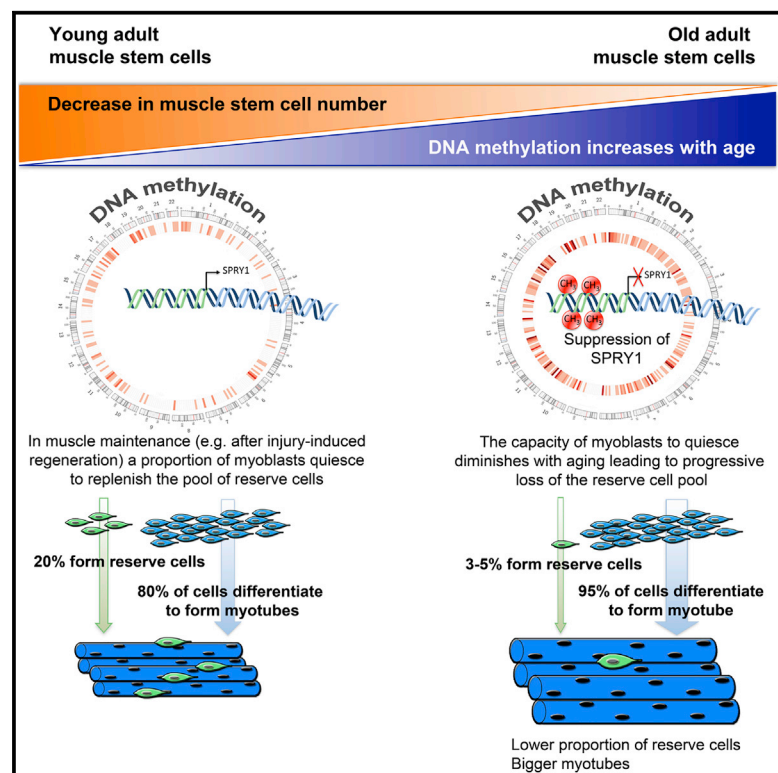


## Age-Associated Methylation Suppresses *SPRY1*, Leading to a Failure of Re-quiescence and Loss of the Reserve Stem Cell Pool in Elderly Muscle

### Graphical Abstract



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### In Brief

Loss of muscle strength in old age is linked to diminution of the muscle stem cell pool. Bigot et al. show that the age-associated increase in global DNA methylation acts through the *SPRY1* pathway to suppress human muscle stem cell entry into quiescence, thus impairing self-renewal of the stem cell pool.

### Highlights

- The capacity of human muscle stem cells to enter quiescence diminishes with age
- This reduced capacity to re-quiesce is associated with increased DNA methylation
- DNA methylation suppresses *SPRY1*, a known regulator of quiescence
- Senescence, a feature of late cell division counts, is not increased with age

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# Age-Associated Methylation Suppresses *SPRY1*, Leading to a Failure of Re-quiescence and Loss of the Reserve Stem Cell Pool in Elderly Muscle

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## SUMMARY

The molecular mechanisms by which aging affects stem cell number and function are poorly understood. Murine data have implicated cellular senescence in the loss of muscle stem cells with aging. Here, using human cells and by carrying out experiments within a strictly pre-senescent division count, we demonstrate an impaired capacity for stem cell self-renewal in elderly muscle. We link aging to an increased methylation of the *SPRY1* gene, a known regulator of muscle stem cell quiescence. Replenishment of the reserve cell pool was modulated experimentally by demethylation or siRNA knockdown of *SPRY1*. We propose that suppression of *SPRY1* by age-associated methylation in humans inhibits the replenishment of the muscle stem cell pool, contributing to a decreased regenerative response in old age. We further show that aging does not affect muscle stem cell senescence in humans.

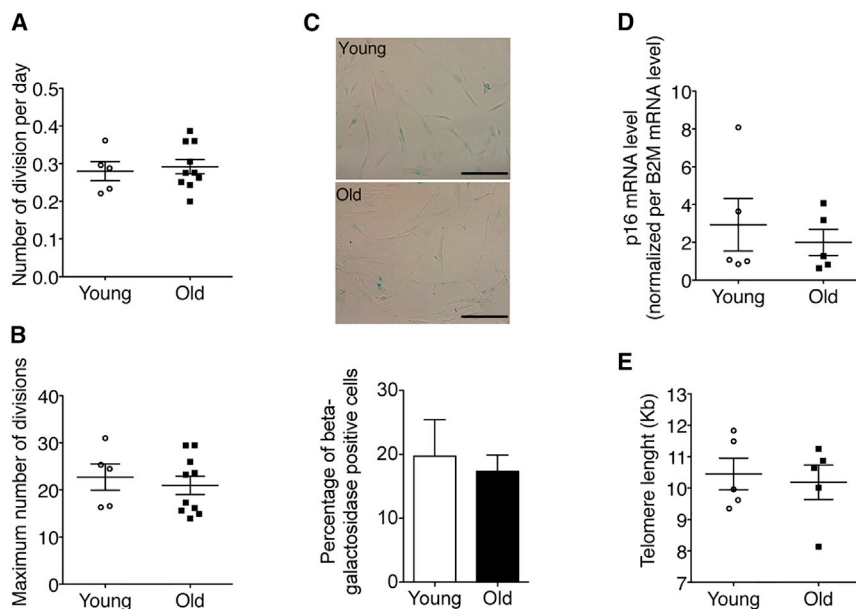
## INTRODUCTION

Aging is characterized by a progressive decline in the physiology and turnover of adult tissues. Tissue renewal requires stem cells, which show declining functional properties with age (Pollina and Brunet, 2011; Signer and Morrison, 2013). Over a lifetime, adult tissues present an accumulation of cellular damage as follows: genomic mutations, epigenetic alterations, mitochondrial dysfunctions, imbalance of protein synthesis and degradation, telomere shortening, accumulation of senescent cells, and altered intercellular communication (see López-Otín et al., 2013 for review). In skeletal muscle, aging is characterized by a decline in mass and strength due to a decrease in the number, size, and quality of contractile myofibers (Klein et al., 2003; Nilwik et al.,

2013), as well as a loss of regenerative capacity (Pollina and Brunet, 2011).

Once activated, the muscle stem cell divides asymmetrically to maintain a pool of muscle precursor cells and produce a progeny that will fuse with damaged myofibers to form new muscle contractile tissue (Conboy and Rando, 2002; Olguin and Olwin, 2004; Zammit et al., 2004). This process is at least partly cell autonomous since muscle stem cells in vitro can fuse to form myotubes while a minority remain undifferentiated as reserve cells (Zammit et al., 2006). The decline of the muscle regenerative capacity with age (Carlson et al., 2008) has been attributed to a decline in the number of muscle stem cells in mouse (Brack et al., 2005; Chakkalakal et al., 2012; Collins et al., 2007; Conboy et al., 2005) and humans (Malmgren et al., 2000; Renault et al., 2002), and also to extrinsic environmental influences and to the intrinsic regenerative potential of the cells themselves (Collins et al., 2007; Conboy et al., 2005). When old murine muscle stem cells are exposed to a young environment or to growth factors, their capacities to proliferate and differentiate are partly restored (Brack et al., 2007; Collins et al., 2007; Conboy et al., 2005), suggesting that functional deregulations with age may be reversible.

Loss of the stem cell population with aging may involve cell death, although this has never been evidenced, or a progressive loss of the cell's potential to self-renew. A decline of muscle stem cell function also was associated with a process of senescence in geriatric mice (Sousa-Victor et al., 2014). Multiple regulatory mechanisms determine stem cell fate (Collas, 2010; Krishnakumar and Bltelloch, 2013), including the epigenetic status, which is defined by DNA and histone methylation as well as the expression of regulatory RNAs (Krishnakumar and Bltelloch, 2013). Although DNA methylation regulates gene expression (Jones, 2012) and correlates with aging (Bocklandt et al., 2011; Horvath, 2013) in many tissues, little is known about muscle stem cell methylation status with aging and the mechanism(s) by which it could regulate stem cell fate. Here we demonstrate that age-associated changes in methylation of a quiescence regulator,



**Figure 1. Muscle Stem Cells from Young and Elderly Subjects Have Similar Characteristics of Proliferation and Senescence**

(A) Rate of division and (B) maximum division number were similar in myogenic precursors derived from young and elderly subjects, as determined by cell counts throughout culture, until proliferative arrest ( $n = 5$ – $10$  subjects per group). (C) Similar levels of  $\beta$ -galactosidase, a marker of senescence. (Top) Representative image shows  $\beta$ -galactosidase staining. (Bottom) Percentage of  $\beta$ -galactosidase-positive cells is shown ( $n = 3$  subjects per group). Scale bar,  $50 \mu\text{m}$ .

(D) Levels of p16 expression, quantified by qRT-PCR and with results normalized to B2M transcript level, are shown ( $n = 5$  subjects per group).

(E) Telomere length, as measured by qRT-PCR at five to ten divisions, is shown ( $n = 5$  subjects per group).

Each data point represents a single muscle progenitor culture derived from one subject. Values are means  $\pm$  SEM. See also [Figure S1](#).

*SPRY1*, cause a failure of re-quiescence in activated stem cells, leading to a decline of the stem cell pool in elderly human muscle.

## RESULTS

### The Replicative Potential of Muscle Stem Cells Is Unaffected by Age

Cultures isolated from muscle biopsies of elderly subjects presented less myogenic cells (desmin-expressing cells; [Figure S1A](#)) than those of young ones. We tested whether this could be explained by earlier replicative senescence or cell death. For this purpose, we enriched the CD56-positive myogenic population up to 82%–99% purity ([Figure S1B](#)), and we investigated the proliferative potential and markers of senescence.

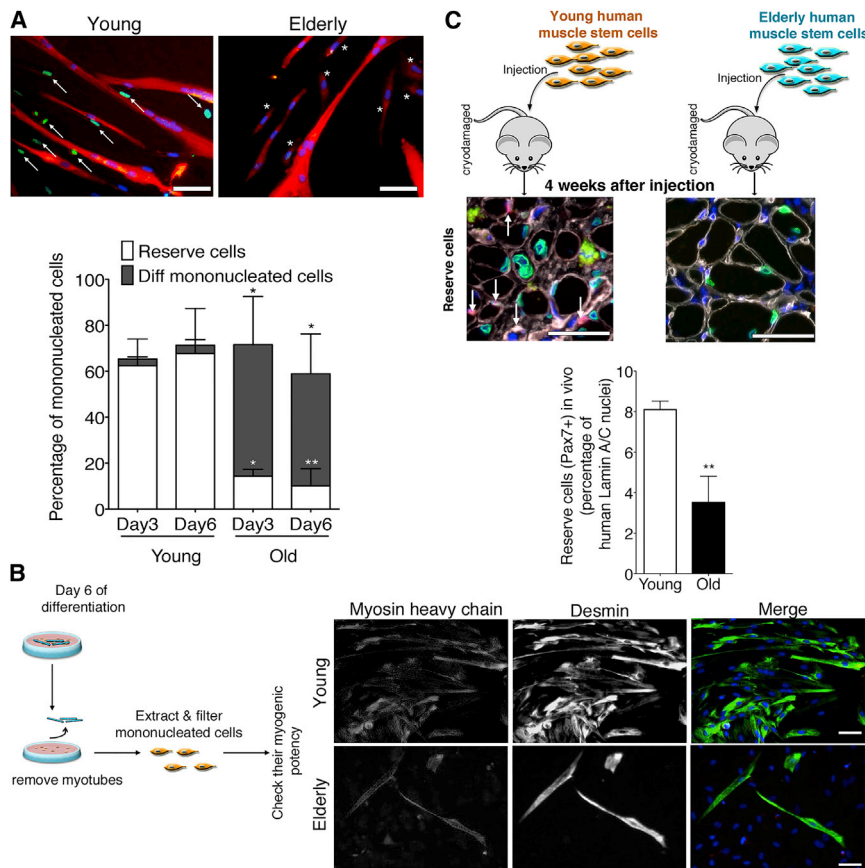
The capacity of elderly muscle precursor cells to proliferate was equal to that of young subjects: muscle precursors underwent the same number of divisions per day ([Figure 1A](#)) and divided homogeneously, reaching a similar number of generations after 5 days (generation 7; [Figure S1C](#)). In contrast to what is described in muscle stem cells of geriatric mice ([Sousa-Victor et al., 2014](#)), there was no greater propensity toward senescence in human elderly cells, as we observed a comparable replicative lifespan ([Figure 1B](#)), a similar percentage of  $\beta$ -galactosidase-positive cells (a marker of senescence; [Figure 1C](#)), an unaltered expression of p16 (which can hamper the proliferative capacity of human myoblasts; [Zhu et al., 2007](#); [Figure 1D](#)), and equal telomere length ([Figure 1E](#)). This is consistent with our previous work ([Barberi et al., 2013](#)) showing that both young and aged human muscle stem cells exhibit increasing expression of p16 and shortening of telomeres throughout their replicative lifespan, which terminates at around 20 cell divisions independently of subject age. Cell death was unaffected by age, both during proliferation (less than

4% of cell death in both age groups) and at the exit of the cell cycle toward differentiation (similar level of PARP-1 cleavage; [Figure S1D](#)).

We conclude that human muscle stem cells maintain their proliferative capacity with aging, and are prone to neither proliferative senescence nor cell death. Importantly, all subsequent analyses were done in the first half of the lifespan, at division counts of less than 12.

### Self-Renewal Is Impaired in Elderly Muscle Precursors In Vitro

Since cell death or senescence was ruled out, we hypothesized that the reduced size of the muscle stem cell population with age is due to a loss of its capacity to self-renew. Reflecting muscle regeneration in vivo, the majority of muscle stem cells in vitro fuse to form differentiated plurinucleated myotubes, while a minority do not fuse but self-renew to constitute the pool of reserve cells that express PAX7, a marker of muscle progenitor fate ([Zammit et al., 2006](#)). We observed that cultures of elderly muscle stem cells generated a significantly lower proportion of reserve (PAX7-expressing) cells at both 3 and 6 days of differentiation ([Figure 2A](#)). Although mononucleated cells were observed in elderly cultures, most of these were positive for myosin heavy chain (MyHC), a marker of engagement in differentiation, and did not express PAX7 ([Figure 2A](#)). To test whether these MyHC-expressing elderly cells retained their capacity to expand and differentiate, fused myotubes were removed by differential trypsinization and mononucleated cells were re-plated. Upon re-plating, elderly mononucleated cells presented a low proportion of desmin-expressing myogenic cells, 2%–20%, in contrast to up to 90% in young cultures ([Figure 2B](#)), indicating that, in elderly culture, mononucleated cells positive for MyHC were terminally differentiated and unable to reattach and proliferate, thus confirming that the pool of reserve cells was depleted.



**Figure 2. Muscle Precursor Pool Is Diminished in Elderly Muscle Stem Cells In Vitro and In Vivo**

(A) Reserve cell marker PAX7 is lost in elderly cultures of differentiated muscle cells. Cultures were labeled for PAX7 (green) and myosin heavy chain (MyHC) (red). Differentiated myotubes (multinucleated cells positive for MyHC) are present in both cultures. Reserve cells (mononucleated and PAX7 expressing) are observed in young-derived (arrows) but rarely in elderly derived cultures, whereas MyHC-expressing mononucleated cells (asterisks) were abundant in elderly cultures. Counts of reserve cells and of MyHC-expressing mononucleated cells are expressed as a percentage of mononucleated cells (bottom; mean  $\pm$  SEM). (B) The mononucleated cell population can give rise to new myotubes. Diagram summarizes workflow to test the myogenic capacity of mononucleated cells. Re-plated desmin-positive cells were able to differentiate and form myotubes, but their numbers were much lower in elderly derived cultures. Desmin, green; MyHC, red; nuclei, blue. (C) Elderly derived human muscle precursors engrafted into mouse muscles participate less in the reserve cell niche. *Tibialis anterior* muscles were harvested 4 weeks after cryodamage and subsequent injection of  $5 \times 10^5$  human cells. Human cells participating in the reserve cell compartment (white arrows) are defined by nuclear expression of PAX7 (red) and human lamin A/C (green). Laminin (white) and nuclei (blue) are visualized. Human reserve cells are counted as a percentage of the total number of human lamin A/C nuclei (bottom). Significant differences with young-derived cells are indicated (\* $p < 0.05$  and \*\* $p < 0.01$ ). Scale bar, 50  $\mu$ m.

### Self-Renewal of Elderly Muscle Stem Cells Is Impaired In Vivo

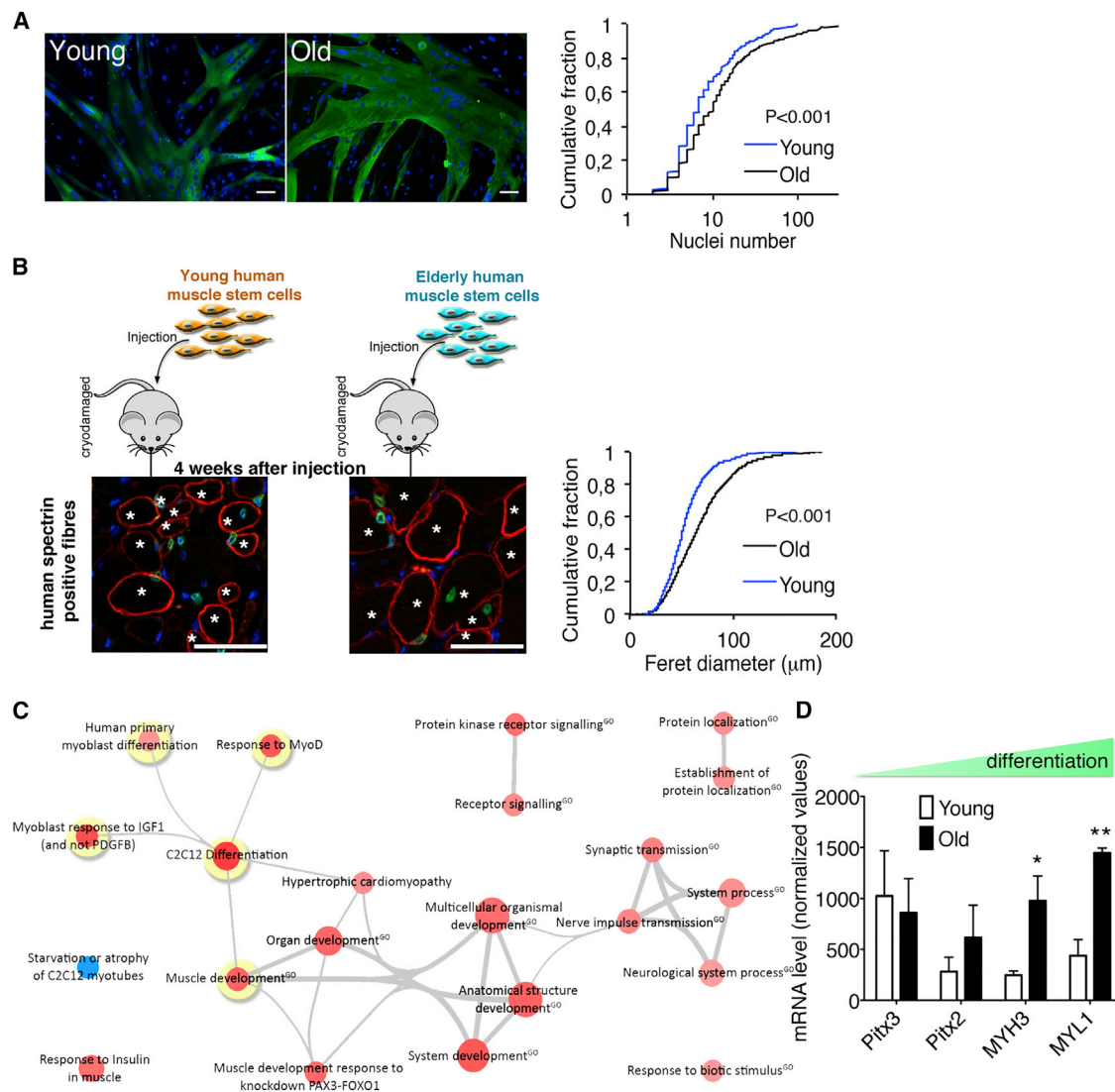
Human muscle stem cells from both young and old subjects were injected into regenerating muscles of immunodeficient mice and able to fuse with the murine muscle host fibers (Figures 2C and 3B). Post-enugraftment, muscle fibers were found expressing human spectrin, and sub-laminar cells containing human nuclei (expressing human lamin A/C) also were detected. While  $8.09\% \pm 0.43\%$  of the nuclei derived from young human muscle stem cells expressed PAX7 and, thus, participated in stem cell renewal, only  $3.53\% \pm 1.29\%$  ( $p < 0.01$ ) of the nuclei derived from elderly human muscle stem cells did this (Figure 2C). Thus, self-renewal of the quiescent stem cell population was impaired in vivo in mouse-engrafted human muscle stem cells derived from elderly subjects.

### Elderly Muscle Stem Cells Strongly Express Markers of Differentiation

Since the late-differentiation marker MyHC was expressed by a higher proportion of mononucleated cells in elderly derived differentiated cultures, we explored further the differentiation fate of these elderly cells. Muscle precursors from elderly cells show a high capacity to fuse, with a fusion index of 88% compared with 71% for young-derived cells (Figure S2A);

in vitro they form larger myotubes containing a significantly greater number of nuclei (Figure 3A); and, following in vivo engraftment, they form significantly much larger fibers than young-derived cells (Figure 3B).

To further explore this observation at the molecular level, transcriptomic analysis was performed on myotubes after 1 and 3 days of differentiation (Tables S1 and S2). Genes that previously were shown to be upregulated during differentiation of human primary myoblasts presented a strongly increased expression in elderly derived as compared with young-derived cells at both day 1 and day 3 (Figure S2B). By day 3 of differentiation, genes that are known to be upregulated during murine  $C_2C_{12}$  myoblast differentiation, in myoblast response to known triggers/enhancers of differentiation (MyoD or IGF-1), or in general muscle development processes were all found to be more strongly expressed in elderly derived compared with young-derived differentiated cells (Figure 3C; muscle differentiation-related gene sets mentioned in the text are highlighted in yellow). Correspondingly, genes induced by starvation or atrophy of myotubes were downregulated in myotubes derived from elderly as compared to those derived from young cells. We confirmed by qRT-PCR that elderly differentiated myoblasts have a higher level of MyHC 3 and myosin light chain 1 mRNA (Figure 3D), markers of late stages of myogenic differentiation.



### Figure 3. Elderly Muscle Stem Cells Strongly Express Markers of Differentiation and Form Large Myotubes

(A) Differentiated myotubes are larger when formed by elderly muscle stem cells. (Left) Representative images show young and elderly myotubes. Nuclei, blue; desmin, green. Scale bar, 50  $\mu\text{m}$ . (Right) Cumulative rank plots show the higher nuclear number per myotube in elderly cultures ( $p < 0.001$ ).

(B) Engrafted elderly muscle stem cells form large myotubes in vivo. (Left) Myofibers (asterisks) formed by the differentiation of engrafted cells are positive for human spectrin (red) and contain human myonuclei (human lamin A/C, green). (Right) Cumulative rank plots show the larger Feret diameters of myofibers formed by elderly compared with young muscle stem cells ( $p < 0.001$ ).

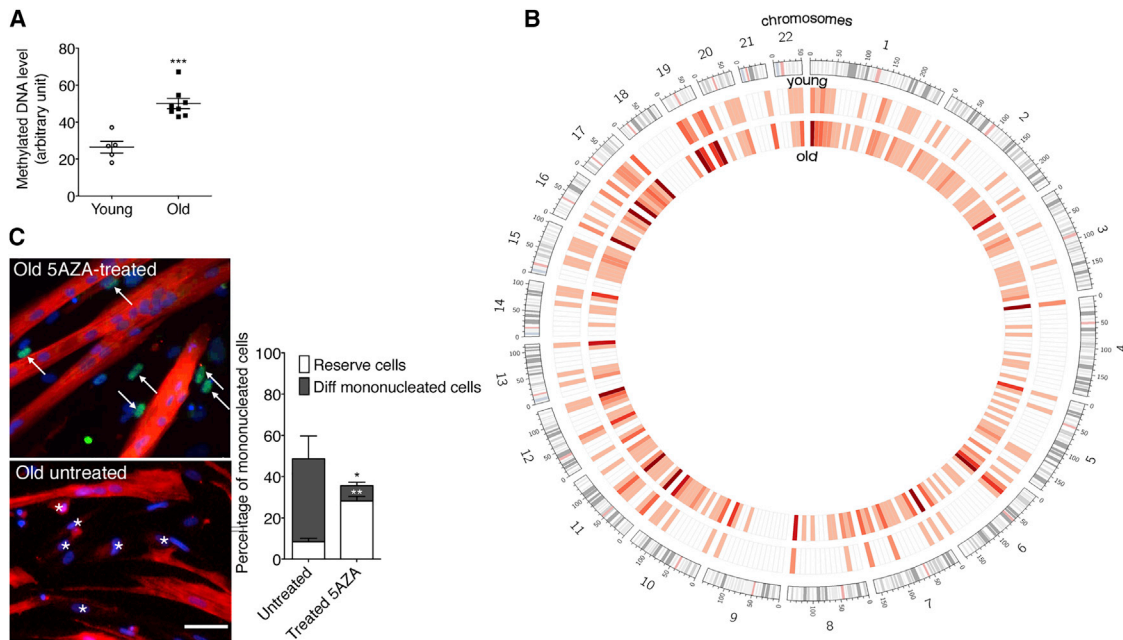
(C) Upregulation of differentiation-related molecular signatures in elderly derived cultures. Of 1,053 gene sets, including all biological processes of the gene ontology, only those whose distributions in the transcriptome profile were significantly non-random ( $p < 0.05$  and  $\text{FDR} < 0.025$ ) are shown, colored red or blue in proportion to up- or downregulation in elderly compared with young cells (differentiation day 3). Node size represents number of significantly altered genes, and edge width represents the number of altered genes that are shared between connected gene sets. References of each gene set are given in Table S3. Differentiation-related gene sets are indicated.

(D) qRT-PCR for markers of early to late myogenic differentiation. The mRNA levels were normalized to desmin. Values are means  $\pm$  SEM ( $n = 3\text{--}4$  per group). Significant differences with young-derived cells are indicated ( $*p < 0.05$  and  $**p < 0.01$ ). See also Figure S2 and Tables S1, S2, and S3.

In addition, western blot analyses confirmed a greater level of MyHC in elderly stem cell cultures (Figure S2C). On the other hand, *PITX3* and *PITX2* mRNA levels, described as markers for proliferation and early stage of differentiation (Knopp et al., 2013), were similar between young and elderly myotubes (Figure 3D).

### Capacity for Self-Renewal Is Regulated by DNA Methylation

Elderly muscle stem cells presented an overall significantly increased level of methylated DNA (Figures 4A and 4B; Figure S3A; Table S4), and a whole DNA methylome array showed that hypermethylated CpGs were distributed throughout the



**Figure 4. DNA Methylation Regulates the Size of the Reserve Cell Pool**

(A) DNA from elderly muscle stem cells is hypermethylated. An ELISA assay was used to measure the level of methylated DNA in muscle progenitors derived from young and elderly subjects (one data point per subject;  $n = 5\text{--}8$  per group). Values are means  $\pm$  SEM. \*\*\* $p < 0.001$ .

(B) Genomic visualization of DNA methylation. Chromosome numbers, chromosomal positions (in units of 1 Mb), and cytogenetic bands are shown at the outermost circle. Within this are displayed counts of hypermethylated CpG sites in muscle stem cells from old compared with young subjects (inner ring), and from young compared with old subjects (outer ring). Darker reds represent higher counts.

(C) Experimental demethylation of DNA increased the number of muscle reserve cells in elderly cultures. Elderly cells were treated with 5-Aza-2'-deoxycytidine. (Left) Representative immunostaining shows treated (top) and untreated (bottom) elderly cells. PAX7, green; MyHC, red; nuclei, blue. Reserve cells (expressing PAX7, white arrows) and mononucleated cells positive for MyHC (asterisks) are indicated. (Right) Percentage of mononucleated cells that are PAX7 or MyHC positive is shown ( $n = 3$  per condition). Values are means  $\pm$  SEM. Significantly different from untreated cells, \* $p < 0.05$  and \*\* $p < 0.01$ . Scale bar, 50  $\mu\text{m}$ . See also [Figure S3](#) and [Table S4](#).

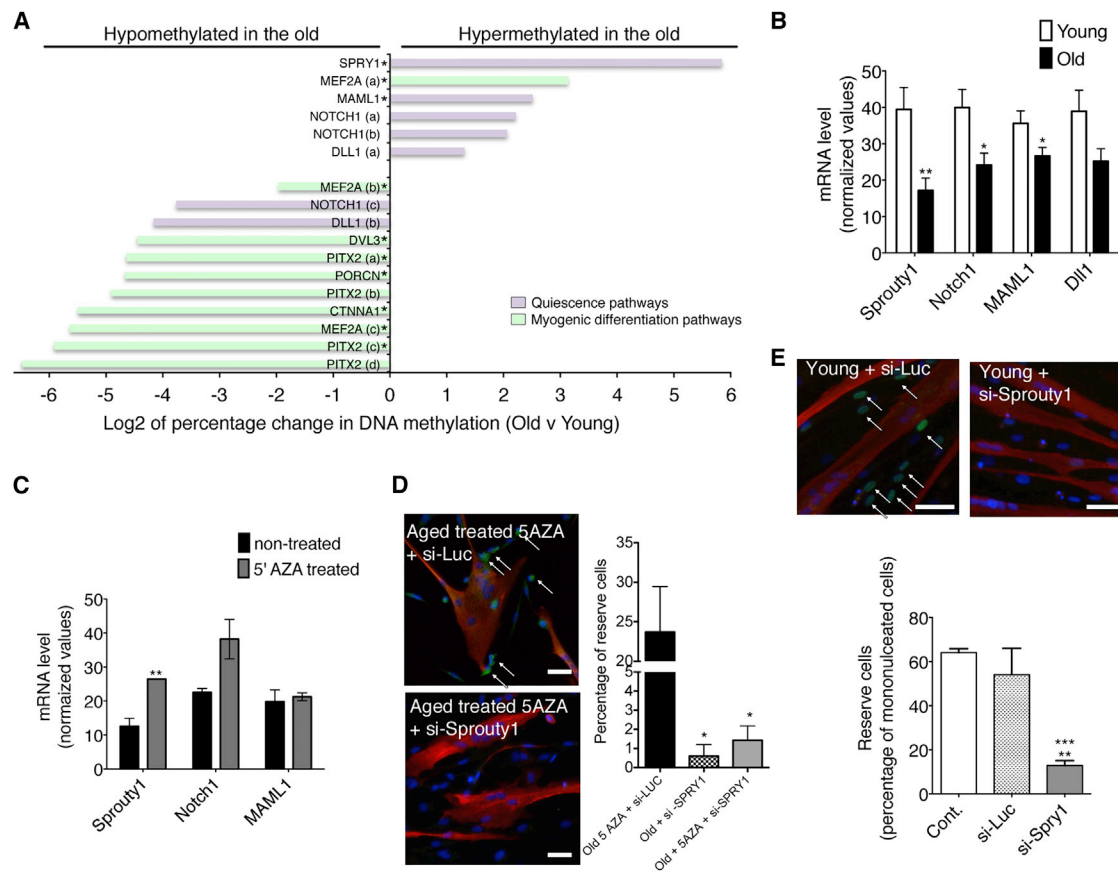
genome ([Figure 4B](#)). Importantly, a higher DNA methylation also was confirmed on human biopsies ([Figure S3B](#)). When we demethylated DNA using 5-Aza-2'-deoxycytidine in *in vitro* cultures up to day 6 of differentiation, the number of reserve (PAX7-expressing) cells generated was restored in elderly cultures (an increase of about 2.7-fold; [Figure 4C](#)), whereas the number of mononucleated cells positive for MyHC was significantly decreased ([Figure 4C](#)). Fusion into myotubes was unaffected since the number of nuclei per myotube was similar in both conditions ([Figure S3C](#)). Thus, DNA methylation is increased in elderly muscle stem cells, inhibiting reserve pool self-renewal, which can be restored by demethylation.

#### Sprouty1 Regulates Self-Renewal in Muscle Stem Cells

The genome-wide methylation analysis revealed a general hypermethylation of gene bodies in elderly muscle stem cells, whereas upstream transcription start sites and promoter regions were hypomethylated ([Figures S4A](#) and [S4B](#)), suggesting that an overall activation of transcription underlies both their engagement into the myogenic differentiation program ([Figure 3](#)) and their reduced quiescence ([Figure 2](#)). Genes involved in myogenic differentiation pathways, such as elements of the WNT3A pathway, were hypomethylated ([Figure 5A](#); [Table S4](#)). This is concordant with results in the previous section showing that

the myogenic program is upregulated in elderly muscle stem cells ([Figures 3C](#) and [3D](#); [Figures S2](#) and [S4C](#)). Conversely, genes involved in self-renewal pathways, such as *SPRY1* and *NOTCH1* ([Brack et al., 2008](#); [Chakkalakal et al., 2012](#); [Conboy and Rando, 2002](#); [Shea et al., 2010](#); [Wen et al., 2012](#)), were hypermethylated ([Figure 5A](#)). Lower mRNA levels of *SPRY1*, *NOTCH1*, and its co-regulator mastermind-like protein 1 (*MAML1*) were detected in elderly cultures; delta-like protein 1 (*DLL1*), a second regulator of *NOTCH1*, was unchanged ([Figure 5B](#)).

5-Aza-2'-deoxycytidine treatment, which rescued self-renewal *in vitro*, also rescued the expression level of *SPRY1* ([Figure 5C](#)), whereas the mRNA level of *NOTCH1* was highly variable and its cofactor *MAML1* remained unchanged ([Figure 5C](#)). Following small interfering RNA (siRNA) knockdown of *SPRY1* to about 5% in elderly muscle stem cells treated with 5-Aza-2'-deoxycytidine, their capacity to renew the reserve cell compartment was abolished ([Figure 5D](#)). Similarly, knocking down the expression of *SPRY1* to 55% in young muscle stem cells diminished their capacity to self-renew the reserve cell pool to a level similar to that observed in elderly cells ([Figure 5D](#)). It is noteworthy that the number of mononucleated differentiated cells (expressing MyHC) remained low (about 3%; [Figure S4D](#)) and the number of nuclei per myotube was unchanged ([Figure S4E](#)), suggesting



**Figure 5. Shutdown of Quiescence Pathways in Elderly Muscle Stem Cells Is Rescued by Demethylation; Knockdown of *SPRY1* Diminishes the Pool of Reserve Cells**

(A) Change in DNA methylation of genes involved in myogenic differentiation and quiescence. Each bar represents a significantly altered CpG site (multiple sites for some genes); asterisks indicate sites within upstream regulatory regions.

(B) Transcript levels and protein localization of genes involved in quiescence pathways. The mRNA levels are means  $\pm$  SEM (n = 5–7 per group) and were normalized to B2M. Significance against young-derived cells is indicated (\*p < 0.05, \*\*p < 0.01).

(C) DNA demethylation with 5-Aza-2'-deoxycytidine in elderly cultures increased the mRNA levels of *SPRY1*. The mRNA levels are means  $\pm$  SEM (n = 3 per group) and were normalized to B2M. Significantly different from young cells, \*\*p < 0.01.

(D) Inhibition of *SPRY1* expression in elderly muscle stem cells treated with 5-Aza-2'-deoxycytidine diminished their capacity to form a pool of reserve cells. In aged cells treated with 5-Aza-2'-deoxycytidine, the pool of reserve cell was diminished at day 3 of differentiation following knockdown of *SPRY1* (si-Sprouty) compared to controls (Cont and si-Luc). White arrows indicate reserve cells. PAX7, green; MyHC, red; nuclei, blue. Scale bar, 50  $\mu$ m. Mean values ( $\pm$ SEM) are as a percentage of the mononucleated cells. Significant differences with controls are indicated (\*p < 0.05 compared with si-Luc).

(E) Inhibition of *SPRY1* expression in young muscle stem cells diminished their capacity to form a pool of reserve cells. In young cells at day 3 of differentiation, the reserve cell pool was diminished following knockdown of *SPRY1* (si-Sprouty) compared to controls (Cont and si-Luc). White arrows indicate reserve cells. PAX7, green; MyHC, red; nuclei, blue. Scale bar, 50  $\mu$ m. Mean values ( $\pm$ SEM) are as a percentage of the mononucleated cells. Significant differences with controls are indicated (\*\*p < 0.01 compared with si-Luc; \*\*\*p < 0.001 compared with Cont). See also Figure S4 and Table S4.

that the sprouty1 pathway, despite its strong regulation of quiescent fate, does not regulate myogenic differentiation. Altogether, our data evidence a downregulation of the sprouty1 self-renewal pathway by DNA methylation in elderly muscle stem cells, which inhibits quiescence and may contribute to the age-associated decrease in the pool of muscle stem cells.

In this context, we wanted to check whether FGF<sub>2</sub>, an antagonist regulator of sprouty1 pathway that is secreted by myofibers (Chakkalakal et al., 2012), was differently expressed between young and elderly murine myotubes. We could not detect any differences in FGF<sub>2</sub> mRNA level between young and elderly myotubes (Figure S4F). However, we observed a significantly higher

level of FGF<sub>2</sub> mRNA in elderly muscle biopsies (Figure S4G), suggesting that FGF<sub>2</sub> is differentially regulated in vivo and may act synergistically with DNA methylation to repress the quiescence pathway in elderly stem cells.

## DISCUSSION

Changes in stem cell content play a key role in tissue homeostasis, regulating the balance between growth and atrophy in skeletal muscle (Brack et al., 2005; Verdijk et al., 2014). However, although a loss of stem cells has been evidenced with aging, little is known about the mechanisms involved. In this study,

performed on human muscle stem cells isolated from seven young and 14 elderly subjects while carefully controlling for division number to avoid artifacts due to senescence, we showed that DNA methylation is an age-sensitive upstream regulator of genes controlling cell quiescence and that it acts chiefly through suppression of the sprouty1 pathway to impair the self-renewal capacity of the elderly muscle stem cell compartment.

Two key features of this work were that, first, we verified the myogenic purity immediately following each thawing of frozen stocks; this was important because contaminant populations such as fibroblasts may senesce more rapidly than myoblasts, thereby giving a false impression of myoblast senescence and potentially introducing bias into measures such as the fusion index. Second, and more importantly, all experiments were carried out at a division number of less than 12. We have shown previously that later divisions begin to show signs of senescence, including a lower proliferation rate even in strong proliferation-inducing media (20% fetal bovine serum [FBS]) (Barberi et al., 2013). It is important to distinguish control of division number (as calculated here and in previous work [Barberi et al., 2013]) from control of passage number: primary isolates from elderly subjects are less numerous, thus initial culture is usually at lower cell density so that more divisions are required to reach the first passage. These two features of our work may be relevant to the fact that, like several previous studies (Alsharidah et al., 2013; Renault et al., 2002) but unlike a recent report by Sousa-Victor et al. (2014), we did not observe senescence in elderly primary myoblasts. We also note that our previous study of these same primary human myoblasts showed that levels of p16 expression, while increasing markedly with division number, remained unchanged between young and elderly cultures (Barberi et al., 2013).

Consideration also should be given to the physiological history of muscle. Muscle growth during early post-natal development results from muscle precursor cells fusing with the muscle fiber, as shown in rat (Moss and Leblond, 1971) and mice (White et al., 2010), but this accretion is completed by 21 days postnatally in mice (Duddy et al., 2015; White et al., 2010). Similarly, muscle growth is complete in young human adults (Verdijk et al., 2014) and subsequent myonuclear turnover is then low during adulthood, with myonuclear turnover being estimated at 15 years (Spalding et al., 2005). Our observations of similarity between cells of elderly and young subjects, in terms of capacity to proliferate, lifespan, p16 expression, and telomere length, confirm that their mitotic age is largely unaffected by aging in accordance with a very slow turnover during adulthood.

Our study shows that the sprouty1 pathway is an important regulator of human myoblast quiescence and that this pathway is suppressed by methylation during aging. This was confirmed by both *SPRY1* knockdown and demethylation in young and old cells and by in vivo transplantations. A further confirmation would have been to transduce young myoblasts with a small hairpin RNA (shRNA) *SPRY1* knockdown construct and then carry out in vivo transplantation. However, the lengthy antibiotic selection required for this assay places it at the technical limits of what could be achieved without reaching cell senescence, potentially leading to artifactual findings.

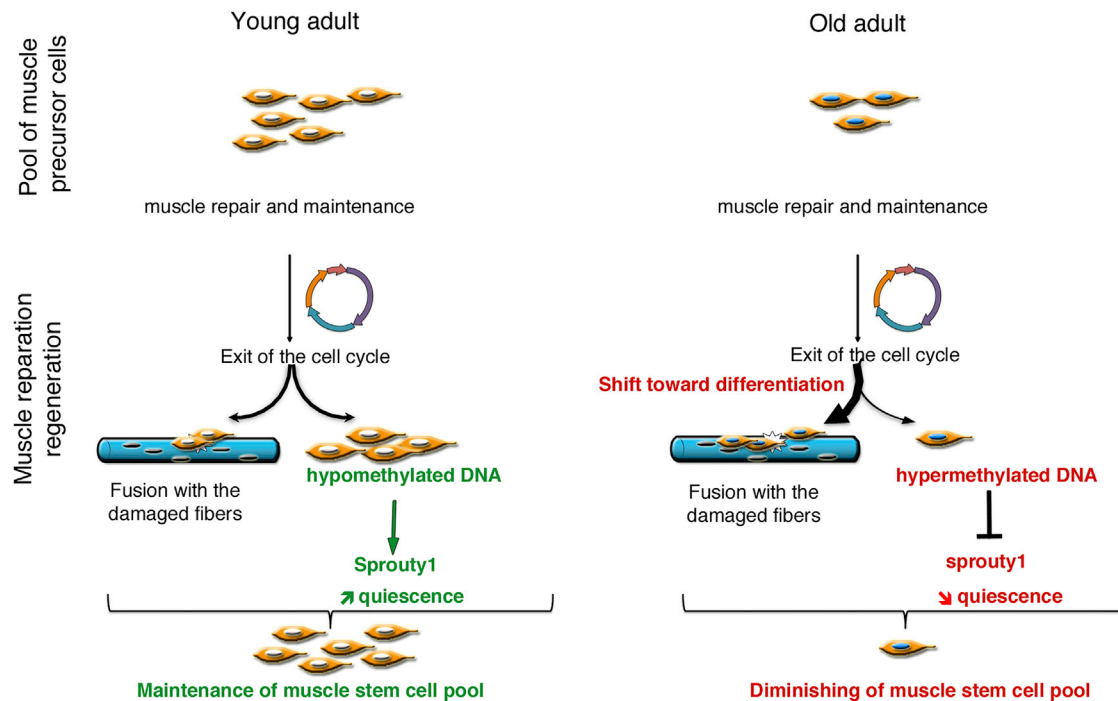
It is important to note that the age-associated changes in stem cell fate identified by our study in no way challenge the existence of asymmetric division in human myoblasts. Our results consistently show that a proportion of reserve cells is maintained in both young and old cultures—what changes is the size of this proportion. In both young and old cultures, a percentage of mononucleated cells continued to express the PAX7 quiescence marker after differentiation was induced (12% of all nuclei in young cultures and 3.6% in elderly cultures). Thus, asymmetric division is present but less frequent in elderly cultures.

Several mechanisms have been proposed to be responsible for muscle stem cell loss with aging, including apoptosis and cellular senescence. Collins et al. (2007) showed that aged murine muscle stem cells are prone to in vitro apoptosis with an accumulation of damaged DNA. In contrast, more recent studies did not observe any sign of apoptosis or cell death in cultured elderly murine and human muscle stem cells (Alsharidah et al., 2013; Cousin et al., 2013). Comparing cultures derived from young and old adult human subjects, we confirmed in vitro that apoptosis is a very rare event.

DNA methylome analysis of elderly compared to young-derived cells identified an increased methylation of certain quiescence pathways, but reduced methylation of a marker of differentiation. This was consistent with observations of downstream processes, such as the following: (1) an overall transcriptomic molecular signature of increased myogenic differentiation (Figure 3; Figure S2B); (2) changes in the expression of myogenic differentiation markers (Figure 3; Figures S2C and S4C); and (3) the downregulation of quiescence pathways, such as that of sprouty1 (Figure 5). This latter finding in human cells is in agreement with the recent report that elderly murine muscle stem cells lose markers of quiescent fate, such as sprouty1 and Pax7 (Chakkalakal et al., 2012). The downregulation of quiescence pathways results in a decreased number of reserve cells in elderly cultures and a failure to self-renew the stem cell pool in vivo. Our results distinguish two major characteristics of the aged muscle progenitors as follows: (1) myogenic potential is preserved in elderly human muscle stem cells, as shown previously in the mouse (Brack et al., 2007; Collins et al., 2007; Conboy et al., 2005) and in humans (Alsharidah et al., 2013); and (2) elderly muscle stem cells have an impaired ability to return to quiescence once they are activated, due to the suppression of quiescence pathways, chiefly *SPRY1*, by DNA methylation. The impact of this on the stem cell population is modeled in Figure 6 and explains its disappearance with age.

Several pathways are implicated in muscle stem cell quiescence, including notch 1 (Brack et al., 2007; Bröhl et al., 2012; Conboy et al., 2003; Wen et al., 2012) and sprouty1 (Abou-Khalil and Brack, 2010; Shea et al., 2010). In this study, the loss of the reserve pool in elderly muscle stem cells correlates with an upregulation of *WNT3A* (Figure S4C), WNT being an antagonist of notch pathways (Brack et al., 2007; Bröhl et al., 2012; Conboy et al., 2003; Wen et al., 2012), and with a downregulation of *NOTCH1* and its co-regulator *MAML1* (Figure 5). Notch has been shown to upregulate Pax7 (Wen et al., 2012). Demethylating the DNA did not markedly rescue expression levels of the notch 1 pathway, despite rescuing reserve cell commitment. FGF<sub>2</sub> is secreted by murine myofibers (Chakkalakal et al.,





**Figure 6. A Model for the Inhibition of Quiescence by DNA Methylation and Its Consequences on the Maintenance of the Stem Cell Pool During Tissue Repair**

DNA methylation increases with aging in muscle stem cells. During regeneration/repair, muscle stem cells are activated in both young and elderly subjects. Once activated, the cells proliferate. In youth, the sprouty1 pathway is activated upon exit from the cell cycle, driving some cells toward quiescence, thus replenishing the pool of reserve cells. In contrast, hypermethylation of DNA in elderly stem cells suppresses sprouty1, inhibiting re-quiescence to prevent full replenishment of the reserve cell population, which results in its gradual loss with aging.

2012) and by murine cardiac fibroblasts (Thum et al., 2008), and it stimulates an antagonist pathway of sprouty1. Our failure to observe any age-associated change in muscle stem cell *FGF2* expression supports methylation as a cell-intrinsic regulator of the sprouty1 pathway, but it does not rule out the possibility that *FGF2* (which we did observe to be increased with age in muscle biopsies) exerts an influence in vivo.

The downstream effects of *SPRY1* demethylation or siRNA knockdown in young human cells (present work), or genetic knockdown in the mouse (Shea et al., 2010), show the importance of this pathway in driving the quiescence fate of muscle stem cells and, thus, in the maintenance of the stem cell pool. As noted above, the expression level of *SPRY1* does not interfere with the differentiation pathway, since knockdown did not change the number of nuclei per myotube (Figure S4E). Similarly, a sprouty1 knockout murine model showed the same capacity as control mice to regenerate, with unchanged myofiber number and cross-section area after induction of the degeneration/regeneration process (Shea et al., 2010). Shea et al. (2010) and Abou-Khalil and Brack (2010) suggested that sprouty1 is required for the self-renewal of muscle stem cells. Based on the results presented in this report, we propose that the role of sprouty1 is to trigger the commitment of cells toward a quiescent fate, without interfering with myogenic differentiation, the latter instead being driven by an aging-associated reinforcement of myogenic specialization, as discussed above.

Our findings follow those of others showing that stem cell behavior is affected by age-related changes in epigenetic status (Liu et al., 2013; Oberdoerffer et al., 2008; Rando and Chang, 2012), but we have linked this to a specific pathway. Modulation of stem cell renewal by methylation may not be restricted to muscle stem cells: we note, for instance, that the number of hematopoietic stem cells increases with aging (Pollina and Brunet, 2011) while their DNA becomes globally hypomethylated (Bocker et al., 2011). A fundamental question is whether the epigenetic status of the stem cell is regulated by its local environmental niche. Accumulated evidence suggests that muscle fibers secrete growth factors, microRNAs, and components of the extracellular matrix into their environment (Le Bihan et al., 2012; Duguez et al., 2013; Henningsen et al., 2010; Roca-Rivada et al., 2012). Another environmental factor is oxidative stress, which can have local effects on the activity of histone-deacetylases and DNA methylases (Cencioni et al., 2013). Oxidative stress in aging muscle is well characterized and is related to mitochondrial dysfunction in muscle fibers (Marzetti et al., 2013) and increased inflammation (Franceschi et al., 2007; see Thorley et al., 2015 for review). We have shown that epigenetic manipulation of the pathways involved in quiescence can modulate the formation of the reserve cell pool. Determining if the muscle secretome changes with aging, and in turn alters the epigenetic status of these stem cells, may suggest strategies to improve human health in age-related diseases associated with stem cell dysfunction.

## EXPERIMENTAL PROCEDURES

See the [Supplemental Experimental Procedures](#) for descriptions of the cell culture conditions, qRT-PCR, methylated DNA quantification, muscle cell injection in mice, telomere length measurement, proliferation and senescence assays, immunolabeling, 5-Aza-2'-deoxycytidine treatment, and knockdown of *SPRY1* using siRNA.

### Participants and Ethical Approval

Seven muscle biopsies were obtained from the Bank of Tissues for Research (BTR, a partner in the EU network EuroBioBank) in accordance with European recommendations and French legislation. Fifteen muscle biopsies were obtained from Leiden University Medical Center (LUMC) after approbation of the local medical ethical committee in the context of a European study (HEALTH-2007-2.4.5-10: Understanding and combating age related muscle weakness "MYOAGE"). Written informed consent was obtained from all patients. All biopsies were isolated from quadriceps muscle. Seven young (15–24 years old) and 14 old (72–80 years old) healthy subjects were included.

### Samples and Cell Culture

Briefly, the muscle biopsies were dissociated mechanically and cultured as previously described (see the [Supplemental Experimental Procedures](#) for more detailed information).

### Gene Expression Profiling

An aliquot of 150 ng high-quality total RNA from each sample was used for the mRNA expression profiling. Samples were analyzed using Illumina Gene Expression BeadChip Array technology. See the [Supplemental Experimental Procedures](#) for more details.

### GSEA and Enrichment Mapping

Gene set enrichment analysis (GSEA) was applied to genes that were significantly altered in elderly versus young muscle stem cells at day 1 and day 3 of differentiation. Probe sets were first filtered for  $p$  value  $< 0.05$ ; then, in cases where multiple probe sets for a given gene were significantly altered, the probe set with the greatest absolute fold change was retained. Fold-change values were  $\log_2$  transformed and subjected to pre-ranked GSEA using the GSEA tool. A total of 1,053 gene sets were tested for their positive or negative enrichment. The majority of these were downloaded from the Molecular Signatures Database (MSigDB, <http://www.broadinstitute.org/gsea/msigdb/>), including 866 gene ontology biological processes and 46 sets identified by a search of the MSigDB for muscle-related sets. Several custom-made sets derived from publications chosen by us also were added, including those listed in the [Results](#). For statistical testing, data were permuted 1,000 times and the "meandiv" normalization algorithm was applied. A gene set was tested if 10–500 genes from the significantly altered list were present in the gene set.

Gene sets that were found by enrichment analysis to be highly significantly enriched ( $p < 0.05$  and false discovery rate [FDR]  $< 0.025$ ) were graphically presented using the enrichment mapping plugin for Cytoscape. The overlap coefficient was set to 0.2 with a combined constant of 0.5.

### Global DNA Methylation Studies

Genomic DNA extracted from young and elderly muscle cells differentiated for 24 hr was used in the DNA methylation studies ( $n = 3$  per group). The Infinium HumanMethylation 450 BeadChip (Illumina), which includes 485,577 cytosine positions in the human genome, was utilized for genome-wide DNA methylation screening. See the [Supplemental Experimental Procedures](#) for more details.

### DNA Methylation Data Analysis

The  $p$  values were calculated to identify failed probes as per Illumina's recommendations and no arrays exceeded our quality threshold of  $>5\%$  failed probes. In addition, we removed CpG sites on the X and Y chromosomes (and removed from the analysis CpG sites that contained a SNP or a SNP within 10 bp of the methylation probe). Raw data were normalized using Illumina's control probe scaling procedure and background subtracted.

The  $\beta$  values were imported into Partek Genomics Suite (version 6.6) and underwent a logit transformation ( $M$  value). The  $M$  value is calculated as the  $\log_2$  ratio of the intensities of the methylation probes versus unmethylated probe. The problem of heteroscedasticity in the high and low ranges of methylation ( $<0.2$  and  $\geq 0.8$ ) is resolved with the transformation of  $\beta$  value to  $M$  value. The data were analyzed using ANOVA models, with  $M$  value for each site as the dependent variable and response (old versus young) as the independent variable. For Circos visualization, counts of significantly altered CpG sites within bins of equal size (10 Mb) were calculated across all chromosomes using R. Bins were then displayed using Circos and colored according to counts, with color incrementally darkening from white to red in increments of 25, up to a maximum darkness of red at counts  $>150$ .

The lists of genes hypermethylated (200%–15%) and hypomethylated (30%–200%) were imported into DAVID functional annotation software developed by [Huang et al. \(2009a, 2009b\)](#) mapped to their official gene symbol. We used the clustering algorithm of DAVID (version 6.7) with high clustering stringency (initial and final group membership, 5; similarity term overlap, 3; multiple linkage threshold, 0.5; and similarity threshold, 0.85). This identified several functional annotation clusters with a Fisher exact  $p$  value  $< 3.7 \times 10^{-2}$  and an enrichment score above 2 (geometric mean in log scale of member's  $p$  values, significance being considered at 1.5). See the [Supplemental Experimental Procedures](#) for more details.

### Statistical Analysis

All values are presented as means  $\pm$  SEM. Student's  $t$  test was used to compare differences between young and elderly samples. A Kolmogorov-Smirnov test was used to compare the distribution of myotube nuclear numbers in young and elderly cell culture and the distribution of the fiber size containing human nuclei from young or elderly donor. One-way ANOVA was used to evaluate the time course changes in reserve cells and in mononucleated cells positive for MyHC, followed by a Newman-Keuls multiple comparison test. Differences were considered to be statistically different at  $p < 0.05$ .

### ACCESSION NUMBERS

The accession numbers for the data reported in this paper are GEO: GSE52699 and GSE53302.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.09.067>.

### AUTHOR CONTRIBUTIONS

S.D., V. Mouly, and G.B.-B. conceptualized the study. V. Mouly and G.B.-B. obtained funding for the project. S.D. performed and analyzed the experiments. W.J.D. performed the bioinformatics analysis. E.N. performed the transplantation experiments. S.G. performed the microarray analysis. B.H. and J. Devaney performed the DNA methylome assay and associated statistical analysis. S.D., Z.G.O., C.L., and A.B. measured the cell division speed. A.W. measured the telomere length. A.B. extracted the cells from human biopsies and measured their lifespans. A.B., V. Mariot, and S.D. performed the 5AZA and siRNA knockdown experiments. S.D. and Z.G.O. performed qRT-PCR and immunostaining. A.B., W.J.D., Z.G.O., G.B.-B., V. Mouly, J. Dumonceaux, and S.D. wrote, discussed, and edited the manuscript. S.D. supervised the project.

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