



Published in final edited form as:

*Nat Rev Mol Cell Biol.* 2016 May ; 17(5): 267–279. doi:10.1038/nrm.2016.7.

## Molecular circuitry of stem cell fate in skeletal muscle regeneration, ageing, and disease

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### Abstract

Satellite cells are adult myogenic stem cells that function to repair damaged muscle. The enduring capacity for muscle regeneration requires efficient satellite cell expansion after injury, differentiation to produce myoblasts that can reconstitute damaged fibers, and self-renewal to replenish the muscle stem cell pool for subsequent rounds of injury and repair. Emerging studies indicate that misregulations of satellite cell fate and function contribute to age-associated muscle dysfunction and influence the severity of muscle diseases, including Duchenne Muscular Dystrophy (DMD). It has also become apparent that satellite cell fate during muscle regeneration, aging, and in the context of DMD is governed by an intricate network of intrinsic and extrinsic regulators. Targeted manipulation of this network may offer unique opportunities for muscle regenerative medicine.

### Introduction

Skeletal muscle is a contractile, post-mitotic tissue composed of multinucleated muscle cells, known as myofibers, which are formed by fusion of differentiated mononuclear muscle cells. Skeletal muscle accounts for 30–50% of body mass in humans, and is one of the few organs that retains a highly adaptive and robust capacity to regenerate throughout most of life. Muscle regeneration depends upon resident muscle stem cells known as satellite cells. These mesoderm-derived cells comprise a heterogeneous population of adult stem cells (Box 1), capable of both self-renewal and myogenic differentiation, which reside in a specialized niche between the muscle sarcolemma and the basal lamina of individual myofibers<sup>1</sup>(Fig. 1). The satellite cell niche is comprised of both acellular and cellular components, including extracellular matrix proteins and growth factors, myofibers, and muscle-resident non-myogenic cells such as fibro-adipogenic progenitors (FAPs), macrophages, and regulatory T-cells (Tregs)<sup>2–9</sup>. Collectively, components of the satellite cell niche create a complex microenvironment that plays a crucial role in maintaining satellite cell identity and ensuring robust regenerative responses to muscle insult<sup>2, 4–9</sup>.

#### Box 1

##### Origin and heterogeneity of satellite cells

Most satellite cells in postnatal muscle originate from a population of embryonic precursors that expresses PAX7 and/or the related Paired box protein, PAX3. These

embryonic precursors of adult muscle are of mesodermal origin and arise from a dorsal structure of the developing somite (known as the dermomyotome)<sup>136, 137</sup>. In the mouse, by embryonic day 16.5 to 18.5, a subset of myogenic progenitors in the dermomyotome migrates to its prospective niche (analogous to the niche of satellite cells in postnatal skeletal muscle), which is positioned between a primitive basal lamina structure and the myotome<sup>136</sup>. Shortly after birth, postnatal satellite cells expand extensively to accommodate organismal growth, and begin acquiring characteristic molecular features, including expression of specific surface markers, and the emergence of distinct high- and low- cycling sub-populations<sup>90</sup>, an aspect of satellite cell heterogeneity in adult muscle that is discussed in more depth below.

We define muscle satellite cells as *bona fide* muscle stem cells, capable of self-renewal and differentiation to produce myoblasts, which can then fuse (with each other as well as with existing fibers) to generate myofibers. Yet, several lines of evidence indicate that satellite cells in postnatal muscle exhibit notable molecular and phenotypic heterogeneity that can influence the fate and function of individual cells within the satellite cell pool. Mouse molecular genetic tools have been particularly useful in delineating subsets of muscle satellite cells, suggesting the coexistence in this compartment of a population of committed progenitors ready for myogenic differentiation and a distinct, self-renewing population that is capable of reconstituting the satellite cell niche<sup>45, 82, 83, 90</sup>. In one of the studies Cre recombinase-mediated lineage tracing was used to distinguish a minority of adult muscle satellite cells (~10% of the total pool) that were not marked by *Myf5*-Cre, suggesting that they had never expressed *Myf5*. This study further showed that this subset of cells repopulates the satellite cell compartment more readily (as compared to *Myf5*-expressing satellite cells) when transplanted into PAX7-null animals, indicating that these cells may retain more robust self-renewal capabilities<sup>83</sup>. On the other hand, satellite cells that did experience *Myf5* expression were more prone to myogenic differentiation in these *in vivo* engraftment assays<sup>83</sup>. In another study, satellite cells that expressed higher levels of PAX7 RNA (Pax7<sup>hi</sup> cells) as determined by flow cytometry using a Pax7-GFP reporter mouse<sup>82</sup>, displayed slower cycling, lower metabolic activity, and the exclusive capacity to replenish the entire complement of Pax7<sup>hi</sup> and Pax7<sup>low</sup> satellite cells upon transplantation. Studies to determine whether satellite cells that have never expressed *Myf5* are enriched in the Pax7<sup>hi</sup> subset, or vice versa, have yet to be reported. Satellite cells have also been functionally segregated based solely on their proliferative history, with several studies indicating that low-cycling satellite cells exhibit a higher engraftment potential than high-cycling satellite cells when both populations are transplanted into injured animals<sup>45, 90, 138</sup>. These data clearly document phenotypic and functional heterogeneity within the satellite cell pool; yet, how cells toggle between these potentially interchangeable states and how each subset is maintained and regulated during homeostasis and throughout regeneration remains an area of very active exploration.

Previous studies have established the central importance of a regulated cascade of transcription factors that mediate satellite cell maintenance, activation, and differentiation<sup>3</sup>. Satellite cells are distinguished from other mononuclear cells in muscle by expression of the canonical satellite cell regulator gene, Paired box protein 7 (PAX7)<sup>10, 11</sup>. Satellite cells exist

in a quiescent (or non-dividing) state in uninjured muscle, a state often referred to as muscle homeostasis (Fig. 1). But, these cells become activated upon muscle trauma, which promotes their subsequent proliferation and, for a subset of activated satellite cells, differentiation into fusion-competent myoblasts. Satellite cell activation is mediated in part by the induced expression of Myogenic determination protein (MYOD) and Myogenic Factor 5 (MYF5)<sup>12–17</sup>. The differentiation of committed myoblasts involves downregulation of PAX7 and de novo expression of myogenin (also known as MYOG), which is followed by fusion of the differentiated myoblasts with each other and with any remaining myofibers to repair damaged muscle<sup>18</sup>. Activated satellite cells that do not commit to differentiation can downregulate MYOD and MYF5 and undergo self-renewing proliferation that replenishes the pool of muscle satellite cells, thereby ensuring that the capacity to respond to future injuries is maintained in the muscle<sup>3, 19–21</sup> (Fig. 1).

Extensive evidence supports the notion that adult satellite cells are required for healthy muscle repair, and severe muscular and regenerative deficits result in their absence<sup>22, 23</sup>. Although there has been debate regarding the requirements for PAX7 in satellite cell maintenance and muscle repair<sup>24</sup>, recent efforts have affirmed an absolute requirement for PAX7 in satellite cell proliferation and self-renewal, and hence, for efficient repair of muscle after injury<sup>25–27</sup>. Functional loss of the satellite cell pool has been observed in association with age-related muscle dysfunction and other muscle degenerative pathologies<sup>28, 29</sup>, highlighting the critical need to maintain this unique cell population in order to sustain effective regenerative function over a lifetime.

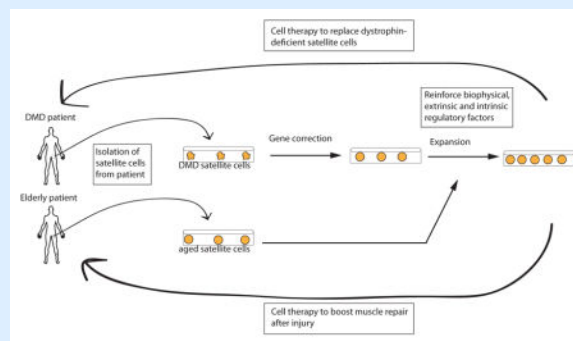
The development of efficient methods to prospectively isolate satellite cells from skeletal muscle has fuelled progress in further delineating their regenerative functions *in vivo*. Satellite cells can be purified from muscle using protocols that combine chemical and mechanical dissociation of muscle and separation of mononuclear cells from fibers with Fluorescence Activating Cell Sorting (FACS) using positive and negative selection markers to enrich for myogenic populations<sup>19, 30–32</sup>. Various surface marker combinations have been reported to positively enrich for highly functional satellite cells including, CD34/ $\alpha$ 7-integrin<sup>19</sup>, VCAM1<sup>30, 33–35</sup>, CXCR4/ $\beta$ 1-integrin<sup>36, 37</sup>, NCAM (CD56)<sup>38, 39</sup>, EGFR<sup>40</sup>, and Syndecan3/4<sup>41</sup>. Whether these different surface marker sorting schemes yield identical myogenic populations has yet to be fully addressed; however, cells enriched by any of these methods can efficiently engraft skeletal muscle when transplanted into either injured or diseased muscle, and can improve muscle strength and function<sup>19, 37, 42</sup>. Satellite cells transplanted into pre-injured, diseased muscle also repopulate the satellite cell niche, and can contribute to future rounds of muscle repair<sup>19, 37, 42</sup>. These observations have raised enthusiasm for the possibility of utilizing satellite cells as a cellular source for regenerative medicine (Box 2). New insights into how these cells regulate self-renewal and differentiation decisions *in vivo*, and how *ex vivo* culture systems impact satellite cell fate will certainly be required to achieve this goal.

**Box 2****Translational applications for modulating satellite cell fate decisions**

There has been tremendous excitement over the years in using satellite cells as a transplantable cell population that could restore the functional stem cell pool in aged and diseased muscle, for instance in patients with Duchenne Muscular Dystrophy (DMD). One could envision a scenario in which satellite cells isolated from the patient are genetically corrected *ex vivo*<sup>139</sup>, expanded, and transplanted back to promote therapeutic muscle repair (Figure). However, a major obstacle to this approach is that these cells are very rare, encompassing only ~1% of the mononuclear cells associated with myofibers<sup>37</sup>. In addition, removal of satellite cells from their endogenous niche rapidly alters their cellular state and, ultimately, their functional capacity<sup>42, 140–142</sup>.

New culture systems that more precisely mimic the *in vivo* environment will likely prove essential for generating sufficient numbers of engraftable satellite cells. For example, it was shown that culturing satellite cells on soft hydrogels to mimic *in vivo* forces and recreate niche signals, promotes enhanced cell survival and self-renewal. Although this approach is a definite improvement over standard culturing conditions, 3-fold more hydrogel-cultured satellite cells are needed to obtain as much as 50% of the engraftment achieved by freshly isolated cells<sup>97, 140</sup>. Reestablishing in culture the full functional potential of satellite cells will likely require a combination of extrinsic and intrinsic signals<sup>57, 58, 143</sup>.

Cell delivery represents a second major impediment to the realization of cell therapeutic approaches in skeletal muscle. Two delivery strategies have been utilized so far: systemic and intramuscular delivery. An ideal stem cell therapy to combat disseminated muscle diseases such as DMD or sarcopenia would be compatible with systemic delivery. However, prior work shows that satellite cells delivered systemically into mice get trapped in other organs (lungs, kidney, spleen).<sup>144</sup> Therefore, methods to achieve more efficient muscle “homing” of satellite cells, similar to the homing properties of circulating immune cells<sup>7</sup>, as well as to improve their mobility within the tissue<sup>145</sup>, will be critical in overcoming this technical hurdle.



In this review, we discuss recent progress in our understanding of the intrinsic molecular mechanisms regulating satellite cell fate during muscle regeneration, aging, and in the

context of Duchenne Muscular Dystrophy (DMD), the most common and severe muscle pathology diagnosed in childhood. As the details of myogenic differentiation and function of satellite cells during embryonic development have been recently addressed elsewhere<sup>3</sup>, we focus here on adult satellite cells and the molecular networks governing their quiescence, activation, and self-renewal as key stages of the satellite cell life cycle. We also highlight the critical influence of extrinsic factors that can modulate satellite cell intrinsic signalling to direct satellite cell fate decisions during muscle regeneration and aging. We anticipate that further elucidation of the gene regulatory circuits that determine satellite cell fate will provide a molecular blueprint that can be used to establish novel therapies to treat the increasing numbers of individuals suffering from age-associated muscle dysfunction and muscular diseases that currently have no cure.

## Satellite cell fate decisions

Careful regulation of satellite cell identity and myogenic commitment are essential for maintaining the muscle satellite cell pool and appropriately activating repair mechanisms in response to muscle damage. We discuss here recent advances that have defined critical new effectors of satellite cell regulation that govern the maintenance of satellite cell quiescence, activation of proliferation, and capacity for self-renewal.

## Satellite cell quiescence

Satellite cell division is restrained in uninjured muscle through mechanisms that appear to enforce satellite cell quiescence during homeostasis. In this section we discuss recent advances in the transcriptional, epigenetic, and signalling pathways controlling the quiescent state.

Quiescent satellite cells (QSCs) express PAX7 but lack MYOD and other markers associated with cellular proliferation<sup>20, 43</sup> (Fig. 1). Satellite cells isolated from fresh muscle by FACS also express PAX7 and have low to undetectable levels of MYOD<sup>19, 37, 42</sup>. Given these molecular similarities, and keeping with convention in the field<sup>19, 33, 37, 42</sup>, this review refers to freshly isolated satellite cells as QSCs, although we note the likelihood that the process of satellite cell isolation from muscle may trigger some initiating events in satellite cell activation. Future studies to provide a better molecular understanding of the very early molecular changes that signal satellite cell activation will help to clarify this issue.

Recent genome-wide RNA expression maps have revealed that QSCs possess a unique transcriptional profile that distinguishes them from their more activated progeny. QSCs express roughly 500 uniquely enriched genes involved in transcriptional control, negative regulation of the cell cycle (including several members of the Sprouty family<sup>44</sup>), and cell-cell adhesion, extracellular matrix, and lipid transporter activity<sup>33, 34</sup>. Although the functional importance of many of these genes is yet to be understood, it seems likely that this transcriptional program acts to inhibit QSC proliferation<sup>44, 45</sup>, to anchor satellite cells in their anatomical niche<sup>46</sup>, and to provide mechanisms for efficient transport and processing of lipids that are required for metabolic reactions characteristic of quiescent cells<sup>47</sup>. At the epigenetic level, nearly half of all protein coding genes are marked by active chromatin, specifically acquiring the histone 3 modification, H3K4me3<sup>33, 48</sup>. Interestingly, genes that

are expressed at low levels in quiescent satellite cells, but induced upon satellite cell activation, exhibit this active histone mark, a likely indicator that these gene regions are in a “poised” state, anticipating release from quiescence<sup>33</sup>. These results emphasize the notion that satellite cell quiescence should not be mistaken as a form of cellular dormancy or inactivity. Rather, it represents an actively maintained state typified by a quiescence-specific gene expression program that is poised to execute the transcriptional and epigenetic transformations that enable a rapid switch to activation in the presence of muscle trauma.

Given the central role of developmental signalling pathways in modulating stem cell behaviours, it is perhaps not surprising that Notch signalling plays a key role in promoting satellite cell quiescence<sup>49–51</sup>. The Notch gene family consists of 4 transmembrane proteins (NOTCH1, 2, 3, and 4) that bind 5 distinct ligands<sup>52</sup>. Upon receptor-ligand binding, Notch undergoes a series of proteolytic cleavages that leads to nuclear translocation of the Notch Intracellular Domain (NICD). In the nucleus, NICD interacts with downstream effector proteins, such as Recombining Binding Protein J $\kappa$  (RBP-J $\kappa$ ), to regulate transcription of Notch target genes<sup>52</sup>. Supporting a role for Notch signalling in QSCs, *Notch 3* mRNA and protein levels decline rapidly upon satellite cell activation<sup>49</sup>, and genetic deletion of RBP-J $\kappa$  causes loss of PAX7-expressing satellite cells in muscle sections, even in the absence of muscle injury. This loss of RBP-J $\kappa$  deficient satellite cells has been attributed to entry of these cells into the cell cycle, and subsequent differentiation<sup>50</sup>. Conversely, ectopic expression of NICD in cultured primary muscle myoblasts slowed cycling kinetics, upregulated PAX7, and suppressed MYOD, indicating that active Notch signalling is sufficient to program at least some of the molecular and phenotypic aspects of quiescence<sup>51</sup>. The molecular mechanisms that link activated Notch with preserved quiescence are far from complete, but insights have been gained from studies indicating that Notch target genes, like the Hes and Hey family of transcription factors, can reduce *MyoD* mRNA levels, and thereby suppress myogenic commitment. In addition, RBP-J $\kappa$  can bind directly to the *Pax7* promoter leading to its transcriptional activation<sup>51</sup>. This enables PAX7 to further reduce MYOD protein stability via transcription-independent mechanisms<sup>53, 54</sup> (Fig 2a).

Mechanisms to regulate Notch signalling include modulating expression of the ligand or the receptor. Recent data indicate that satellite cells lacking the Forkhead Box protein 3 (FOXO3) transcription factor exhibit reduced levels of the *Notch1* and *Notch3* receptor RNAs. FOXO3-deficient satellite cells also proliferate faster and differentiate more readily in culture, consistent with a failure to preserve cellular quiescence. Mice carrying an inactivating *Foxo3* deletion in satellite cells display markedly impaired muscle regeneration after two consecutive muscle injuries, perhaps due to a failure of satellite cells to self-renew after the primary injury. These studies also raise the possibility that FOXO3 might promote either proliferation and/or survival of satellite cells during the transient amplification phase, since fewer PAX7-positive satellite cells were associated with centrally nucleated fibers one week after injury in these mice. Further supporting a potentially direct link between FOXO3 and Notch signalling in QSCs, ectopic expression of NICD in FOXO3-deficient satellite cells partially recovered QSC number in single fiber assays<sup>55</sup>. Together, these results suggest that a key function of FOXO3 in satellite cells is to maintain Notch receptor expression, which is in turn essential for maintaining the quiescent state (Fig 2a). Future work should build upon these and other findings discussed elsewhere<sup>56–58</sup> to fully characterize critical



transcription factors, non-coding RNAs, epigenetic regulators, and the translational regulators that are required to enforce satellite cell quiescence.

### Satellite cell activation

Proper satellite cell activation is crucial to ensuring an adequate regenerative response after muscle injury. Upon muscle insult, QSCs are exposed to extrinsic, pro-myogenic stimuli, including Fibroblast Growth Factor (FGF) proteins<sup>59, 60</sup>, Hepatocyte Growth factor (HGF)<sup>61, 62</sup>, insulin-like growth Factor (IGF-1) factors<sup>63</sup>, and Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ )<sup>64, 65</sup>, which activate intrinsic pathways that stimulate proliferation. It has been reported that the first cell division is the rate-limiting step in satellite cell conversion to a highly proliferative state<sup>66</sup>, which likely explains why the peak of satellite cell expansion occurs roughly 3–4 days after muscle injury *in vivo*<sup>44</sup>. In addition, the long delay in initiation of satellite cell division may hint at the possibility that QSCs may transition through one or more intermediate cell states on the path to full activation (Box 3).

#### Box 3

##### $G_{Alert}$ state of satellite cells

Recently, a new satellite cell state – the  $G_{Alert}$  state was revealed when QSCs were exposed to injury at a distance<sup>66</sup>. In this study, animals were subjected to muscle injury in one limb, and then Activated Satellite Cells (ASCs) were isolated from the injured limb muscle and Contralateral Satellite cells (CSCs) were isolated from the contralateral, uninjured limb muscle. Interestingly, as compared to QSCs, CSCs exhibited a shortened time until their first division, were slightly larger in size, had higher quantities of functional mitochondria and increased mtDNA content. CSCs also exhibited a higher propensity to differentiate and fuse with damaged fibers *in vivo* when compared to QSCs<sup>66</sup>. However, CSCs were still less activated, according to most of the phenotypic criteria described above, than ASCs that were proximal to muscle injury<sup>66</sup>. Thus, it was proposed that CSCs had entered an “alert” state, representing a discrete stage of the cell cycle. This state is characterized by an intermediate cellular program that can be reversibly adopted by satellite cells as they anticipate a pending need for a rapid transition from quiescence to activation to enable efficient muscle repair<sup>66</sup>. Interestingly, entry into the  $G_{Alert}$  state appears to require mTORC1, since genetic deletion of one of its inhibitors, TSC1 (tuberous sclerosis complex 1), induced QSCs in uninjured animals to adopt a  $G_{Alert}$  phenotype. Conversely, genetic deletion of RAPTOR (Regulatory-Associated Protein of mTOR), an adaptor protein required for mTORC1 activity<sup>149</sup>, impaired the  $G_{Alert}$  phenotype in CSCs. Abrogation of HGF signalling inhibited the  $G_{Alert}$  phenotype, revealing that secreted HGF in the local microenvironment<sup>62</sup> may be required for this alternative satellite cell state. Together, these data suggest that QSCs are highly responsive to extracellular damage cues and can execute somewhat preemptive alterations to their cellular phenotype that prime them for activation, enabling them to respond more rapidly to subsequent perturbations of muscle homeostasis in their vicinity. It will be interesting in future experiments to test whether the  $G_{Alert}$  state is also required as an intermediate step for satellite cells responding directly to local muscle injury.

From an epigenetic perspective, the transition of satellite cells to full activation requires the implementation of a highly specialized, epigenetic and regulatory gene expression program<sup>33, 34</sup>. For example, freshly isolated ASCs exhibit enriched expression of gene pathways involved in metabolic processes (see Box 4), cell cycle progression, chemotaxis, and immune system regulation<sup>33, 34</sup>, reflecting the need for satellite cells to grow in size, proliferate, migrate, and interact with muscle-resident immune cells in the context of muscle repair. In addition, the chromatin of ASCs is uniquely altered from QSCs, with ASCs having more genes associated with the repressive H3K27me3 chromatin mark<sup>67</sup>. H3K27me3 is deposited by EZH2, the enzymatic unit of the Polycomb repressive complex 2 (PRC2), which functions during development to restrict alternative cell fates during lineage commitment<sup>68</sup>. Interestingly, pro-inflammatory cytokines secreted during muscle injury and repair can stimulate EZH2, which (in complex with the transcriptional regulator, YY1) acts to repress PAX7 expression in cultured satellite cells that have already begun downregulating PAX7, suggesting that EZH2 can play a role in mediating myogenic differentiation<sup>69</sup>. However, a subsequent study from the same group showed that genetic deletion of *Ezh2* in PAX7-expressing satellite cells prior to injury hampers their proliferation and predisposes them to differentiate, ultimately impairing muscle repair after injury<sup>70, 71</sup>. These studies highlight the complex activities of epigenetic regulators at multiple stages in the satellite cell life cycle, and the need to confirm findings from ex vivo systems using *in vivo* animal models. Nonetheless, these findings clearly support the importance of chromatin reorganization and repression of key target genes in satellite cells during the transition from a QSC to ASC.

#### Box 4

##### Metabolic sensing and control of satellite cell quiescence and activation

Muscle satellite cells undergo extreme changes in size and metabolic activity as they transition from a quiescent to a proliferating state<sup>47</sup>. In fact, even during homeostasis, heterogeneities in the metabolic status of quiescent satellite cells exist. For example, freshly isolated muscle satellite cells expressing lower levels of *Pax7* transcripts (suggestive of a more activated state) have higher levels of active mitochondria, ATP, and mitochondrial transcription factor RNAs, indicating that an increase in oxidative phosphorylation (OXPHOS) may be required for satellite cell activation and proliferation<sup>47, 82</sup>. Increased mitochondrial activity is also found in satellite cells from mice subjected to short-term calorie restriction, a dietary regimen shown to enhance muscle regenerative activity<sup>146</sup>.

A direct functional link between metabolic state and the transition from satellite cell quiescence to activation has been unclear. However, it was recently reported that autophagic flux in quiescent satellite cells provides key bioenergetic resources needed for cell growth and proliferation during activation<sup>147</sup>. Interestingly, deletion of the NAD-dependent deacetylase *Sirt1* in satellite cells led to a reduction in autophagy and consequently less active mitochondria, leading to impairments in satellite cell proliferation<sup>147</sup>. The activities of SIRT1 in satellite cells are likely complicated, since it has also been reported that proliferating satellite cell-derived myoblasts in culture, exhibit reduced NAD<sup>+</sup> levels rendering SIRT1 inactive, and that *Sirt1* deletion *in vivo* led to



precocious satellite cell differentiation<sup>148</sup>. As these two studies utilized different genetic systems for *Sirt1* ablation (inducible versus constitutive), it may be that the timing of expression and cellular distribution of SIRT1, as well as potential compensatory mechanisms, underlie these seemingly contradictory observations.

In addition to modulating satellite cell entry and exit from the cell cycle, metabolic status may also directly influence the ability of these cells to support recovery from muscle damage as well as the effectiveness of their transplantation. As noted above, satellite cells from calorie-restricted mice displayed enhanced oxygen consumption and mitochondrial activity. They were also more abundant, and engrafted host muscle more efficiently upon transplantation<sup>37</sup>. These functional alterations suggest that metabolic reprogramming of quiescent satellite cells may promote their survival as well as induce their proliferation and/or differentiation – all properties that would increase the capacity of these cells to contribute to new and existing fibers. These data hint at exciting possibilities for enhancing muscle regenerative function through metabolic modulation. However, further investigations are needed to clarify the precise mechanisms through which metabolic shifts alter satellite cell function and to delineate additional key regulators of this process.

MyoD and MYF5 are “master” transcription factors that are critical for enforcing the unique regulatory gene expression program ascribed to ASCs<sup>72, 73</sup>. MYOD and MYF5 are induced in satellite cells *in vivo* within 3 hours after cardiotoxin-mediated muscle injury, implicating them in the early stages of satellite cell activation<sup>16</sup>. Indeed, *ex vivo* knockdown of MYOD in satellite cells associated with muscle fibers reduced the expression of *Cdc6*, a regulator of DNA replication, and blocked satellite cell entry into S-phase<sup>74</sup>. The activities of MYOD and MYF5 may only partially overlap, since MYOD-null animals have a severe myogenic differentiation defect<sup>14</sup>, while in MYF5-null animals, proliferation is primarily impaired<sup>15, 17</sup>. Future studies should focus on elucidating the mechanisms by which MYOD can enable both proliferative expansion and myogenic differentiation in ASCs, and whether co-expression of MYF5 influences these cell fate decisions.

There has been great interest in identifying the relevant upstream regulators of MYOD and MYF5. Although MYOD protein is undetectable in QSCs, PAX7 and FOXO3 induce transcriptional activation at the *MyoD* locus in these cells<sup>75</sup>, further highlighting that satellite cells are primed for activation, but also hinting towards post-transcriptional mechanisms to regulate MYOD protein levels in QSCs. Insight into such regulation was revealed when it was shown that p38  $\alpha$  and  $\beta$  MAPK (p38 $\alpha/\beta$ -MAPK) is crucial for awakening QSCs into the proliferative state<sup>76</sup>. p38 $\alpha/\beta$ -MAPK are increased in proliferating satellite cells, where they induce MYOD protein expression by inhibiting the RNA destabilizing protein, Tristetraprolin (TTP)<sup>76, 77</sup> (Fig. 2b). The activities of TTP in QSCs ensure that *MyoD* mRNA is stabilized for satellite cell activation. Like MYOD, MYF5 also can be regulated both transcriptionally and post-transcriptionally, and its regulation is critical for a rapid transition to the full ASC state<sup>78–80</sup>. For example, CARM1, an arginine methyltransferase enzyme, methylates PAX7 protein in satellite cells committed to myogenesis leading to association of PAX7 with the Histone Methyl-Transferase (HMT) complex (which is comprised of Absent, Small, or Homeotic – like (ASH2L); Mixed-lineage

leukemia 1 or 2 (MLL-1 or -2); WD Repeat Domain 5 (WDR5); Retinoblastoma Binding Protein 5 (RBBP5)) and subsequent gene activation of *Myf5*<sup>79</sup>. The HMT complex, which functions as a chromatin-interacting unit that recruits elements required for active transcription, has previously been shown to collaborate with PAX7 to induce target gene expression<sup>81</sup> (Fig. 2b). Another study showed that *Myf5* mRNA is post-transcriptionally repressed by miRNA-31 in QSCs, via a mechanism involving mRNA sequestration in cytoplasmic RNA granules<sup>80</sup>. These RNA granules disassemble upon satellite cell activation leading to *Myf5* mRNA release and subsequent robust protein expression<sup>80</sup>(Fig 2b).

Altogether, these studies highlight the complex regulation involving signalling pathways, transcription factors, epigenetic regulators, RNA-binding proteins, and non-coding RNAs that are required for the transition from quiescence to the full ASC program. Future studies should focus on a more detailed understanding of the earliest stages of satellite cell activation, to define more completely the complexity of how QSCs awaken and prepare for cell division. New investigations into these unexplored areas may reveal insight into mechanisms that can be exploited to prime satellite cells for enhanced muscle repair *in vivo*, such as the newly described poised ( $G_{Alert}$ ) state of these cells (Box 4)

### Satellite cell self renewal

The satellite cell population must self-renew to ensure that a sufficient number of reserve cells can seed future rounds of muscle repair. Current data indicates that most resident satellite cells enter the cell cycle in response to muscle injury, as the majority Pax7-positive satellite cells in an injured muscle incorporate BrdU within the first 7-days of muscle regeneration<sup>44</sup>. Thus, to achieve self-renewal, a sub-population of proliferating satellite cells must first avoid myogenic differentiation and then subsequently return to  $G_0$ , or the QSC state, to replenish the available pool of stem cells. In this section, we discuss the various modes of cellular division that have recently been shown to influence these crucial cell fate decisions, and then highlight recent progress in identifying novel regulators that drive the self-renewal process during muscle regeneration.

Adult muscle stem cells can undergo either symmetric or asymmetric cell divisions (Figure 2c). The mode of division depends on the distribution of cellular components, signalling molecules, and genetic material and is paramount in balancing the maintenance and differentiation of the muscle stem cell pool<sup>76, 82–86</sup>. For example, upon satellite cell activation, the template DNA strand used for DNA replication, the asymmetric determinant regulator, NUMB, and p38 $\alpha$ / $\beta$ -MAPK signalling molecules can segregate asymmetrically to daughter cells, with those daughters that receive p38 $\alpha$ / $\beta$ -MAPK acquiring a more committed/differentiated state (Figure 3a); those daughters that acquire NUMB and the template DNA strand (and not p38 $\alpha$ / $\beta$ -MAPK) are destined to self-renew to replenish the QSC pool<sup>76, 82, 84, 87</sup>. The evolutionarily conserved PAR (Partitioning-defective) complex and the Scrib (Scribbled) cell polarity complex<sup>77, 88</sup> are likely drivers of the asymmetric distribution pattern of p38 $\alpha$ / $\beta$ -MAPK. It has also been suggested that satellite cells that have never expressed *Myf5*, as determined by Cre-based lineage tracing, represent a satellite stem cell population that divides asymmetrically, with the daughter that maintains contact with the basal lamina eventually acquiring a self-renewed state (remaining *Myf5*-negative), and

the apical daughter becoming *Myf5*-positive and committing to differentiation<sup>83</sup>. On the other hand, satellite cells can also self-renew using symmetric cell division through the activities of Wnt7a signalling<sup>83, 89</sup>, or when the JAK-STAT signalling pathway is genetically or pharmacologically impaired<sup>85, 86</sup>. Further studies will be needed to decipher the molecular determinants that regulate asymmetric versus symmetric satellite cell division, to determine whether these modes of cellular division occur concurrently *in vivo*, and to identify additional intrinsic gene regulatory factors that must be distributed selectively to self-renewing satellite cells to enable their stalled differentiation and return to quiescence.

Some insight into the crucial factors that may promote satellite cell self-renewal comes from recent studies of a slow-dividing sub-population of satellite cells that is established after birth and requires the cell-cycle inhibitor, CDKN1B (p27<sup>kip1</sup>), for maintenance<sup>90</sup>. Sprouty1 (SPRY1), a membrane-associated inhibitor of receptor tyrosine kinase signalling<sup>91</sup>, is expressed specifically in this slow cycling sub-population of satellite cells<sup>45, 90</sup>, and its genetic deletion in PAX7-expressing satellite cells led to a 50% reduction in the quiescent PAX7+MYOD-population present after muscle regeneration<sup>44</sup>. Thus, Sprouty1 function in satellite cells appears to be required for efficient return of a subset of ASCs to quiescence after muscle repair is complete (Fig. 2c). Sprouty1 likely acts to slow cell division through inhibition of FGF signalling, which normally stimulates proliferation<sup>45, 60</sup>. FOXO3 also may play a role in the suppression of myogenic differentiation and the return to quiescence (Fig. 2c), since FOXO3-deficient satellite cells were more prone to spontaneous myogenic differentiation, and mice carrying a *Foxo3* deletion displayed reduced satellite cell numbers after two rounds of muscle regeneration<sup>55</sup>. Collectively, these data suggest a model whereby both FOXO3 and Sprouty1, independently or in concert, act to suppress myogenic differentiation in self-renewing satellite cells and return these cells to the G<sub>0</sub>, QSC state (Fig. 2). It is now important to identify upstream regulators of FOXO3 and Sprouty1 and define the mechanism by which these factors are induced in only self-renewing satellite cells during muscle regeneration. It will also be beneficial to pinpoint exactly when during the regeneration process these cell fate decisions, to self-renew or to differentiate, are made.

## Satellite cell fate in aging muscle

Muscle aging is characterized by a decline in muscle repair and progressive loss of muscle mass and function due to muscular atrophy, a process commonly referred to as sarcopenia. As elderly individuals represent the fastest growing demographic in many national populations, there is an urgent need to identify new therapeutics to boost muscle repair and prevent muscle wasting and dysfunction. Although satellite cell dysfunction alone may be insufficient to drive the emergence of sarcopenia, misregulation of this cell population may promote increased muscle fibrosis and certainly impedes recovery after injury in older individuals<sup>92–94</sup>. It is increasingly apparent that intrinsic misregulation of gene pathways in aging satellite cells leads to alterations in their proliferation and differentiation *in vivo*, impeding muscle regeneration. Likewise, extrinsic signals from the local niche and even circulating, systemic factors can greatly influence muscle regenerative function and contribute to the decline of satellite cell regenerative function in aged muscle.

### Intrinsic changes in aging satellite cells

Recent studies have revealed critical changes in the transcriptional and epigenetic landscape of QSCs as they age<sup>33, 86, 95</sup>. These analyses generally indicate that aged QSCs feature an increase in repressed chromatin states, with widespread H3K27me3 across the genome<sup>33</sup>, so it would not be surprising if these chromatin re-organization events contribute to the misregulation of key signalling pathways that are required for a robust regenerative response. Work in this area recently converged to highlight the critical influence of overactive p38 $\alpha$ / $\beta$ -MAPK signalling in aged satellite cells, which leads to a drastic decline in satellite cell self-renewal and impairs satellite cell function<sup>96, 97</sup>.

A recent study showed that when as few as 10 freshly isolated satellite cells from aged or young mice were intramuscularly transplanted into immuno-compromised recipient mice, aged satellite cells engrafted (as defined by bioluminescence and histology) skeletal muscle at two-thirds the rate of younger cells<sup>97</sup>. This difference was lost when higher numbers of muscle satellite cells were transplanted (between 100 and 300), indicating that a sub-population of aged satellite cells may retain high functionality<sup>98</sup>. Interestingly, aged satellite cells exhibited a reduced proliferative output and were associated with elevated levels of p38 $\alpha$ / $\beta$ -MAPK and senescence regulator genes, which could be reversed by pharmacological inhibition of p38 $\alpha$ / $\beta$ -MAPK<sup>97</sup>. A parallel study demonstrated that the normal asymmetric partitioning of p38 $\alpha$ / $\beta$ -MAPK signalling to satellite cell daughters is disrupted in aged cells and that this disruption causes symmetric inheritance of p38 $\alpha$ / $\beta$ -MAPK and impedes satellite cell self-renewal (Fig. 3a), which could be recovered by treatment with a small molecule inhibitor of p38 signalling<sup>96</sup>.

Another recent study found elevated levels of p16<sup>Ink4a</sup>, an inhibitor of cyclin-dependent kinases (CDKs)<sup>99</sup>, in “geriatric” satellite cells from 28–32 month old mice. The elevation of p16<sup>Ink4a</sup> in “geriatric” satellite cells led to their conversion from a reversibly quiescent to an irreversibly senescent state<sup>95</sup>. Paradoxically, in addition to its known role in satellite cell activation<sup>76</sup>, p38  $\alpha$ / $\beta$ -MAPK signalling can also activate p16<sup>Ink4a</sup> to promote cellular senescence<sup>99</sup>. A genetic link between p38 $\alpha$ / $\beta$ -MAPK and p16<sup>Ink4a</sup> was recently demonstrated in skeletal muscle, when it was found that p16<sup>Ink4a</sup> protein levels were reduced in the gastrocnemius muscle of animals expressing a dominant-negative p38 $\alpha$  allele, although future studies will need to verify this connection in satellite cells<sup>100</sup>. Cumulatively, these data suggest that an age-regulated intrinsic p38 signalling-axis exists in the satellite cell pool. Under this model, while p38  $\alpha$ / $\beta$ -MAPK is asymmetrically distributed to daughter cells in young satellite cells, the switch to symmetric p38  $\alpha$ / $\beta$ -MAPK inheritance leads to a depletion of self-renewed satellite cells and through unknown mechanisms activates p16<sup>Ink4a</sup>, driving cellular senescence (Fig 3a).

Finally, in two parallel studies, it was shown that JAK-STAT signaling and effector target genes play a pivotal role in myogenic commitment and differentiation, and that this pathway is misregulated in satellite cells of aged muscle<sup>85, 86</sup>. JAK-STAT target genes are elevated in 18-month old mice, relative to 3-month old animals, and genetic perturbation of JAK-STAT members in freshly isolated satellite cells from aged animals improved their functional engraftment when transplanted into host skeletal muscle. SiRNA knockdown of JAK2 and STAT3 in satellite cells still associated with aged single fibers led to increases in the

symmetric expansion of the *Pax7+Myf5*-satellite stem cell population<sup>86</sup>. STAT3 is required for MYOD-mediated myogenic commitment *ex vivo*, and a transient pharmacological inhibition of STAT3 in aged animals *in vivo* increased the population of proliferating satellite cells and improved muscle regeneration<sup>85</sup>. Altogether, these data support a model in which elevated JAK-STAT signaling in aged satellite cells leads to their rapid differentiation and failure to undergo efficient symmetric expansion in response to muscle injury (Fig. 3a).

In future studies, it will be useful to determine whether senescence is a state that all satellite cells will eventually reach, or rather, a condition that only a subset of satellite cells will experience during muscle aging. It will also be helpful to clarify whether p38 $\alpha$ / $\beta$ -MAPK directly influences the senescent phenotype in aging satellite cells. Furthermore, the role of JAK-STAT signaling in regulating cell fate decisions of muscle stem cells is probably context dependent and likely varies for different JAK-STAT family members, since JAK-STAT proteins have been reported to promote satellite cell proliferation and prevent myogenic differentiation<sup>101</sup>, in addition to their role in promoting myogenic differentiation (discussed above)<sup>85, 86</sup>. Future studies to generate genomic binding profiles and identify interacting partners of the various STAT transcription factors in young and aged satellite cells should shed light on their important functions during muscle regeneration and aging.

### Regulation by local factors

Satellite cells are greatly influenced by their local microenvironment, as muscle fibers and other non-myogenic cells secrete growth factors and cytokines that influence their behaviour during regeneration<sup>3</sup>. As satellite cells age, so does the niche in which they reside, and these structural, and molecular alterations can impede satellite cell function. One example comes from a recent study showing that aged muscle secretes higher levels of FGF2 (Fibroblast growth factor 2) into the satellite cell niche, leading to a considerable depletion of the low-cycling satellite cell population. This study also reported that inhibition of FGF receptor 1 (FGFR1) can rescue age-related defects in satellite cell cycling and apoptosis<sup>45</sup>. Importantly, FGF2 was shown to inhibit the activities of Sprouty1, a seminal regulator of satellite cell quiescence, explaining the shift towards more high-cycling satellite cells. Curiously, a different study found that the activity of FGFR1 was attenuated in aged satellite cells, and that ectopic expression of a constitutively active FGFR1 recombinant protein enhanced the asymmetric inheritance of p38 $\alpha$ / $\beta$ -MAPK<sup>102</sup>. As many signalling pathways exhibit bimodal or multimodal effects, which can be influenced by cellular context, timing, and dosage, it is likely that these seemingly contradictory results reflect a critical need to maintain FGF signalling in satellite cells within a tight, physiological window, as overactivation or attenuation of this pathway can perturb muscle homeostasis and contribute to satellite cell dysfunction.

Altogether, these data provide an intriguing example of how aging-related misregulation of extrinsic factors in the local niche can influence critical intrinsic pathways that modify satellite cell fate.

## Regulation by systemic signals

Evaluation of potential systemic influences on muscle regenerative function has been undertaken by a number of research groups using a 150 year-old technique known as parabiosis<sup>103</sup>, which involves the surgical joining of the circulatory systems of two mice. Remarkably, heterochronic parabiotic pairings of young and aged mice led to the restoration of satellite cell activation and a more robust regenerative response after injury in the aged partner of the pair<sup>104, 105</sup>. These data provided initial support for the notion that young blood may contain “rejuvenation” factors capable of regulating muscle repair in older animals. Importantly, the physiological impacts of heterochronic parabiosis are not limited to the aged partner, and exposure of a young animal to an aged circulation through such pairing has been shown to slow regeneration in young muscle<sup>92, 106, 107</sup>. Thus, in addition to loss of pro-regenerative factors, aged blood also appears to accumulate suppressive factors that can actively impair healthy function in the muscle and other organs.

Molecular identification of circulating molecules that regulate cell function in aged animals has been a major emphasis in recent years. Published reports implicate increases in TGF $\beta$ 1<sup>108, 109</sup> and Wnt<sup>92, 110</sup> signalling as key contributors to the active suppression of satellite cell function in aged muscle, and these factors could potentially contribute to rare, age-related cell fate conversion events, in which satellite cells transition from a myogenic to a fibrogenic state<sup>92, 111</sup>. A few “rejuvenating” molecules have also been identified. For example, it has been recently reported that Oxytocin, a hormone produced by the hypothalamus with a demonstrated role in social behavior<sup>112</sup>, is reduced 3-fold in the blood of aged mice, and that its receptor is down-regulated by nearly 50% in aged satellite cells<sup>113</sup>. Administration of Oxytocin to aged mice relieved the proliferation and differentiation defects of old satellite cells, and enhanced the regenerative response to muscle injury<sup>113</sup>. In an intriguing and still unexplained link to the studies of cell intrinsic alterations in aged satellite cells discussed above, Oxytocin induced the activation of the MAPK/ERK pathway in satellite cells and improved their proliferation potential. Similar beneficial effects on muscle repair following cryoinjury in aging mice have been reported for the circulating cytokine Growth Differentiation Factor-11 (GDF11), a member of the TGF $\beta$  superfamily that has significant structural and functional homology to Myostatin (MSTN)<sup>114</sup>. Several studies suggest that expression of *Gdf11* declines with age in key GDF11-producing organs, and that the combined pool of GDF11 and MSTN is diminished in the blood of aged animals and humans<sup>115–117</sup>. Moreover, supplementation of recombinant GDF11 protein in older mice yielded improvements in muscle ultrastructure, satellite cell function and muscle regeneration<sup>118</sup>, supporting the notion that this protein, in particular, promotes healthier function in aged muscle. It is important to note, however, that these results were recently challenged by another group, who reported that GDF11 levels may increase with age in some species and that GDF11 supplementation in old mice has no effect and in young mice may impede muscle regeneration in response to cardiotoxin injury<sup>119</sup>. These apparently discrepant results may relate to cross-reactivity of reagents used to track GDF11 protein levels, which also react with GDF8<sup>119–121</sup> and immunoglobulin light chain<sup>116</sup>, as well as to differences in experimental design, including differences in the modes of muscle injury employed, in the doses of GDF11 injected, in the strategies for analysis of muscle regeneration employed, and even in the sources of GDF11 protein and aged animals used by



the different groups. While further studies have clarified some of the reported discrepancies<sup>116</sup>, additional experiments will be needed to fully reconcile these observations and determine how the levels of circulating GDF11 protein change throughout life as well as how such changes influence satellite cells.

## Satellite cells in muscular dystrophy

Duchenne muscular dystrophy (DMD) is a severe, genetic disorder that causes progressive muscle wasting in affected individuals and currently has no cure. Dystrophin, the defective gene product that causes DMD, plays a pivotal structural role in anchoring the muscle fiber to the extracellular matrix in the muscle niche. Numerous studies in mice have shown that dystrophic fibers rupture and die after repeated muscle contractions, and are eventually replaced with fibrotic tissue<sup>122</sup>. Fibrosis arises due to an excess of connective tissue deposited in muscle during regenerative processes, and is thought to be mediated in part by muscle-resident fibroblasts<sup>123</sup>. Most of the physiological and functional deficits exhibited by DMD patients have been attributed to abnormalities in mature muscle fibers, where a known role for Dystrophin has been documented. However, recent work has shown that dystrophic satellite cells are impaired in various aspects of the satellite cell life cycle, which impact their muscle regenerative functions<sup>37, 111, 124–129</sup>. We discuss here emerging data suggesting that intrinsic misregulation of satellite cell fate in dystrophic muscle may impair muscle regenerative function and contribute directly to the DMD pathology.

Dystrophin functions near the cell membrane and acts as a structural component of the muscle cell that regulates the flow of signalling molecules. Therefore, it would not be surprising if lack of dystrophin, which normally is expressed by satellite cells, would directly influence their downstream cell-intrinsic, signalling pathways to alter their fate, and ultimately, function. Initial evidence that functional impairments in the satellite cell pool contribute to the DMD phenotype derives from a recent study that created a telomerase and dystrophin double knockout animal, mainly as a tool to impair the functional muscle stem cell pool<sup>130</sup>. These mice exhibit severe molecular and physiological defects in skeletal muscles, including the diaphragm<sup>130</sup>, similar to human DMD patients. Interestingly, satellite cells from these mice showed reduced proliferation both *in vivo* and during *ex vivo* culturing, and they were functionally impaired when transplanted into host animals<sup>130</sup>. These data suggested, for the first time, that the severity of DMD can be influenced by the regenerative functions of endogenous muscle stem cells.

Evidence for a direct role of Dystrophin in satellite cells remained elusive, however, until a recent study showed that the Dystrophin protein has a highly specialized function in regulating cell division mechanisms in muscle stem cells<sup>129</sup>. Specifically, it was shown that the presence of Dystrophin enhances MARK2(Par1b) protein levels and that MARK2 then mediates the asymmetric localization of the PARD3 polarity protein, which functions to properly guide asymmetric cell division in proliferating satellite cells. Accordingly, in response to injury, dystrophic satellite cells undergo a prolonged mitosis and their ability to undergo apico-basal cell divisions is perturbed, leading to impaired asymmetric division and a smaller population of progenitors capable of contributing to muscle repair<sup>129</sup>(Fig. 3b). In line with this, after culturing dystrophic muscle fibers for 72h an increase in the subset of

*Pax7+* satellite cells that have never expressed *Myf5* (discussed above<sup>83</sup>) and a reduction in the number of MYOG-expressing progenitors was observed, further suggesting that dystrophic satellite cells may display enhanced self-renewal and that some may be blocked with respect to myogenic differentiation<sup>129</sup>. These findings may explain why dystrophic muscle or single fibers contain more PAX7-positive satellite cells than control muscle or wildtype fibers<sup>128, 131</sup>. Interestingly, a prior report suggested that dystrophin-deficient mice exhibit defects in satellite cell self-renewal, with reduced numbers of *Pax7+Myf5*-satellite cells associated with single fibers from pre-injured animals<sup>128</sup>. As this effect was more pronounced in 6 month old, as compared to 2 month old mice (the approximate age used in the study discussed above), these data may suggest that the impact of dystrophin deficiency on satellite cell self-renewal can vary with age. Alternatively, it is possible that these differences may be explained by the different activation models used - isolation of single fibers from pre-injured animals<sup>128</sup> versus single fibers from uninjured animals cultured for 72 hrs<sup>129</sup>. Regardless, further studies are needed to harmonize these results.

Impaired self-renewal of the *Pax7+Myf5*-sub-population of dystrophic satellite cells have also been associated with reduced Notch signalling, and enforced activation of this pathway restored satellite cell self-renewal<sup>128</sup>. Given the role that Notch plays in promoting satellite cell quiescence (discussed above), reduced Notch signaling in dystrophin deficient satellite cells may also contribute to the reported increase in the high-cycling satellite cell subset in these animals<sup>90, 132</sup>. The notion that modulation of Notch signalling may improve the dystrophic phenotype has gained further support from a very recent analysis of two dystrophin-deficient dogs presenting a milder DMD phenotype<sup>133, 134</sup>. Intriguingly, these “escaper” animals were found to possess a single mutation in the promoter of the Notch ligand Jagged-1, which creates a novel Myogenin binding site and causes upregulation of Jagged1 expression<sup>134</sup>. Myogenic cells from escaper dogs overexpressing Jagged-1 also showed enhanced proliferation, and further studies in dystrophic zebrafish confirmed that overactivation of Jagged-1 ameliorates the dystrophic phenotype<sup>134</sup>. These results demonstrate that studies aimed at revealing the mechanisms underlying the effects of Jagged-1 in dystrophic muscles, as well as to delineate additional genetic suppressors of dystrophic phenotypes, may open novel treatment avenues for DMD.

Finally, it has been recently demonstrated that, apart from changes regarding their self-renewal and differentiation potential, a subset of dystrophic satellite cells may initiate a Wnt3a and TGF $\beta$ -dependant fibrogenic-like cellular program (Fig. 3b). This, over time, may serve to deplete functional muscle stem cells and impede effective muscle repair. It also may help to explain the increase in fibrotic tissue seen in DMD muscle<sup>111</sup>.

Collectively, the data discussed above strongly suggest that the loss of satellite cell regenerative function exacerbates DMD symptoms, and that dystrophin deficiency directly influences satellite cell fate. More studies will be required to fully understand the cellular and molecular mechanisms driving these apparently context-dependant effects<sup>135</sup>. It will also be important to decipher the transcriptional and epigenetic changes that may underwrite functional misregulation in dystrophin-deficient satellite cells and to determine the contribution that extrinsic signals may play in modulating these intrinsic changes to the muscle stem cell fate.

## Conclusion/perspective

An emerging theme from the work discussed here is that the intrinsic gene regulatory circuits that control adult muscle satellite cell behaviour in healthy muscle are misregulated in aging and disease. Elucidating the transcriptional and epigenetic “drivers”, and their targets, that act downstream of dystrophin-deficiency or become altered in aged satellite cells, will continue to reveal a molecular roadmap of potentially drug-able targets that could be manipulated with small-molecule intervention to enable the modulation of stem cell activity. Furthermore, reinstating self-renewal pathways in cultured satellite cells may also enable their efficient expansion in culture, a barrier that currently impedes their use in the clinic (see Box 2). There is no better time to be studying gene regulation, muscle stem cell fate, and translational medicine, and we anticipate that novel discoveries in basic science will improve the prognosis and treatment of age associated muscle dysfunction and currently incurable muscle pathologies.

## Acknowledgments

We wish to thank all reviewers of this manuscript for their critical evaluation and their insightful feedback in preparing this manuscript. This work was supported in part by a Burroughs Wellcome Fund Postdoctoral fellowship Award and a T32 NIH training grant (5T32DK007260-38) to A.E.A., and National Institutes of Health (NIH) RO1 ES024935 and R56 AG048917 to A.J.W. Content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

## Glossary

<b>Satellite cell niche</b>	A structural and growth-factor rich environment above the muscle sarcolemma but beneath the basal lamina, consisting of cellular and acellular components that support and regulate muscle satellite cells.
<b>Muscle sarcolemma</b>	Outer cell membrane of a myofiber.
<b>Basal lamina</b>	Inner layer of the basement membrane, composed of extracellular matrix proteins, that is adjacent to the muscle sarcolemma.
<b>Fibro-adipogenic progenitors (FAPs)</b>	A bipotent progenitor population that can adopt both fibrogenic and adipogenic cell fates, located in the interstitial space of muscle fibers.
<b>Cellular senescence</b>	A cellular state induced by extensive proliferation, induction of oncogene expression, inflammatory signals, or other stimuli, in which cells irreversibly exit the cell cycle.

<b>Cyclin-dependent kinases (CDKs)</b>	A family of kinases that regulate cell cycle transitions.
<b>Gastrocnemius muscle</b>	A skeletal muscle located at the back of the lower legs.
<b>Somite</b>	A division in the developing embryo consisting of paired blocks of paraxial mesoderm.
<b>Myotome</b>	The dorsal part of the somite in the developing embryo that gives rise to the skeletal muscle.
<b>Cre recombinase-mediated lineage tracing</b>	A molecular genetic technique used to trace each cell that has ever expressed a gene-of-interest, through gene-specific Cre-mediated recombination that leads to expression of a fluorescent reporter gene.
<b>Oxidative phosphorylation (OXPHOS)</b>	A metabolic pathway in which mitochondria oxidize nutrients to form ATP.
<b>Autophagic flux</b>	A measurement to quantify cellular autophagic activity.
<b>NAD<sup>+</sup></b>	The oxidized form of Nicotinamide Adenine Dinucleotide (NAD) that can accept an electron becoming reduced to its NADH form.
<b>Quiescence</b>	A reversible state of dormancy characterized by withdrawal from the cell cycle, or lack of proliferation.
<b>Activation</b>	A reversible state characterized by entry into the cell-cycle, or transition to a proliferative state.
<b>Symmetric cell division</b>	The partitioning of cellular components during cell division that leads to two daughter cells being molecularly and functionally identical.
<b>Asymmetric cell division</b>	The partitioning of cellular components during cell division that leads to two daughter cells being molecularly and functionally distinct.

<b>Pax3</b>	A paired box protein family member expressed in satellite cells in a few adult muscle groups with a prominent role in embryonic myogenesis.
<b>Sprouty1</b>	A receptor tyrosine kinase inhibitor that restricts entry to the cell cycle by abrogating cell signalling pathways, like the Fibroblast Growth Factor Receptor (FGFR).
<b>mTORC1</b>	A gene product encoding the mammalian Target of Rapamycin Complex 1 that is part of a protein complex that controls protein synthesis by sensing nutrients, energy, and metabolic products in the cell.
<b>TTP</b>	Tristetraprolin is an RNA-binding protein that binds the 3' untranslated region of target RNAs, leading to their rapid decay through the recruitment of cytoplasmic RNA degradation machinery.
<b>Active chromatin</b>	Regions of the genome that are actively transcribed and are flanked by histone H3 tri-methylation at lysine 4 (H3K4me3).
<b>p38<math>\alpha</math>/<math>\beta</math> MAPK</b>	Two isoforms of the p38 family of MAPKs, which plays a seminal role in detecting extracellular signals from the outside of the cell and directing a cascade of phosphorylation events that activates downstream effectors and ultimately target gene expression.
<b>Parabiosis</b>	The surgical joining of the circulatory systems between two animals.
<b>Heterochronic parabiosis</b>	The surgical joining of the circulatory systems between a young and old animal.

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## Biographies

**Albert E. Almada** is a Burroughs Wellcome Fund postdoctoral fellow in the laboratory of Amy J. Wagers at the department of Stem Cell and Regenerative Biology at Harvard University, where he is investigating the transcriptional and epigenetic mechanisms that

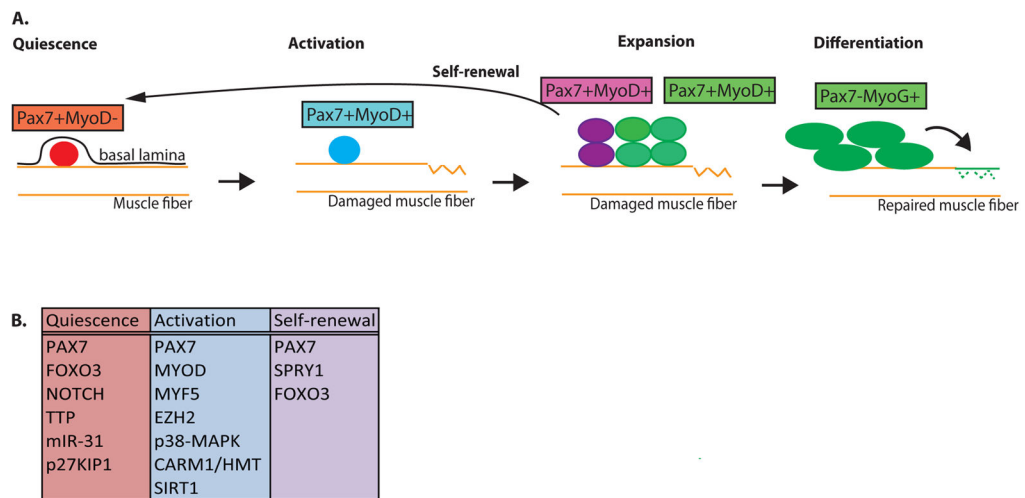


program muscle stem cell identity and function. He obtained his Ph.D. in Biology at the Massachusetts Institute of Technology (MIT) under the tutelage of Dr. Phillip A. Sharp, where he studied the biogenesis and transcriptional regulation of a novel class of promoter-associated non-coding RNAs in embryonic stem cells.

**Amy J. Wagers** is the Forst Family Professor of Stem Cell and Regenerative Biology at Harvard University, a Senior Investigator in the Section on Islet Cell and Regenerative Biology at the Joslin Diabetes Center and a member of the Harvard Stem Cell Institute and Paul F. Glenn Center for the Biology of Aging at Harvard University and Harvard Medical School. She received her Ph.D. in Immunology and Microbial Pathogenesis from Northwestern University, and completed postdoctoral training in stem cell biology at Stanford University. Dr. Wagers has studied muscle stem cell biology for more than 15 years and her research seeks to understand how changes in stem cell activity impact tissue homeostasis and repair throughout life and how these cells may be harnessed for regenerative medicine.

### Online summary

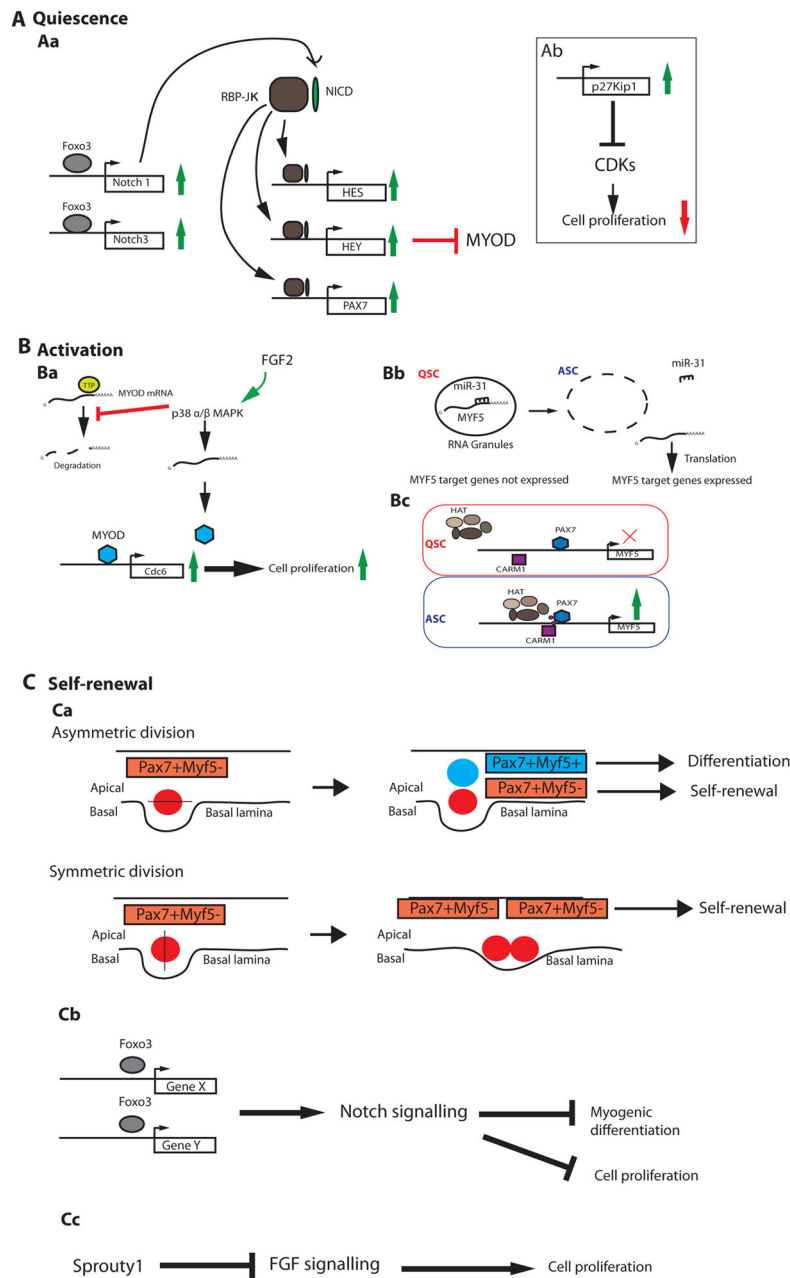
- Satellite cell quiescence, activation, and self-renewal are coordinated by extrinsic and intrinsic regulators.
- Quiescent satellite cells are molecularly and functionally heterogeneous and have a unique transcriptional and epigenetic profile that distinguishes them from activated satellite cells.
- Aged satellite cells misregulate intrinsic factors like p38- $\alpha/\beta$ -MAPK, JAK-STAT, and p16<sup>Ink4a</sup>, leading to impairments in self-renewal and eventually, conversion to a senescent state.
- Local and systemic factors influence satellite cell fate decisions, and systemic delivery of youthful blood-borne factors can ameliorate some aspects of the aging phenotype in skeletal muscle.
- Dystrophin-deficiency directly misregulates the satellite cell state and function, which further exacerbates DMD symptoms.



**Figure 1. Classical view of muscle myogenesis**

**(A)** During muscle homeostasis, satellite cells are maintained in a quiescent state (red) beneath the basal lamina and above the muscle sarcolemma of the muscle fiber. Quiescent satellite cells express Pax7 and lack MyoD. Upon muscle insult (ruptured fiber), satellite cells become activated (blue) and proliferate. Proliferating satellite cells maintain expression of Pax7 and induced expression of MyoD. A subset of these dividing satellite cells commit to differentiation (green), through the expression of Myogenin (MyoG) and downregulation of Pax7, ultimately producing myoblasts that exit the cell cycle and fuse to repair muscle. Alternatively, another subset of activated satellite cells will self-renew (purple) by inhibiting myogenic determination and re-instating quiescence. This ensures that the muscle retains a sufficient number of functional muscle stem cells to support future rounds of muscle repair.

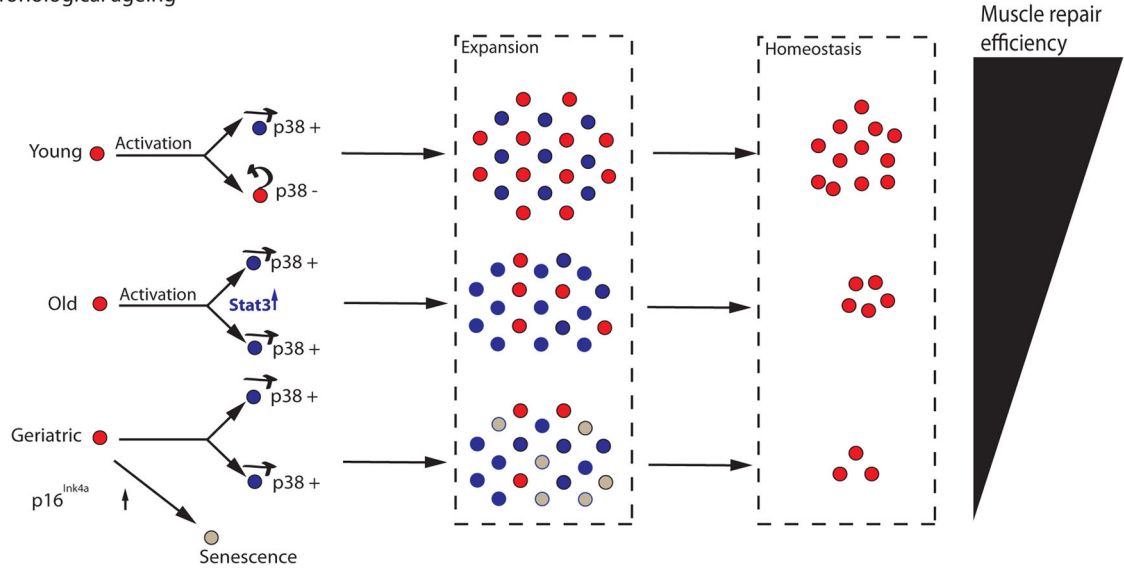
**(B)** Cell-intrinsic factors discussed in this review having roles in satellite cell quiescence, activation, and self-renewal.



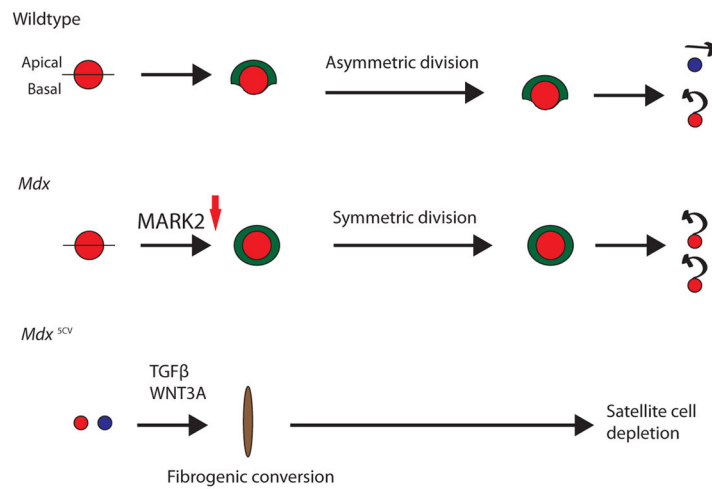
**Figure 2. Emerging regulators of satellite cell quiescence, activation, and self-renewal**  
 (A) During homeostasis most satellite cells are quiescent. (Aa) The Notch signalling pathway plays a key role in enforcing satellite cell quiescence. Upon ligand/receptor binding, the Notch Intracellular Domain (NICD, pink oval) undergoes several proteolytic cleavages (not shown), and then translocates to the nucleus where it interacts with the Recombining Binding Protein J- $\kappa$  effector protein (RBPJ- $\kappa$ , blue circle). The NICD:RBPJ- $\kappa$  complex activates expression of target genes, including the Hes and Hey family of transcription factors, which function to inhibit MYOD. RBPJ- $\kappa$  also binds the promoter of *Pax7* and induces its expression. PAX7 induction further downregulates MYOD via transcription-independent mechanisms (not shown). The Forkhead box 3 (FOXO3, green circle)

transcription factor can induce expression of NOTCH1 and NOTCH3 trans-membrane proteins, which facilitates Notch signalling by ensuring an adequate number of receptors are produced and shuttled to the cell membrane where they can interact with available ligands in the local environment. (Ab) Satellite cell entry into the cell cycle is inhibited through the activities of p27<sup>Kip1</sup>, which inhibits cyclin-dependant kinases (CDKs). (B) After muscle injury, activated satellite cells detect pro-myogenic stimuli from the local environment and begin proliferation. (Ba) Exposure to FGF2 induces p38 $\alpha$ / $\beta$  mitogen-activated protein kinase (MAPK) activity, which inhibits the RNA-binding destabilizing protein, Tristetraprolin (TTP), leading to stabilization of the *MyoD* mRNA. This allows MYOD protein to accumulate (purple circle) and induce expression of *Cdc6*, a DNA replication gene that promotes cell cycle entry and thus proliferation. (Bb) Another activation regulator, MYF5, is post-transcriptionally repressed by miRNA-31 (miR-31), which sequesters *Myf5* mRNA into RNA granules in quiescent satellite cells (QSCs) and prevents activation of MYF5 targets. Upon muscle trauma, the RNA granules dissociate, miRNA-31 levels decrease, and MYF5 protein is produced and can activate target genes. (Bc) The *Myf5* gene is also transcriptionally regulated through the activities of CARM1 (pink circle), an arginine methyl-transferase that methylates PAX7 (small purple circles on the light blue circle) leading to PAX7 association with the Histone Acetyl-Transferase complex (HAT, purple complex), and the induced expression of target genes (such as MYF5) in activated satellite cells (ASCs). (C) Satellite cells are capable of self renewal. (Ca) A balance between self-renewal and differentiation of satellite cells is achieved by the mode of cell division. Apico-basal divisions generate one self-renewing (red, *Myf5*<sup>+</sup>) and one differentiating cell (blue, *Myf5*<sup>-</sup>). On the other hand, planar divisions (parallel to the muscle fiber) are symmetric and generate two daughters that retain self-renewal capabilities. (Cb) Self-renewal of satellite cells can be followed by their return to quiescence. FOXO3 induces expression of Notch signalling genes, enabling the cascade of molecular events leading to the inhibition of MYOD-mediated differentiation and blockade of cell cycle progression. (Cc) The return to quiescence also requires Sprouty1 (Spry1), a tyrosine kinase inhibitor that inhibits FGF signalling, thereby restricting cellular proliferation.

## a Chronological ageing



## b Muscular dystrophy



**Figure 3. Intrinsic cell fate regulators are misregulated in aged and dystrophic satellite cells**  
**(A)** In youth and adulthood (age 2–6 months in mice), quiescent satellite cells (red circles) asymmetrically distribute p38 to daughter cells, such that one daughter receives p38 and is competent for myogenic commitment and differentiation (blue circle with arrow pointing forwards to denote lineage commitment), while the other daughter self-renews and returns to the quiescent state (red circle with arrow pointing backwards to denote their self-renewal and return to quiescence). Asymmetric satellite cell divisions in young animals yield a healthy balance between satellite cells that are competent for myogenic differentiation and cells that self-renew during the expansion phase, which ensures the efficient repair of muscle and the return of a sufficient number of cells to the satellite cell niche to support future demands for muscle regeneration (homeostasis). However, in aged animals (18–24 months



in mice), p38 is symmetrically partitioned to daughter cells and JAK-STAT signalling is elevated in proliferating cells, leading to an overall increase in myogenic commitment within the satellite cell population, and reducing the number of quiescent satellite cells at homeostasis. In “geriatric” muscle (28–32 months of age in mice), p16<sup>Ink4a</sup> is additionally elevated in the satellite cell population, leading to a block in satellite cell activation and directing these cells to an irreversible state of cellular senescence (grey circles). The intrinsic deficits in aged and “geriatric” satellite cells deplete the self-renewing satellite cell population at homeostasis, and thus impair the regenerative response in skeletal muscle as animals age (Black triangle). **(B)** Satellite cells from 2-month old animals (adult) carrying a loss-of-function mutation in the Dystrophin gene (*mdx*) display defects in asymmetric division and a stark reduction in apico-basally oriented divisions due to reduced levels of polarity kinase MARK2 (also known as Par-1). This alters the localization of PARD3 (shown in green) and impairs mitosis, leading to a failure to properly expand the lineage committed satellite cells, and a consequent inefficiency of muscle repair in response to injury. In mice carrying an alternative *mdx* allele (*mdx<sup>5cv</sup>*), dystrophic satellite cells (either QSC or ASC) can undergo a cell type transition towards a fibrogenic state (brown oval) via overactivation of Wnt and TGFβ signalling. This may over time and in a context-dependant manner lead to depletion of highly functional muscle stem cells.