muscle (Ctrl 23.00 ± 2.65; Prmt5sKO 13.33 ± 4.93, each n = 3; Fig. 2e), indicating that Prmt5 is required for MuSC expansion during regeneration and needed to replenish the MuSC niche during physiological ageing.
To investigate the cellular mechanisms responsible for the loss of muscle regeneration under chronic disease conditions.

**Figure 1 | Identification of regulators of stem cell homeostasis in a MuSC proteome-based shRNA screen.**

- **a** Mass spectrometry (MS)-based identification of MuSC-enriched proteins (Proteome SC) using samples from fractionated skeletal muscles including purified myotubes, Pax7-GFP- mononuclear cells, percoll-gradient purified and Pax7-GFP+ MuSC.  
  - **b** Immunofluorescence validation of identified proteins (red) counterstained with Pax7 antibody (green) and DAPI (blue; scale bar, 20 μm).  
  - **c** Schematic outline of the shRNA screen against corresponding genes of the satellite cell proteome. Phenotypic scores are calculated as ratios of Pax7+/DAPI+ nuclei for each well.  
  - **d** Poisson distribution of relative Pax7 expression for all 2,226 shRNAs targeting 419 genes. Red and green lines indicate phenotypic scores lower or higher than 0.25 percentiles normalized to plko.1 empty vector control. Knockdown of Pax7 reduces proliferation and enhances differentiation of MuSC, whereas knockdown of Nf1 increases the numbers of Pax7 expressing cells.  
  - **e** shRNA knockdown identifies 90 and 30 candidate genes causing down- and upregulation of Pax7+/DAPI+ ratios, respectively. P.i., post infection; Pos, positive.

**Prmt5 prevents depletion of the MuSC pool in mdx mice.** To further explore the role of Prmt5 in replenishing the MuSC pool, we utilized mdx mice, which lack functional dystrophin resulting in continuous degeneration/regeneration of myofibres, accompanied by repeated activation and enhanced turnover of satellite cells. Treatment of 8-week-old Prmt5sKO/mdx compound mutant mice for 3 weeks with TAM (Fig. 3a) resulted in progressive loss of body weight, whereas Prmt5-deficient and mdx mice gained weight similar to wild-type littermates (Fig. 3b,c). Moreover, we found a virtually complete absence of the formation of myogenic colonies on single myofibres from FDB muscles of Prmt5sKO/mdx mice, we first analysed FACS-purified MuSCs from Prmt5sKO and control mice in vitro. Prmt5-deficient MuSCs showed a virtually complete arrest of cell proliferation as reflected by a marked reduction of the number of Pax7+/5-ethyl-2'-deoxyuridine (EdU)-incorporating cells (Ctrl 32.55±5.94%; Prmt5sKO 8.29±4.24%, each n=6), which is in line with the results from Prmt5 knockdown experiments (Fig. 4a). Conversely, lentiviral overexpression of human Prmt5 stimulated proliferation of MuSC indicated by increased EdU incorporation (Ctrl1 36.90±2.52%, n=8; Prmt5OE 43.43±4.13%, n=6; Fig. 4b). Moreover, we found a virtually complete absence of the formation of myogenic colonies on single myofibres from FDB muscles of TAM-treated Prmt5sKO mice (1.00±1.00, n=3) despite the presence of Pax7+ satellite cells (Fig. 4c). Genetic labelling of MuSC using a Rosa26lacZ reporter in which removal of a stop-lox cassette by Pax7CreERT2 resulted in activation of lacZ expression uncovered a marked reduction of lacZ-positive MuSC in regenerating muscle of Prmt5sKO mice 3 days after CTX injection (Ctrl 925±104; Prmt5sKO 212±6, each n=3; Fig. 4d), indicating that Prmt5 is required for MuSC proliferation during the early phase of injury-induced muscle regeneration. Additional lineage tracing of MuSC using a Rosa26YFP reporter revealed that Prmt5-deficient MuSC cells activated MyoD expression both on isolated myofibres and in single-cell cultures despite the failure to proliferate, suggesting that activation and proliferation of MuSC are not necessarily linked (Fig. 5a,b). However, activated, non-proliferative Prmt5-mutant MuSC failed to differentiate properly.
of isolated Prmt5\(^{\text{K}0}\) MuSC and induction of differentiation. Although we observed expression of the early differentiation marker MyoG in this experimental setting (Fig. 6b), differentiation of MuSC into MF20\(^{+}\) myotubes was essentially abrogated (Fig. 6c). We concluded that Prmt5 plays an additional role at a late stage of myogenic differentiation, independent of its function in proliferation and regulation of MyoG expression. Intriguingly, we also detected an increase of apoptosis in isolated Prmt5\(^{\text{K}0}\) MuSCs after induction of differentiation (Ctrl 0.27 ± 0.12%; Prmt5\(^{\text{K}0}\) 9.00 ± 1.75%, each \(n = 3\)), but not under conditions stimulating proliferation (Ctrl 0.08 ± 0.07%; Prmt5\(^{\text{K}0}\) 0.11 ± 0.11%, each \(n = 3\); Fig. 6d), suggesting that either Prmt5 promotes cell survival during differentiation of MuSC or that lack of proliferation before differentiation favours apoptosis.

**Prmt5 represses the cell cycle inhibitor p21 in MuSC.** To gain a better mechanistic understanding of the action of Prmt5 in MuSC and to identify genes that might be directly regulated by Prmt5, we performed transcriptome analysis in 4-OH-treated MuSC from control and Prmt5\(^{\text{K}0}\) mice by RNA-sequencing (RNA-seq; Supplementary Data 4). Gene ontology (GO)-term analysis revealed an up- or downregulation of ~500 genes (false discovery rate < 0.05) involved in cell cycle control, DNA metabolism and replication after inactivation of Prmt5 (Supplementary Fig. 3a,b), which is consistent with the proliferation defects observed in Prmt5-deficient MuSC\(^{27,21}\). The upregulation of the cell cycle inhibitor p21 attracted our particular attention\(^{22–24}\). qRT–PCR analysis of freshly isolated FACS-sorted MuSC confirmed a transcriptional inhibition of p21 by Prmt5 (Fig. 7a). In addition, we detected a clear upregulation of p21 in 4-OH-treated MuSC from Prmt5\(^{\text{K}0}\) mice (Fig. 7b) together with downregulation of CyclinB1, a p21 target gene, while transcription of myogenic factors including Pax7, MyoD and Myf5 was not altered (Fig. 7b). To investigate a potential direct repression of the p21 gene by Prmt5, we performed chromatin immunoprecipitation (ChIP) assays concentrating on four well-characterized regulatory regions of the murine p21 gene: upstream enhancer like region (En), p53 binding site (p53BS), transcriptional start site (TSS) and downstream intronic CpG island (CpG; Fig. 7c; refs 25,26). In control MuSC, Prmt5 was highly enriched at the En and p53BS but not at the TSS and CpG sites, which was lost after treatment of Prmt5\(^{\text{K}0}\) MuSC with 4-OH (Fig. 7d). Loss of Prmt5 binding caused a significant reduction of H3R8me2s at the p53BS site in Prmt5-deficient MuSCs (Fig. 7e), a significant loss of nucleosome occupancy at the TSS site (Fig. 7f) and increased H3K4 trimethylation at the p53BS (Fig. 7g), which is all consistent with suppression of p21 by Prmt5. Binding of Prmt5 to the p53BS prompted us to ask whether Prmt5 suppresses p21 expression by preventing recruitment of p53. Surprisingly, inactivation of Prmt5 prevented binding of p53 to the p53BS in the p21 locus, thereby suggesting p53-independent upregulation of p21 in Prmt5-deficient MuSCs (Fig. 7h). This conclusion was also supported by normal p53 mRNA and protein levels in Prmt5 mutant compared with control MuSCs, although we detected accumulation of Mdm4 splicing variants that were shown to stabilize p53 in Prmt5-deficient NPCs (Fig. 7i; ref. 19).

To investigate whether impaired proliferation of Prmt5-mutant MuSC is mediated by p21, we generated Prmt5\(^{\text{K}0}/\text{p}21{\text{K}0}\) compound mutant mice\(^{22}\). Intriguingly, we observed a significant increase of proliferation of MuSC isolated from Prmt5\(^{\text{K}0}/\text{p}21{\text{K}0}\) mice (25.25 ± 1.47%, \(n = 3\)) compared with Prmt5\(^{\text{K}0}\) mice (11.34 ± 1.50%, \(n = 3\); Fig. 8a). Furthermore, we found an increase of the number of myogenic colonies on 3-day cultured
Pax7CreERT2 Prmt5sKO/mdx cryosections. Error bars represent s.d.'s of the mean (diaphragm differences are apparent10. Hence, we wanted to know whether related by similar molecular cues, although a number of important widely assumed that embryonic and adult myogenesis are regul-
direct regulation of the p21 gene (Fig. 8c,d).

Prmt5 is also involved in the formation of skeletal muscles during embryonic muscle development. Similarly, lack of Prmt5-dependent mechanisms play a preeminent role in the
FDB myofibres in Prmt5sKO/p21−/− mice (22.40 ± 7.30%, n = 5) compared with Prmt5sKO mice (6.00 ± 2.16%, n = 4; Fig. 8b). However, inactivation of p21 in Prmt5 mutant mice failed to restore skeletal muscle regeneration, indicating that Prmt5 controls MuSC expansion and differentiation not exclusively by direct regulation of the p21 gene (Fig. 8c,d).

Prmt5 is dispensable for embryonic muscle development. It is widely assumed that embryonic and adult myogenesis are regulated by similar molecular cues, although a number of important differences are apparent10. Hence, we wanted to know whether Prmt5 does not only control MuSC and muscle regeneration but is also involved in the formation of skeletal muscles during development, in particular, since Prmt5 has been claimed to regulate expression of Myf5, MyoD, Myogenin and Mef2c during zebrafish myogenesis27. Therefore, we deleted the Prmt5 gene in the myogenic lineage using the constitutively active Pax7Cre knock-in mouse strain (Pax7Cre14). qRT–PCR analysis of FACS-sorted Pax7EGFP+ myogenic cells from Pax7Cre/Prmt5sKO/Prmt5loxP/loxP mutant embryos (hereafter referred to as Prmt5mKO) verified efficient inactivation of Prmt5 expression in embryonic muscle progenitor cells (data not shown). Analysis of control and Prmt5mKO mutant embryos at E9.5, E12.5 and E16.5 revealed no obvious defects in skeletal muscle formation (Fig. 9a). The normal presence of Pax7+ , MyoG+ and MF20+ cells in embryonic forelimbs of E12.5 and E14.5 Prmt5mKO embryos (Fig. 9b,c) suggested that loss of Prmt5 in Pax7+ myogenic progenitor cells neither affects their expansion nor differentiation during embryonic muscle development. Similarly, lack of Prmt5 had no effects on Pax7, MyoD and MyoG expression in forelimb and hindlimb muscles at E16.5 when Pax7+ muscle progenitor cells play an essential role for fetal muscle growth28 or on prenatal muscle growth until birth (Fig. 9d,e). Despite the absence of an apparent skeletal muscle phenotype, most Prmt5mKO mutants died around birth, which we attributed to the activity of Pax7Cre and consecutive loss of Prmt5 in the central nervous system (CNS)19.

Discussion
Our screen identified several novel potential regulators together with molecules that have already been documented to control the fate of MuSC. Prominent examples include Smad3 (ref. 29) and syndecan-4 (ref. 30). We also identified several epigenetic modifiers including Wdr91, the poly (ADP-ribose) polymerase Parp12 and Ash2l, a component of the Mll2 complex that mediates H3K4 methylation, which has been shown to form a complex with the transcription factor Pax7 to regulate Myf5 expression and satellite cell proliferation12,31,32. The histone arginine methyltransferase Prmt5 attracted our particular attention also because we identified several known interaction partners of Prmt5 in the screen including Myd88 (ref. 33) and Mapk13 (also known as p38delta, a component of the mitogen-activated protein (MAP) kinase pathway34, suggesting that Prmt5-dependent mechanisms play a preeminent role in the regulation of MuSC proliferation and differentiation. During adulthood MuSC mostly exist in a resting, quiescent state but must be able to expand rapidly in order to regenerate damaged muscle tissue. Relaxed control of quiescence might lead

**Figure 3 | Continuous muscle regeneration depletes the MuSC pool of Prmt5sKO mice.** (a) Schematic outline of the TAM administration and analysis of wild-type (WT), Prmt5sKO, mdx and Prmt5sKO/mdx mice (n = 3, each). (b–d) Prmt5sKO/mdx mice (n = 3) show lower body weight (b,c) and thinner diaphragm (d) compared with WT (n = 3), mdx (n = 3) and Prmt5sKO (n = 3) littermates 3 months after TAM administration. Scale bar, 100 μm (d). (e) MRI measurements of decreased and increased muscle volume (brown colour) in Prmt5sKO/mdx and mdx mice, respectively, 3 months after TAM administration (WT and mdx, n = 5 each; Prmt5sKO, n = 3; Prmt5sKO/mdx, n = 4). Quantification of muscle volume normalized to tibia length is shown on the right. Error bars represent s.d.’s of the mean (t-test: ***P < 0.0001; **P < 0.001; NS, P > 0.05). (f) Decreased numbers of Pax7+ cells in TA muscles of Prmt5sKO/mdx mice (n = 5) compared with WT (n = 5 and mdx (n = 3) littermates. The number of Pax7+ cells per 10 mm2 area was counted on cryosections. Error bars represent s.d.’s of the mean (t-test: *P < 0.01; NS, P > 0.05). Control (Ctrl): Pax7CreERT2+/−/Prmt5sKO/Prmt5sKO/mdx; Pax7CreERT2+/−/Prmt5sKO/loxP, NS, not significant.
to over-proliferation, depletion of the stem cell pool and might favour tumour formation. Failure to respond appropriately to proliferative cues will impair self-renewal of MuSC and compromise regeneration. Prmt5 seems to be a decisive component of the regulatory network that maintains this intricate balance and keeps MuSC in a poised standby mode (Fig. 10). In contrast, embryonic myogenesis is characterized by the rapid expansion of myogenic progenitor cells, which need to form skeletal muscles in a relatively short time period alleviating the need to enter a quiescent, non-proliferative state. Hence, it makes sense that the role of Prmt5 in the regulation of cell proliferation differs significantly between embryonic and adult myogenesis, whereas the control of muscle lineage determination and differentiation seems to follow a similar pattern.

A major function of Prmt5 for conferring a reversible resting state to MuSC is apparently the restriction of p21 expression. Reduced expression of Prmt5 in MuSC will result in upregulation of p21, which increases the threshold for cell cycle re-entry (Fig. 10). Although we detected Prmt5 by immunofluorescence in virtually all MuSC, its level of activity and hence regulation of p21 might vary, thereby contributing to the heterogeneity of MuSC. MuSC with lower Prmt5 activity might constitute a reserve population that is only activated under severe stress conditions. Alternatively, differential regulation of Prmt5 activity in asymmetrically dividing MuSC might distinguish cells returning to quiescence from those that undergo rapid expansion. Careful quantitative evaluation of Prmt5 activity in single MuSC will solve these questions in the future.

We do not claim that the epigenetic repression of p21 is the only mechanism by which Prmt5 arrests MuSC proliferation, in particular, since RNA-seq analysis identified several additional cell cycle regulators that might also be regulated by Prmt5 either directly or indirectly. Inactivation of p21 in Prmt5-deficient MuSC failed to rescue muscle regeneration fully, although proliferation of MuSC could be partially restored, indicating different modes of action of Prmt5 independent of p21. In fact, additional functions of Prmt5 in the regulation of progenitor cell behaviour have been reported previously. In embryonic stem (ES) cells, Prmt5 promotes pluripotency by modulating the cytoplasmic LIF/Stat3 signalling pathway, indirectly suppressing genes...
Figure 5 | Prmt5 is required for differentiation of MuSC. (a,c) Immunofluorescence analysis of double YFP+/MyoD+ cells (a) and double YFP+ and MyoG+ cells (c) on isolated FDB myofibres of control and Prmt5sKO mice after 3-day culture (n = 3, each). Scale bar, 50 μm. Total numbers of Pax7+ colonies from 100 myofibres are counted. Quantifications are shown on the right. Error bars represent s.d.’s of the mean (t-test: **P < 0.01, ***P < 0.001). (b) Immunofluorescence staining of FACS-purified YFP+ cells cultured for 3 days in proliferation medium for MyoD (n = 3, each). Scale bar, 50 μm. The percentage of MyoD+ cells is shown on the right. Error bars represent s.d.’s of the mean (t-test: NS, P > 0.05). (d) Immunofluorescence staining for MyoG of FACS-purified YFP+ cells cultured for 3 days in proliferation medium followed by 2 days in differentiation medium (n = 4, each). Scale bar, 50 μm. The percentage of MyoG+ cells is shown on the right. Error bars represent s.d.’s of the mean (t-test: ****P < 0.0001). (e) Immunofluorescence images of MF20+ myotubes differentiated from FACS-purified YFP+ cells cultured for 3 days in proliferation medium followed by 2 days in differentiation medium (n = 3, each). Scale bar, 50 μm. The area of MF20+ cells is shown on the right. Error bars represent s.d.’s of the mean (t-test: ***P < 0.001). Control (Ctrl): Pax7CreERT2+/+/Prmt5+/loxP, Prmt5sKO: Pax7CreERT2+/−/Prmt5loxP/loxP.
that are associated with ES cell differentiation. In NPCs, Prmt5 regulates alternative splicing of Mdm4 that in turn stabilizes p53, which causes upregulation of p21 and inhibition of cell cycle progression. Superficially, the findings in NPC appear to partially recapitulate the situation in MuSC, but a more careful analysis reveals fundamental differences in the mode of action. Inactivation of Prmt5 in MuSC does not change the mRNA and protein level of p53, although alternative splicing of Mdm4 was altered. Furthermore, we found that binding of p53 to the p21 locus was lost after inactivation of Prmt5, indicating that activation of p21 in Prmt5-deficient MuSC does not depend on p53.

Although suppression of MuSC expansion by upregulation of p21 dominated the phenotype of Prmt5KO mice, lineage-tracing experiments revealed that Prmt5-deficient MuSC failed to express myogenin, indicating that Prmt5 mutant MuSCs are unable to differentiate and form myofibres in vivo. This conclusion was also supported by the failure of MuSC to form myotubes even when Prmt5 was deleted after initiation of MyoD expression, a phenomenon that was also observed in C2C12 myoblasts. In addition, the timed inactivation of Prmt5 in differentiating MuSC suggests an additional role for terminal myogenic differentiation after expression of MyoG has commenced. Interestingly, induction of differentiation of Prmt5 mutant MuSC triggered apoptosis, which might be related to the differentiation block and contribute to the loss of MuSC during regeneration and ageing.

Our study revealed that inactivation of Prmt5 in MuSC of mdx mice resulted in a severe loss of muscle volume and recapitulated several symptoms of human Duchenne muscular dystrophy within 90 days. The findings emphasize the pivotal role of MuSC in maintaining muscle mass under disease conditions. A similar phenotype was described recently using mdx mice completely lacking telomerase activity (mdx/mTR2G mice). However, in mdx/mTR2G mice, a massive atrophy of the diaphragm was only visible after 60 weeks, indicating that the lack of Prmt5 had more severe consequences in MuSC function than loss of telomerase activity. We believe that Prmt5KO/mdx mice might serve as a valuable model to study effects of therapeutic interventions on dystrophin-deficient myofibres without the interference of MuSC constantly replenishing lost or damaged myofibres.

Remarkably, muscle mass remained rather stable in Prmt5KO mice under physiological conditions for at least 3 months despite a significant decline of the number of MuSC and the failure of MuSC to expand. This finding allows two conclusions: (i) under physiological conditions, MuSC contribute only to a minor degree to the maintenance of muscle mass; and (ii) a significant proportion of MuSC undergoes self-renewal during a 3-month period. However, the second conclusion has to be viewed with caution, since it is possible that the lack of Prmt5 induces cell death of MuSC without prior activation and induction of proliferation, although we did not find evidence for such a scenario in our experiments. In the future, it will be interesting to further exploit the Prmt5KO model (Fig. 10) to study the role of
Figure 7 | Epigenetic silencing of the cell cycle inhibitor p21 by Prmt5. (a) RT-qPCR analysis of p21 upregulation in isolated quiescent satellite cells of Prmt5<sup>−/−</sup> mice (n = 4, each). (b) RT-qPCR analysis of Prmt5, p21, cyclinB1 and myogenic factors after Prmt5 inactivation by addition of 4-OH-TAM (n = 3, each). Expression levels of different mRNAs were normalized to GAPDH mRNA. Error bars indicate s.d. of the mean (*P < 0.05; **P < 0.01; NS, P > 0.05). (c) Schematic outline of the localization of four important regulatory regions in the p21 gene locus: enhancer-like (En), p53 binding site (p53BS), transcriptional start site (TSS) and intronic CpG island (CpG). Primer pairs used for ChIP assays are indicated by arrows. The p53 consensus motif of the murine and human p21 gene locus is shown. (d–h) Quantitative PCR analyses of ChIP using antibodies against Prmt5 (d), H3R8me2s (e), histone H3 (f), H3K4me3 (g) and p53 (h) at indicated regulatory regions of the p21 gene locus in MuSC of Prmt5<sup>−/−</sup> and control mice (n = 3, each). Relative (Rel.) enrichment of Prmt5, H3 and p53 was normalized to input DNA. Enrichment of H3R8me2 and H3K4me3 was normalized to histone H3. Error bars represent s.d.’s of the mean (*P < 0.01; **P < 0.05; NS, P > 0.1). (i) RT-qPCR analysis of p53 mRNA levels in MuSC of Prmt5<sup>−/−</sup> mice and control littermates (n = 3, each). Error bars represent s.d.’s of the mean (*P < 0.05). (j) Semi-quantitative RT-PCR analysis of different Mdm4 splicing isoforms in MuSC of Prmt5<sup>−/−</sup> mice and control littermates. RT-PCR using primers in the 3′ untranslated region of Mdm4 detecting both Mdm4fl and Mdm4s was used as loading control. RT-PCR-mediated detection of m36B4 served as an additional loading control. Control (Ctrl): Pax7CreERT2<sup>+/−</sup> / Prmt5<sup>+/loxP</sup>; Prmt5<sup>−/−</sup>: Pax7CreERT2<sup>+/−</sup> / Prmt5<sup>loxP/loxP</sup>. NS, not significant; WT, wild type.
MuSC in muscle dystrophies, analyse their function in muscle hypertrophy or to block proliferation of tumour cells in rhabdomyosarcomas.

Methods

Animals. The Prmt5loxPLOxP mouse strain was obtained from EUCOMM. Rosa26RosaYFP and C57BL/10ScSn-Dmdmdx/J (mdx) mouse strain was obtained from The Jackson Laboratory (Bar Harbor, ME). Generation of Pax7ICN(Rosa26nlacZ and Pax7CreERT2(TA (CTX lower panel) muscles of control, **p < 0.01; NS, P > 0.05). (b) Increased numbers of Pax7+ colonies on Prmt5KO/loxP/loxP mice (n = 3, each). Error bars represent s.d.'s of the mean (t-test: ****P < 0.0001; **P < 0.01; NS, P > 0.05). (c) Representative macroscopic images of non-injured and injured TA muscles of control, p21−/−, Prmt5KO and Prmt5KO/p21−/− mice (n = 3, each) 14 days after CTX injection. Scale bar, 1 cm. (d) Haematoxylin and eosin (H&E) staining of muscle section from non-injured (upper panel) and injured TA (CTX lower panel) muscles of control, p21−/−, Prmt5mice (n = 3, each) 14 days after CTX injection. Control (Ctrl): Pax7CreERT2+/+/Prmt5+/+YFP, Prmt5KO; Pax7CreERT2+/−/Prmt5+/+YFP. Scale bar, 50 μm.

Myofibre isolation and MuSC purification. The FDB muscles were isolated and digested with 0.2% collagenase P (Roche) in DMEM medium. The isolated myofibres were either fixed directly with 4% paraformaldehyde (PFA) or fixed after 3-day culturing in DMEM medium with 20% fetal calf serum (FCS) and basic fibroblast growth factor (bFGF) (5 ng ml−1). Satellite cell isolation and purification were performed according to established methods6. Briefly limb and trunk muscles were minced, digested with 100 CU Dispase (BD) and 0.2% type II collagenase (Worthington Biochemicals), and consecutively filtered through 100-, 70- and 40-μm cell strainers (BD). Cells were applied to a discontinuous Percoll gradient consisting of 70% Percoll overlayed with 30% (vol/vol) Percoll. Mononuclear cells were collected at the 70/30 interphase and subjected to FACS (BD FACSAriaII) assays to monitor apoptosis were carried out with the In Situ Cell Death Detection Click-iT EdU kit (Invitrogen) according to the manufacturer’s protocol. TUNEL assays to monitor apoptosis were carried out with the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s protocol. For Cre recombinase-mediated in vitro ablation of Prmt5, cultured satellite cells were treated with 4-OH-TAM (0.4 mM, Calbiochem) for 4 days and were analysed 5 days later. Lentiviruses expressing the coding region of human Prmt5, cultured satellite cells were treated with 4-OH-TAM (0.4 mM, Calbiochem) for 4 days and were analysed 5 days later. Lentiviruses expressing the coding region of human Prmt5 were generated with a modified lentiviral vector derived from pIko.1 (Sigma-Aldrich) in HEK293T cells using the helper plasmids pMD2.G and psPAX2, and used for infection of MuSC.

Immunofluorescence and morphological analysis. Cultured cells and myofibres were fixed in 4% PFA. Frozen muscle sections (5–10 μm) were fixed in cold 4% PFA. Frozen muscle sections (5–10 μm) were fixed in cold acetone. Primary antibodies for immunohistochemical staining are shown in
Figure 9 | Prmt5 is dispensable in embryonic Pax7\(^{+}\) muscle progenitor cells. (a) Representative images of control and Prmt5\(^{\text{mKO}}\) embryos at embryonic day E12.5, E14.5 and E16.5. (n = 3, each). Scale bar, 10 mm. (b,c) Immunofluorescence images of cryosectioned forelimbs and hindlimbs. (b) E12.5 frontal sections: upper panel; E14.5 sagittal sections: lower panel, Pax7 (green), myogenin (red), DAPI (blue). Scale bar, 50 \(\mu\)m. (c) E12.5 frontal sections: upper panel; E14.5 sagittal sections: lower panel, MF20 (green), DAPI (blue). Scale bar, 50 \(\mu\)m. (d,e) Immunofluorescence of cryosections from hindlimbs. E16.5 transverse sections: upper panel and forelimbs. E16.5 frontal sections: lower panel, Pax7 (green), myogenin (red; d), and Pax7 (green) and MyoD (red; e). DNA is stained by DAPI (blue). Scale bars, 50 \(\mu\)m. Scale bars in inserts 20 \(\mu\)m. Control (Ctrl): Pax7CreERT2\(^{+/−}\)/Prmt5\(^{+/−}\)/Prmt5\(^{loxP/loxP}\). Pax7CreERT2\(^{+/−}\)/Prmt5\(^{loxP/loxP}\).
Prmt5 in proliferating MuSC does not suppress MyoG expression but blocks differentiation, thereby suggesting an additional function of Prmt5 for muscle cell differentiation. Proliferating fetal muscle progenitor cells are defined by expression of Pax7 and MyoD, quiescent MuSC by expression of Pax7 and lack of MyoD expression, proliferating MuSC by the concomitant expression of Pax7 and MyoD, and differentiating muscle cells by expression of MyoG. Differentiated muscle cells are marked by expression of myosin heavy chain (MyHC).

**Figure 10 | Model of the role of Prmt5 during fetal myogenesis and adult muscle regeneration.** Prmt5 controls proliferation of adult MuSC by direct epigenetic silencing of the cell cycle inhibitor p21 independent of p53, but is dispensable for proliferation and differentiation of Pax7⁺ muscle progenitor cells during fetal myogenesis. Prmt5 does not affect the initial activation of MuSC resulting in MyoD expression but enables proliferation of MuSC, thus generating a poised state, which keeps MuSC in a standby mode allowing rapid MuSC amplification under disease conditions. Inactivation of Prmt5 during fetal myogenesis. Prmt5 does not affect the initial activation of MuSC resulting in MyoD expression but enables proliferation of MuSC, thus generating a poised state, which keeps MuSC in a standby mode allowing rapid MuSC amplification under disease conditions. Inactivation of Prmt5 during fetal myogenesis.
GenElute HP 96-Well Miniprep Kits (Sigma-Aldrich) and tested for integrity with a P200 digest. High-throughput production of lentivirus was performed by 
(P300) transfection of HEK293T cells with helper plasmids pMDGX G and pSPAX2. Four-hundred fifty freshly isolated satellite cells per well were seeded into 384-well tissue culture plates freshly coated with Matrigel (Greiner). Twenty-four hours later, cells were transfected with lentiviral supernatants supplemented with 8 μg/mL polybrene for 6 h. After media exchange, cells were incubated for 72 h. Each 384-well plate contained 56 controls including 12 individual GFP-producing lentiviruses to monitor transduction efficiency, four positive controls for Pax7 knockdown (shRNA Pax7) and four positive controls for Nf1 knockdown (shRNA Nf1). Cells were fixed in 4% PFA and whole-well images were acquired and analysed using an ImageXpress Micro automated high-throughput fluorescence microscope and MetaXpress software (Molecular Devices). Pax7/DAPI ratios were determined for each individual well. Only plates with Z’ values > 0.5 according to 1-2(POSZ’ + 2μAPL/POS - μNEG)/2μNEG were used for further processing. Values as reference for Pax7 expression in percentage were then calculated according to z = (x̄/μ) − (1) (x, value of particular sample; μ, mean of plko1 empty vector; n = 4). Target genes qualified as hits if percentiles were higher or lower than 25% compared with control.

Magnetic resonance imaging. All MRI experiments were performed on a 7.0-T superconducting magnet (Bruker Biospin, Pharmascan, 70/16, 16 cm; Ettlingen, Germany) equipped with an actively shielded imaging gradient field of 300 mT m⁻¹ (ref. 50). The frequency for the ¹H isotope is 300.33 MHz. A 60-mm inner diameter linear-polarized ¹H volume resonator was used for RF pulse transmission and signal reception (Bruker Biospin). Localized images were acquired using a spin-echo sequence and corrections of slice angulation were performed, if necessary. RARE (Rapid Acquisition with Relaxation Enhancement) sequences (repetition time (TR) = 2,500 ms, echo time (TE) = 36.7 ms, slice thickness = 1 mm) in axial and coronal orientation were used to determine exact positioning of the lower part of the mouse body. A coronal MSME (Multi-Slice Magnetic Resonance Magnification echo-sequences with an echo time TE = 8.6 ms, repetition time TR = 453 ms, a field of view FOV = 7 × 7 cm², matrix size MTX = 512 × 256 and a slice thickness of 1 mm was recorded. Volumetric quantification of fat and muscle tissue from images was processed by software ImageJ. A list of anatomically defined landmarks was used to derive tissue-specific signal intensity thresholds and to define the region of interest for intensity sensitive region growing segmentation. The resulting tissue voxel volumes inside the region of interest were determined as cubic millimetres for each tissue class. Mice were measured under volatile anaesthesia; the body temperature was maintained at 37 °C by a thermostatically regulated water flow system during the entire imaging protocol.

Statistics. For statistical analysis, the two following tests (two-tailed) were used: (1) three or more groups: one factorial ANOVA; (2) two groups: unpaired t-test. P values <0.05 were considered statistically significant. Data were analysed using GraphPad Prism v5.03 (GraphPad Software, San Diego, CA).

References

1. Wilson, A. Heteroplastic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 135, 1118–1128 (2008).
2. Brack, A. S. & Rando, T. A. Tissue-specific stem cell lessons from the skeletal muscle satellite cell. Cell Stem Cell 10, 504–514 (2012).
3. Schienda, J. et al. Somitic origin of limb muscle satellite and side population cells. Proc. Natl Acad. Sci. USA 103, 945–950 (2006).
4. Seale, P. et al. Pax7 is required for the specification of myogenic satellite cells. Nature 435, 948–953 (2005).
5. Gunther, S. et al. Myf5-positive satellite cells contribute to Pax7-dependent long-term maintenance of adult muscle stem cells. Cell Stem Cell 13, 590–601 (2013).
6. Lepper, C., Conway, S. J. & Fan, C. M. Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements. Nature 460, 627–631 (2009).
7. Oustanina, S., Hause, G. & Braun, T. Pax7 is required for the specification of myogenic satellite cells. Nature 460, 627–631 (2009).
8. Montarras, D. et al. Direct isolation of satellite cells for skeletal muscle regeneration. Science 309, 2064–2067 (2005).
9. Oustanina, S., Lepper, C., Coffin, C. M. & Braun, T. M. Pax3/Fkh3 function: implications for alveolar rhabdomyosarcoma cell of origin. Genes Dev. 18, 2608–2613 (2004).
10. Conboy, M. J., Cerletti, M., Wagers, A. J. & Conboy, I. M. Immuno-analysis and FACS sorting of adult muscle fiber-associated stem/precursor cells. Methods Mol. Biol. 621, 165–173 (2010).
11. Amacher, S. L. & Lassar, A. B. Pax7 directs postnatal renewal and differentiation of muscle stem cells. Genes Dev. 22, 2772–2777 (2010).
12. Reimann, A. et al. Regulation of constitutive and alternative splicing by PRMT5 reveals a role for Mdm4 pre-mRNA in sensing defects in the spliceosomal machinery. Gene Dev. 27, 903–1906 (2013).
13. Sacco, A. et al. Short telomeres and stem cell exhaustion model Duchenne muscular dystrophy in mdx/mTR mice. Cell 143, 1059–1071 (2010).
14. Dacwag, C. S., Ohkawa, Y., Pal, S., Sif, S. & Imbalzano, A. N. The protein arginine methyltransferase Prmt5 is required for myogenesis because it facilitates ATP-dependent chromatin remodeling. Mol. Cell. Biol. 27, 384–394 (2007).
15. Spencer, S. L. et al. The proliferation-quiescence decision is controlled by a bimaf2 activity with mitotic exit. Cell 155, 369–383 (2012).
16. Parker, S. B. et al. P53-independent expression of p21cip1 in muscle and other terminally differentiating cells. Science 267, 1024–1027 (1995).
17. Kanade, S. R. & Eckert, R. L. Protein arginine methyltransferase 5 (PRMT5) control differentially myogenesis in zebrafish. PLoS ONE 6, e25427 (2011).
18. Dacwag, C. S. et al. P53 signaling is required for satellite cell function and myogenic differentiation of myoblasts. Cell 121, 1591–1604 (2011).
19. Anderson, C. P. et al. Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration. Genes Dev. 18, 2231–2238 (2004).
20. McKinell, I. W. et al. Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. Nat. Cell Biol. 10, 77–84 (2008).
21. Kawabe, Y., Wang, Y. X., McKinell, I. W., Bedford, M. T. & Rudnicki, M. A. CARM1 regulates Pax7 transcriptional activity through MLL1/2 recruitment during asymmetric satellite stem cell divisions. Cell Stem Cell 11, 333–345 (2012).
22. Wang, T. et al. Adult muscle fiber-associated stem/precursor cells. BMC Dev. Biol. 4, 238 (2004).
23. Shi, X. & Garry, D. J. Muscle stem cells in development, regeneration, and disease. Genes Dev. 20, 1692–1708 (2006).
24. Shen, H. et al. P53 signaling suppresses protein kinase Cdelta- and p38delta-dependent signaling and keratinocyte differentiation. J. Biol. Chem. 287, 7313–7323 (2012).
25. Law, T. J. & Gaultel, M. Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis. Nat. Rev. Mol. Cell Biol. 12, 349–361 (2011).
26. Shi, X. & Garry, D. J. Muscle stem cells in development, regeneration, and disease. Genes Dev. 20, 1692–1708 (2006).
27. Dacwag, C. S., Bedford, M. T., Sif, S. & Imbalzano, A. N. Distinct protein arginine methyltransferases promote ATP-dependent chromatin remodeling function at different stages of skeletal muscle differentiation. Mol. Cell. Biol. 29, 1909–1921 (2009).
28. Sherwood, R. I. et al. Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafing skeletal muscle. Cell 119, 543–554 (2004).
41. Dahl, J. A., Reiner, A. H. & Collas, P. Fast genomic muChIP-chip from 1,000 cells. *Genome Biol.* **10**, R13 (2009).
42. Davis, M. P., van Dongen, S., Abreu-Goodger, C., Bartonicek, N. & Enright, A. J. Kraken: a set of tools for quality control and analysis of high-throughput sequence data. *Methods* **63**, 41–49 (2013).
43. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
44. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
45. Dennis, Jr. G. *et al.* DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.* **4**, P3, 2003.
46. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V. & Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **1**, 2856–2860 (2006).
47. Rappsilber, J., Ishihama, Y. & Mann, M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* **75**, 663–670 (2003).
48. Cox, J. *et al.* A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat. Protoc.* **4**, 698–705 (2009).
49. Moffat, J. *et al.* A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* **124**, 1283–1298 (2006).
50. Biesemann, N. *et al.* Myostatin regulates energy homeostasis in the heart and prevents heart failure. *Circ. Res.* **115**, 296–310 (2014).
51. Kossler, N. *et al.* Neurofibromin (Nf1) is required for skeletal muscle development. *Hum. Mol. Genet.* **20**, 2697–2709 (2011).

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**Author contributions**

T.Z. performed the research and analysed the data; T.Z., Y.Z. and J.K. designed and performed experiments, analysed the data and wrote the manuscript together with T.B.; S.G. assisted in the design of the experiments and performed experiments; M.K. provided the mass spectrometry analysis; M.L. and C.K. performed the bioinformatics analysis and analysed RNA-sequencing data; J.K. and T.B. conceived and supervised the project together with Y.Z.; and T.B. edited the final version of the manuscript.

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