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Skeletal muscle aging: stem cell function and tissue homeostasis

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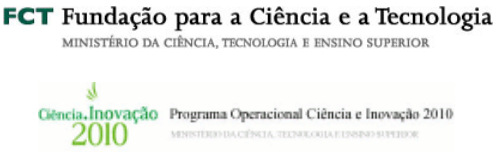

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Summary

Aging is accompanied by a decline in the homeostatic and regenerative capacity of all tissues and organs and it is generally associated with a decline in stem cell function. Muscle aging, in particular, is characterized by the reduction of tissue mass and function, which are particularly prominent in geriatric individuals undergoing sarcopenia. The age-associated muscle wasting is also associated with a decline in regenerative ability and a reduction in resident muscle stem cell (satellite cell) number and function. Although sarcopenia is one of the major contributors to the general loss of physiological function, the mechanisms involved in age-related loss of muscle homeostasis and satellite cell activity are yet poorly understood. To understand the processes that contribute to the phenotype associated with skeletal muscle aging, it is necessary to identify molecular regulators of stem cell functional decline and novel regulators of adult muscle growth and atrophy

Using a microarray-based transcriptome analysis of muscle stem cells isolated from young and physiologically aged/geriatric mice, we uncovered specific changes in the gene expression profile that highlighted key biological processes and potential molecular markers associated with satellite cell aging, which included p16INK4a. We used Bmi1-deficient mice to further explore the implications of p16INK4a up-regulation in satellite cell function. Bmi1 is a member of the Polycomb group family and defects in Bmi1-/- mice have been, in great part, associated with derepression of the INK4a/p16 locus. We found premature p16INK4a up-regulation in young/adult Bmi1-deficient satellite

cells correlating with defects in satellite cell number, proliferation and self-renewal capacity. In addition we have identified a number of overlapping biological processes dysregulated in physiologically aged and Bmi1-deficient satellite cells, suggesting that Bmi1-dependent epigenetic regulation may underlie many of the intrinsic changes taking place in chronologically aged satellite cells. In addition, we show that Bmi1 loss causes defects of late postnatal/adult muscle growth characterized by reduced muscle mass with smaller muscle fibers, typical of atrophying senescent/sarcopenic muscle. Since p16INK4a expression is specifically up-regulated in muscle satellite cells of geriatric, sarcopenic mice and in a mouse model of accelerated senescence/sarcopenia (SAMP8), we propose that the Bmi1/p16INK4a axis might be particularly operative in muscle stem cells from the elderly.

Muscle wasting is one of the physiological consequences of sarcopenia and the identification of novel factors regulating muscle growth and atrophy is of potential relevance for therapeutical applications. We have uncovered a new role for Sestrins as skeletal muscle growth promoting factors in the adult. We found Sestrins expression regulated in mouse models of skeletal muscle atrophy and hypertrophy and in human myopathies. Through a gain of function approach we show that Sestrins induce skeletal muscle growth, by activating the IGF1/PI3K/AKT pathway.

Resumen

El envejecimiento se acompaña de una reducción en la capacidad regeneradora y homeostática de todos los tejidos y órganos, y se asocia generalmente con una reducción en la función de las células madre. El envejecimiento del tejido muscular está caracterizado concretamente por una reducción global de la masa muscular y un empeoramiento de la función de tejido, particularmente prominentes en individuos muy viejos (geriátricos) que padecen sarcopenia. La pérdida muscular asociado a la edad, se acompaña de una reducción en la capacidad de regeneración del músculo y en una reducción del número y la función de las células madre residentes en el músculo (células satélite). Aunque la sarcopenia sea una de las causas principales de la pérdida general de función fisiológica del músculo, los mecanismos implicados en la reducción de la homeostasis muscular y de actividad de las células satélite no han sido completamente caracterizados. Para entender los procesos que contribuyen al fenotipo asociado con el envejecimiento del músculo esquelético, es necesario identificar los reguladores moleculares implicados en el declive de la función de las células madre y en los procesos de crecimiento y atrofia muscular.

Mediante el análisis comparativo del transcriptoma de células madre musculares aisladas de ratones jóvenes y de ratones viejos (geriátricos), hemos encontrado cambios específicos en su perfil de expresión génica que apuntan a los procesos biológicos dominantes y a los marcadores moleculares potencialmente asociados con el envejecimiento de las células satélite, entre los que destaca p16INK4a. Por ello, hemos utilizado ratones deficientes en Bmi1 para explorar más profundamente las implicaciones de la sobreexpresión de p16INK4a en la función de las células satélite. Bmi1 es un miembro de la familia de proteínas del grupo Polycomb y los defectos

encontrados en ratones Bmi1$^{-/-}$, se han asociado en gran parte a la desrepresión del locus de INK4a/p16. Hemos encontrado que células satélite jóvenes del ratón Bmi1$^{-/-}$ presentan sobrexpresión de p16INK4a, que correlacionan con una reducción en el número de la células, y en su capacidad de proliferación y autorenovación. Además hemos identificado un grupo de procesos biológicos comunes entre las células satélite viejas y las deficientes en Bmi1, sugiriendo que la regulación epigenética mediada por Bmi1 puede ser la base de muchos de los cambios intrínsecos que ocurren en células envejecidas fisiológicamente. Además, demostramos que la pérdida Bmi1 causa defectos en el crecimiento postnatal/adulto del músculo, caracterizado por pérdida de masa muscular con fibras más pequeñas, típico del músculo atrofiado senescente o sarcopénico. Puesto que la expresión de p16 está aumentada específicamente en el músculo de ratones viejos, sarcopénicos y en un modelo del ratón con envejecimiento (senescencia) acelerado (SAMP8), proponemos que el eje Bmi1/p16 puede actuar particularmente en las células madre musculares de los ancianos.

La pérdida de masa muscular es una de las consecuencias fisiológicas de la sarcopenia y la identificación de nuevos factores que regulen el crecimiento y atrofia del músculo es de gran importancia para aplicaciones terapéuticas. Hemos descubierto un nuevo papel de las Sestrinas como factores promotores del crecimiento del músculo esquelético en el adulto. Hemos encontrado que la expresión de las Sestrinas se regula en modelos del ratón de atrofia y de hipertrofia muscular y en miopatías humanas. Mediante experimentos de ganacia de función hemos demostrado que las Sestrinas inducen el crecimiento del músculo esquelético, activando el ruta de señalización de IGF1/PI3K/AKT.

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Introduction

Introduction

Skeletal muscle and myogenesis: Overview

Skeletal muscle represents a large fraction of the body mass and it is involved in a number of vital functions, such as movement, breathing and metabolism. Skeletal muscle tissue is composed of elongated multinucleated cells of cylindrical shape, the **myofibers**, made of a network of myofibrils (sarcomeres of actin and myosin) responsible for the skeletal muscle striated pattern.

The formation of myofibers, a process referred to as **myogenesis**, occurs throughout life and follows a stereotyped series of events: proliferation of muscle cell progenitors, determination and commitment of progenitors to myoblasts, differentiation of post-mitotic mononuclear myocytes, and fusion of myocytes into multinucleate myofibers (Murphy and Kardon, 2011).

During embryonic development, myogenic progenitors reside in the medial and lateral lips of the dorsal portion of the somites, the dermomyotome, from where they delaminate prior to undergoing myogenesis. In the limb of the mouse, they undergo embryonic myogenesis between E10.5 and E12.5 to establish the basic muscle pattern (Murphy and Kardon, 2011).

Fetal (E14.5–P0; P, postnatal day) and neonatal (P0–P21) myogenesis are critical for muscle growth and maturation. Fetal progenitors are mononuclear cells lying in the

intersticium of the myofibers while the neonatal and adult progenitors (or muscle stem cells) adopt a unique anatomical position and lie in between the plasmalemma and basement membrane of the myofibers and they are referred to as **satellite cells** (Biressi et al., 2007). The neonatal population of satellite cells is highly proliferative, entering into the quiescent state around P21, remaining in this dormant state thereafter. **Regeneration** of injured adult muscle tissue strictly depends on the activation of the quiescent satellite cell pool, and subsequent transition into the proliferative and differentiation stages leading to formation of new muscle fibers. Instead, the process of **physiological growth** of the adult muscle tissue mostly relies on the balance between anabolic and catabolic processes taking place in the preexisting myofiber, with the contribution of satellite cells to this (non-regenerative) growth process remaining debatable.

Aging of the skeletal muscle is characterized by a significant decline in overall tissue mass (atrophy) and functionality. Maximal muscle wasting (**sarcopenia**) occurs in very old/geriatric individuals, coursing with dramatic loss of muscle mass and increased myofiber denervation. Interestingly, the age-associated muscle decline is accompanied by a reduction in the regenerative capacity which is associated with reduced satellite cell number and function (Conboy and Rando, 2005; Thompson, 2009).

Satellite cells

During postnatal growth, accretion of new myonuclei by the myofibers is supplied by satellite cells: resident muscle stem cells located on the surface of a myofiber.

Satellite cells were first identified in electron micrographs by their unique anatomical position, situated between the basal lamina and cell membrane of mature myofibers, and by chromatin and organelle characteristics, suggesting mitotically and metabolically quiescent cells (Mauro, 1961). In the mouse, satellite cells expressing Pax7 appear at approximately embryonic day 16.5 (E16.5) in the developing limb muscle (Kassar-Duchossoy et al., 2005). Satellite cells account for about 30% of total muscle nuclei in the early postnatal period, when their main function is to provide myonuclei for growth of the neonatal myofibers. The proportion falls over time and satellite cells constitute only 2-7% of total muscle nuclei by adulthood, changing also their role to one of providing myonuclei exclusively for tissue homeostasis or in response to demands for myofiber repair and regeneration (Seale and Rudnicki, 2000).

Satellite cells are in fact a heterogeneous population, composed of noncommitted stem cells, which maintain self-renewal ability, and committed myogenic progenitors, which undergo lineage-specific differentiation. It is clear now that by P21 satellite cells enter into a quiescent state, remaining as

dormant (or very slow cycling) stem cells throughout adult life. Once activated by environmental signals, such as those produced through injury or stress, satellite cells undergo asymmetric cell division that serves both to maintain the satellite cell population and to give rise to committed myogenic cells (Kuang et al., 2007; Shinin et al., 2006). These latter cells (myoblasts) proliferate, migrate and differentiate, subsequently fusing to form new fibers (Buckingham, 2006; Kuang et al.; Sambasivan and Tajbakhsh). The ability of adult satellite cells to transit to a reversible quiescent state after providing a source of progeny is critical for homeostasis of tissue-resident stem cells and presumably the maintenance of the tissue during numerous rounds of damage caused by various insults throughout life (Figure 1).

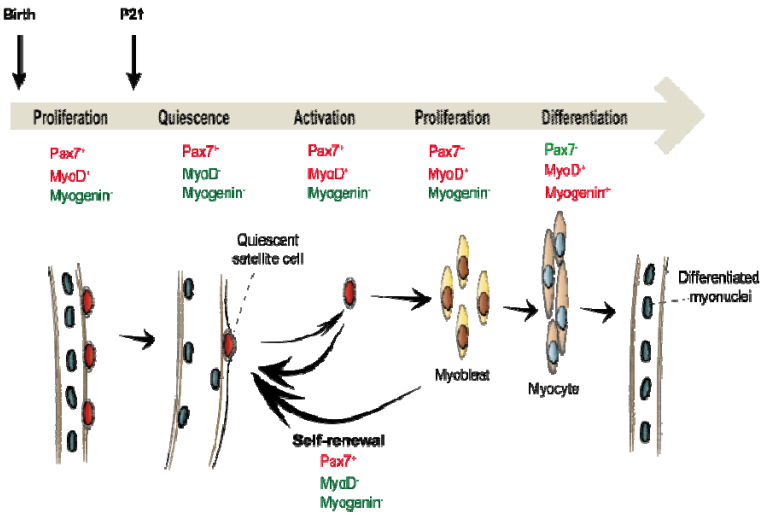


Figure 1: Satellite cell differentiation and self-renewal from birth to the adult age.

With age, there is a gradual decline in the effectiveness of the regenerative response of skeletal muscle to damage (Grounds, 1998). Following a necrotic injury, aged muscle responds with a delayed, partially impaired myogenic response and an increase in fibrous connective tissue (Conboy et al., 2005). Estimates of changes on the number of satellite cells with age vary depending on the species and the specific muscle group examined, but, in general, there seems to be a gradual age-related decline in satellite cell numbers (Shefer et al., 2006). Although this quantitative decrease in the resident stem cell population may have an influence on the loss of regenerative potential of aged skeletal muscle, it seems that the major contribution comes from the decline in satellite cell function (Carosio et al., 2009).

Satellite cell performance is a result of an interplay between external cues from the surrounding environment and the inherent capacity of the cells to respond to those cues and undergo myogenesis. Therefore, the age-related impairment of satellite cell function may be due to the intrinsic aging of the resident stem cells, which renders them less responsive to environmental cues, or due to age-related decline in those environmental signals that instruct the response of the satellite cells. Although there are studies supporting each of these hypotheses independently, it is likely that stem cell aging is a result of the effects produced by the combination of

intrinsic and extrinsic factors (Carlson et al., 2008; Conboy et al., 2003; Day et al., 2010).

One common strategy to compare satellite cell functionality in old and young animals has been the induction of muscle regeneration through injury by chemical or physical means. While there is a decline of tissue regenerative potential with increased age, exposing injured old muscle to a young systemic environment greatly improves its capacity to regenerate (Conboy et al., 2005). These studies indicate a strong contribution of extrinsic factors to the observed decline in satellite cell performance in response to injuries.

Additional studies have focused on the intrinsic properties of the satellite cells, through the isolation of single myofibers from the muscle tissue (and its associated stem cell population) followed by ex vivo analysis, or isolation and in vitro culture of satellite cells (Collins et al., 2007; Day et al., 2010; Shefer et al., 2006). These studies indicate that aged satellite cells lose their intrinsic capacity to produce an optimal reserve progeny, which can be an underlying factor in the depletion of the resident stem cell population with age.

Signaling pathways and transcription factors regulating satellite cell function

Many environmental cues can activate satellite cells, including those mediated by cell adhesion, or by growth

factors and cytokines released by neighboring cells. Signaling cascades transmit extracellular cues to the cell nucleus and, in muscle cells, the p38 mitogen-activated protein kinase (MAPK) and insulin-like growth factor 1 (IGF1)-protein kinase B (PKB/AKT) pathways are thought to be particularly important (Keren et al.; Lluis et al.; Ornatsky et al.; Perdiguero et al.; Serra et al.; Suelves et al.; Wu et al.; Zetser et al.). Such signals can cause the down-regulation of genes associated with the proliferation of myoblasts in response to external activating signals and they can also activate muscle-specific networks of transcription factors. Those are principally comprised of four muscle-specific regulatory factors (MRFs) that belong to the basic helix-loop-helix (bHLH) family of transcription factors: Myf5, MyoD, Myogenin and MRF4.

While quiescent satellite cells expressing Pax7 are negative for MRFs, MyoD and Myf5 transcription factor expression is induced in activated/proliferating satellite cells (myoblasts), whereas Myogenin and MRF4 are subsequently expressed at the early and late stages of differentiation, respectively [Sartorelli and Caretti, 2005; Tapscott, 2005]. Proliferation and differentiation are mutually exclusive processes in myogenesis. Indeed, cessation of proliferation by downregulation of cyclin D1 and dephosphorylation of pRb are required for initiation of muscle differentiation-specific gene expression. The p38 MAPK pathway, by antagonizing the proliferation-promoting cJun N-terminal kinase (JNK)

pathway, leads to downregulation of cyclin D1 expression, thus promoting cell-cycle exit and allowing muscle differentiation to commence (Perdiguero et al., 2007). Initiation of the differentiation program requires the association of MyoD with E proteins (belonging to the bHLH family of transcription factors) and the binding of the MyoD/E protein heterodimers to the E boxes of the muscle gene promoters. A major mechanism for maintaining a proliferative state in the myoblasts in the presence of Myf5 and MyoD is through Id (inhibitor of differentiation), an HLH protein lacking the basic DNA-binding domain, which is expressed at high levels in proliferating myoblasts. Association of Id to MyoD or E proteins results in the formation of non-functional E protein/Id and MyoD/Id heterodimers that cannot bind to E boxes, thus preventing anticipated myogenic differentiation. The downregulation of Id expression when myoblasts exit the cell cycle at the onset of differentiation thus allows the formation of functional heterodimers and muscle differentiation-specific gene expression to proceed (Puri et al., 2000; Sartorelli and Caretti, 2005; Tapscott, 2005). Other proteins (like the HLH factors Twist, MyoR and Myst-1, and ZEB and I-mfa proteins) are also repressors of the MRFs through direct association or sequestration of the E proteins (Puri et al., 2000).

In addition to its role in the myoblast cell cycle exit, several studies have unambiguously shown that the p38 MAPK pathway is essential for the onset of differentiation (Lluis et

al., 2006) by regulating the activity of transcription factors and epigenetic regulators on muscle loci: i) induction of MyoD/E47 heterodimer formation by phosphorylation of E47; ii) recruitment of the SWI/SNF chromatin remodeling complex – probably via phosphorylation of the BAF6Oc subunit; iii) induction of MEF2 transcriptional activity by direct phosphorylation; iv) recruitment of the Trithorax group TrxG/Ash2L complex through p38-phosphorylated MEF2d (Guasconi and Puri, 2009; Lluis et al., 2005; Rampalli et al., 2007). Finally, a p38-dependent phosphorylation of the mRNA decay-promoting factor KSRP has also been shown to control the stability of specific myogenic transcripts.

The basal muscle-specific transcriptional machinery also seems to be controlled epigenetically during myogenesis. The epigenetic regulation appears to respond to environmental signals and it predominantly influences the classical transcriptional machinery through p38 MAPK and AKT signaling [Guasconi and Puri, 2009]. Hence, at myogenic loci, these signaling pathways would appear to forge the connection between transcription factors and chromatin-associated activities.

Epigenetic regulation of satellite cell function

Recent findings have uncovered an epigenetic control layer over the basal muscle-specific transcriptional machinery during myogenesis. Indeed, the activation of satellite cells is caused by a combination of genetic and epigenetic events, ranging from covalent modification of histones and transcription factors, to chromatin remodeling, which in conjunction drives the complete program of muscle-specific gene expression (Buckingham, 2006; Kuang et al., 2008; Sambasivan and Tajbakhsh, 2007; Sartorelli and Caretti, 2005).

Chromatin is generally repressed, and changes in its structure are necessary not only to access target sequences but also to endow local chromatin with specific states of transcriptional competence. There are two main enzymatic activities that modify chromatin and regulate access to DNA: chromatin-modifying complexes and chromatin-remodeling complexes (Kadam and Emerson, 2003; Narlikar et al., 2002). Chromatin-modifying complexes contain the subunits of different histone-modifying enzymes that catalyze reversible posttranslational modification of histones and other factors. Histone modifications are associated with both active gene expression, such as acetylation of histones H3 and H4 (acetyl H3, acetyl H4) and trimethylation of lysine 4 of histone H3 (H3K4me3), and gene repression, including trimethylation

of lysines 9 and 27 of histone H3 (H3K9me3; H3K27me3) and trimethylation of lysine 20 of histone H4 (H4K20me3). The combination or sequential addition of various posttranslational modifications to the terminal amino acid residues of histones have different functional consequences for gene activity and chromatin organization, and they are thought to form a histone code (Strahl and Allis, 2000). Additionally, chromatin-remodeling factors use the free energy released by ATP hydrolysis to loosen DNA-histone contacts and thus facilitate the movement of the nucleosomes along a particular DNA sequence (Simone, 2006). At the DNA level, CpG dinucleotide methylation is a major source of epigenetic information and the methylation of CpG-containing promoters represses the expression of specific genes. This mechanism is closely linked to the histone-modifying machinery and, for instance, it is accepted that the Polycomb group (PcG) of proteins, which catalyze trimethylation of H3K27, target DNA methylation at specific sites (Lande-Diner et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007).

In quiescent and proliferating satellite cells/myoblasts, the histones associated with the promoters of genes specifically involved in muscle differentiation are hypoacetylated, containing H3K9me2 and H3K27me3 residues. This repression is catalyzed by the HDACs, PcG, Suv39H1 and histone lysine methyltransferases (HKMT) (Palacios and Puri, 2006; Sartorelli and Caretti, 2005; Sousa-Victor et al., 2011).

Transcriptional repression by PcG proteins is thought to be the default state and it is counteracted by TrxG proteins that specifically activate target genes (Pietersen and van Lohuizen, 2008; Schuettengruber et al., 2007).

Polycomb repressive complexes and Bmi1

An important group of proteins that regulate gene activity at the chromatin level is constituted by the Polycomb group (PcG) of transcriptional repressors, initially discovered in Drosophila melanogaster as regulators of Hox genes. PcG proteins form at least two distinct multiprotein complexes, the Polycomb-repressive complexes 1 and 2 (PRC1 and PRC2). Although their molecular function is different, both complexes mediate gene silencing by regulating chormatin structure, mostly through post-trasnslational modificatios of histones (Margueron and Reinberg, 2011; Simon and Kingston, 2009). The PRC2 complex is responsible for the methylation (di- and tri-) of Lys 27 of histone H3 (H3K27me2/3) through its enzymatic subunits EZH1 and EZH2, whereas the PRC1 complex monoubiquitylates Lys 119 of histone H2A (H2AK119ub) via the ubiquitin ligases RING1A and RING1B (Schuettengruber and Cavalli, 2009; Simon and Kingston, 2009).

The HKMT Ezh2 is recruited to inactive muscle gene promoters by the transcriptional regulator Ying Yang 1 (YY1), where it represses transcription through H3K27 trimethylation

(Caretti et al., 2004). Recent studies show that PRC2 can also mediate Pax7 repression in response to external cues that induce myoblast differentiation. p38 promotes the interaction between PRC2 and YY1, by direct phosphorylation of Ezh2, leading to the formation of repressive chromatin on the Pax7 promoter (Palacios et al., 2010). While it remains unclear whether this PRC2-mediated repression is general to all muscle-specific genes or if distinct genes are regulated by different methylation complexes, it is clear that certain promoters are methylated by Suv39H1 at H3K9. This HKMT associates with heterochromatin protein 1 (HP1) and HDAC4/5 proteins (Ait-Si-Ali et al., 2004; Mal and Harter). It should also be noted that methylated residues recruit MBD2, a methyl-CpG-binding domain (MBD) protein that acts as a repressor at the *Myogenin* promoter (Luo et al., 2009).

The repressive landscape changes rapidly when signals that promote differentiation are received. Initially, the repressive PRC2 H3K27me3 marks are substituted by TrxG H3K4me3, which is thought to mark actively transcribed genes, while those poised for transcription are marked by H3K4me2 (Guenther et al., 2007). In activated myoblasts derived from satellite cells, Pax7 binds to the regulatory elements of target genes with H3K4me2 (such as Myf5), and it recruits the TRxG histone methyltransferase complex that contains the Ash2L and Wdr5 subunits. These events induce strong H3K4 trimethylation around the transcription start site that

establishes a transcriptionally active domain (McKinnell et al., 2008; Rampalli et al., 2007). The TrxG complex may also be recruited to muscle-specific promoters in differentiating myoblasts, through its association with p38-phosphorylated MEF2D (Rampalli et al., 2007). Interestingly, the histone demethylase UTX targets muscle-specific genes and provokes local demethylation of H3K27me3 within their promoter/enhancer (Seenundun et al., 2010).

The PRC1 component Pc (known as CBX in mammals) binds specifically to the product of PRC2 catalysis, H3K27me3, leading to the hypothesis that PRC1 functions downstream of PRC2. Although this premise is still cited in the literature, its operational status is equivocal as there are genes targeted by PRC2 that lack H2AK119ub and genes targeted by PRC1 in the absence of PRC2 (Ku et al., 2008; Schoeftner et al., 2006).

B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi1), which is one of the core members of the PRC1 complex, was isolated as an oncogene that cooperates with c-myc in the generation of mouse lymphomas (Haupt et al., 1991; van Lohuizen et al., 1991). Bmi1 is composed of a central helix-turn-helix-turn-helix-turn motif (H-T-H-T) and an N-terminal RING finger domain. The first is required for the induction of telomerase activity and human epithelial cell immortalization while the latter interacts with RING1B (Alkema et al., 1997; Alkema et al., 1993; Itahana et al., 2003; Li et al., 2006). The E3 ubiquitin ligase activity of

RING1B is dependent upon binding to Bmi1 and in Bmi1-/- mouse embryonic fibroblasts (MEFs), global levels of H2AK119Ub1 are decreased (Cao et al., 2005).

Mice deficient in Bmi1 display a number of defects including a progressive decrease in the number of hematopoetic cells, severe neurological abnormalities and markedly shortened lifespan. Bmi1-deficient mice that survive birth are significantly smaller than their control littermates and with increasing age display waves of sickness that finally result in death before 20 weeks (van der Lugt et al., 1994). Some of the neurological and hematopoetic abnormalities hint at a defect in proliferation. In particular, Bmi1-/- MEFs undergo premature senescence after only a few passages in culture, which is accompanied by the accelerated accumulation of p16INK4a, p19ARF and p15INK4b. Conversely, ectopic expression of Bmi1 extends the proliferative lifespan of both mouse and human fibroblasts (Jacobs et al., 1999).

Several studies indicate that Bmi1 is required for the self-renewal and post-natal maintenance of hematopoetic stem cells (Lessard and Sauvageau, 2003; Park et al.) and neural stem cells (Molofsky et al., 2003). In each of these tissues Bmi1-deficient stem cells exhibit a post-natal self-renewal defect that leads to their depletion by early adulthood. This also seems to be the case for stem cells in other tissues, including the gastrointestinal tract and breast tissue (Liu et al., 2006; Tateishi et al., 2006).

In most cases, the defect in self-renewal in the absence of Bmi1 was associated with increased p16INK4a expression and Ink4a deficiency partially rescues hematopoietic and neural stem cell self-renewal of Bmi1-/- mice (Molofsky et al., 2005; Smith et al., 2003). Bmi1 was found strongly enriched at the INK4a promoter, together with PRC2 members and the associated H3K27me3 mark (Bracken et al., 2007).

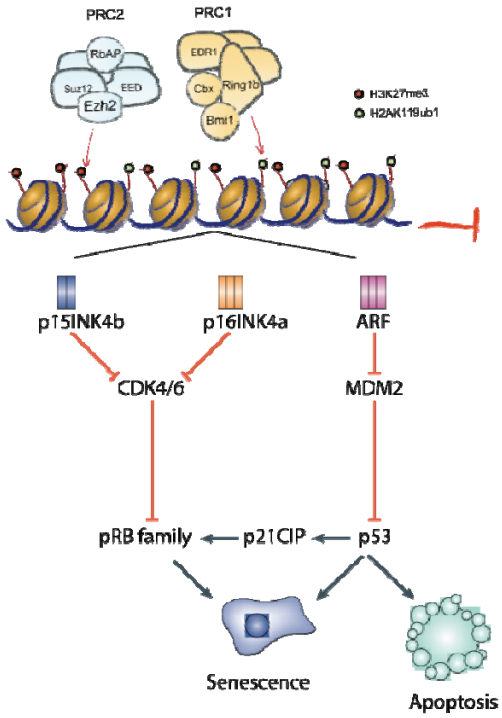


Figure 2: Epigenetic regulation of the INK4/ARF locus and molecular regulation of cellular senescence.

p16INK4a expression in aging

INK4a refers to a chromosomal locus encoding for two alternative reading frame proteins described as tumor suppressors: p16INK4a and ARF (p19ARF in mouse and p14ARF in human) (Figure 2). Each of these proteins has been shown to have a critical role in the senescence, cell cycle control and tumor suppression, and this locus is often functionally inactivated in human tumors. p16INK4a is a cyclin D-dependent kinase (cdk) inhibitor acting upstream of retinoblastoma protein (pRb), preventing its phosphorylation and functional inactivation by cdks. ARF acts upstream of p53 and activates its function by sequestering MDM2, a p53-antagonist (Lowe and Sherr, 2003).

p16INK4a gene expression increases with age in a variety of tissues (Krishnamurthy et al., 2004) and induction of p16INK4a expression can cause the senescence of a variety of cell types in culture and in vivo (Lowe and Sherr, 2003). Although a universal marker that is exclusively expressed in senescent cells has not been identified, most senescent cells seem to express p16INK4a, making it a generally accepted biomarker of senescence. Removal of senescent cells using a strategy of inducible elimination of p16INK4a-expressing cells upon drug administration delayed the onset of some of

the most common age-related phenotypes in adipose tissue, skeletal muscle and eye (Baker et al., 2011).

Physiological adult skeletal muscle growth: hypertrophy versus atrophy

During embryonic and neonatal development, muscle growth is mainly a consequence of satellite cell proliferation and integration into the preexisting myofibers. However, in the adult tissue, myogenesis is only associated with regenerative processes, while adult skeletal muscle mass is mainly maintained through a balance of catabolic and anabolic processes. An imbalance towards anabolic processes results in hypertrophy, an increase in the adult muscle mass. Conversely, if catabolic processes predominate, muscle mass decreases, a process referred to as muscle wasting or atrophy (Glass; Otto and Patel, 2010).

Muscle wasting is associated with many pathological conditions, such as muscular dystrophies, cancer (cachexia), diabetes, renal failure and heart failure. Aging itself is associated with a slow, progressive loss of muscle mass. Sarcopenia (extreme loss of muscle mass accompanied by loss of motor neurons and myofiber denervation) is specifically associated to very old (geriatric) individuals (typically after 75 years of age in humans, and 28-30 months in mice) (Childs, 2003; Doherty, 2003).

Several signaling pathways are involved in the regulation of muscle growth and atrophy and their tight regulation is required for the maintenance of muscle homeostasis.

Signaling pathways regulating muscle hypertrophy and atrophy

One of the main signaling pathways involved in the regulation of muscle mass is the IGF1/PI3K/AKT pathway. Signaling through the IGF1 receptor induces the stimulation of different signaling cascades that are both highly interconnected and tightly regulated. IGF signals through the IGF1 receptor via direct interaction with IGF-1 binding proteins (IGFBPs). Upon binding to its ligand, the IGF1 receptor is autophosphorylated, generating docking sites for insulin receptor substrate (IRS). This results in the recruitment and activation of phosphatidylinositol-3-kinase (PI3K) which phosphorylates membrane phopholipids and provides a membrane-binding site for AKT and PDK1. AKT is activated by phosphorylation after translocation to the membrane, a function mediated by the kinase PDK1 or mTORC2 (Glass, 2005; Otto and Patel, 2010; Sandri, 2008; Schiaffino and Mammucari, 2011).

There are three major branches of the AKT pathway involved in the regulation of muscle mass. AKT promotes protein synthesis through the positive regulation of the mTOR pathway and the negative regulation of glycogen synthase kinase 3β (GSK3β). In addition, AKT prevents protein degradation through the negative regulation of the FoxO pathway (Figure 3).

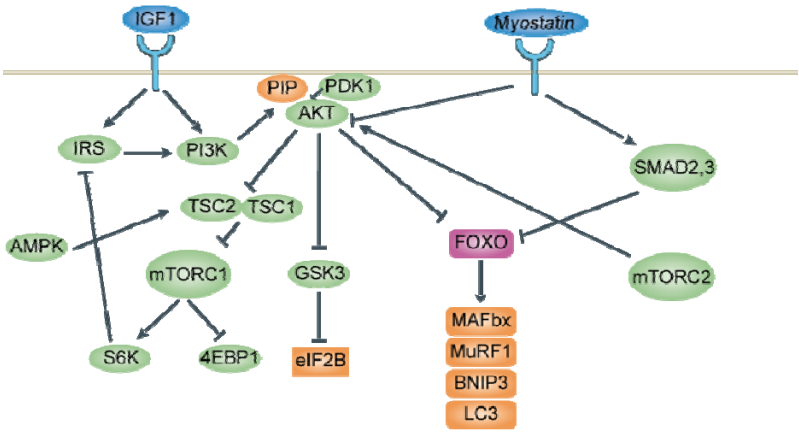


Figure 3: Signalling network regulating muscle growth and atrophy.

The mammalian TOR protein (mTOR) forms two distinct signaling complexes: mTORC1 and mTORC2. In addition to mTOR, the mTORC1 complex contains raptor, PRAS40 and mLST8, while the mTORC2 is composed by rictor, Sin1, PROTOR and mLST8 (Jacinto et al., 2004; Sarbassov et al., 2004; Wullschleger et al., 2006). The effect of AKT on mTORC1 is indirect and it requires the phosphorylation of tuberous sclerosis complex 2 (TSC2) on multiple sites. The TSC1 and TSC2 proteins form a heterodimeric complex that acts as a functional unit in the suppression of mTORC1 activity. Through its direct phosphorylation, AKT releases the inhibitory effect of TSC1-TSC2, thereby activating mTORC1 (Inoki et al., 2002). The rapamycin-sensitive mTORC1 is responsible for the phosphorylation of 4EBP1, a repressor of the cap-binding protein eIF4E, and for the phosphorylation

and activation of the S6 kinase (S6K), which subsequently will phosphorylate the ribossomal protein S6 (Hay and Sonenberg, 2004). S6K is an important effector of the AKT pathway. S6K deletion in mice reduces their skeletal muscle mass and S6K-deficient myotubes have a blunted hypertrophic response to IGF1 and activated AKT (Ohanna et al., 2005). S6K also inhibits IRS through its direct phosphorylation, which blocks its binding to insulin receptors and reduces IRS signal to PI3K (Harrington et al., 2004). This constitutes a negative feedback loop that limits AKT signaling. mTORC2, which is rapamycin insensitive, controls the activity of serum glucocorticoid-induced kinase (SGK) and contributes to the full activation of AKT, through its direct phosphorylation (Sarbassov et al., 2005).

AKT also promotes protein synthesis through the direct phosphorylation and consequent inhibition of GSK3β (Cross et al., 1995). Over-expression of dominant-negative forms of GSK3β, or inhibitors against its function, both lead to hypertrophy in skeletal myotubes. This effect seems to be independent of mTOR, suggesting that the two pathways act separately and downstream of AKT to control muscle growth (Rommel et al., 2001).

AKT inhibits protein degradation by phosphorylating and repressing the function of the transcription factors of the FoxO family (FoxO1, FoxO3 and FoxO4). AKT phosphorylates FoxO transcription factors at multiple sites, leading to the exclusion of phosphorylated FoxO proteins

from the nucleus and inhibition of their transcriptional functions. FoxO factors are required for the transcriptional regulation of two muscle-specific ubiquitin ligases, muscle ring finger1 (MuRF1) and muscle atrophy F-box (MAFbx) (Sandri et al., 2004; Stitt et al., 2004). These are considered to be master genes for muscle wasting, found upregulated in different models of muscle atrophy and responsible for the increased protein degradation through the ubiquitin-proteasome system (Bodine et al., 2001; Gomes et al., 2001). FoxO factors also act on another major pathway of protein degradation: the autophagy-lysosome system. FoxO3 controls the transcription of autophagy-related genes, including microtubule-associated protein 1 light chain 3 (LC3) and BCL2/adenovirus E1B interacting protein 3 (BNIP3), and the transfection of a constitutively active form of FoxO3 is sufficient to induce autophagy, while the transfection of a dominant-negative FoxO3 blocks fasting-induced autophagy (Mammucari et al.).

The effect of AKT on muscle hypertrophy is striking and can be rapidly achieved through the in vivo delivery of a constitutively active form of AKT1 (Pallafacchina et al., 2002) or with inducible transgenic models that express the same constitutively active AKT1 protein (Lai et al., 2004). Muscle hypertrophy induced by AKT is accompanied by increased strength, but does not involve changes in fiber-type distribution, i.e. oxidative slow or glycolitic/fast fibers. It also

does not induce satellite cell activation or new myonuclei incorporation (Blaauw et al., 2009).

The activity of the AKT pathway can be modulated by a variety of factors acting at different steps. Myostatin, also called growth differentiation factor 8 (GDF-8), is a member of the transforming growth family-β (TGF-β) family and a negative regulator of muscle mass. Myostatin acts through the activin receptor IIB (ActRIIB) on Smad2 and Smad3 and is capable of inhibiting the AKT-PI3K protein synthesis pathway and activating FoxO1, thus allowing increased expression of atrogin-1 (Lee and McPherron, 2001; McFarlane et al., 2006). The effect of myostatin in the regulation of muscle mass was demonstrated in studies with genetically-modified mice null for the myostatin gene. These mice present a stricking phenotype of muscle hypertrophy, with some myostatin-deficient muscles doubling in size (McPherron et al., 1997).

mTORC1 also integrates a variety of signals from nutrients and cellular energy status, with several factors directly impacting on its activity. The AMP-activated protein kinase (AMPK) acts as an energy sensor and is a key regulator of anabolic and catabolic processes. AMPK is activated allosterically by AMP as well as by phosphorylation on its activation loop Thr172 residue. A high amount of AMP in the cell reflects a low energy status, and the AMP/ATP ratio in the cell is much higher during energy stress. In response to low energy, AMPK phosphorylates several targets to enhance

catabolism and suppress anabolism (Carling et al., 2011). Through the phosphorylation of TSC2 (Inoki et al., 2003) and raptor (Gwinn et al., 2008), AMPK inhibits the mTOR pathway. AMPK can also act directly on the FoxO-dependent protein degradation pathway by phosphorylating FoxO factors at several regulatory sites distinct from AKT phosphorylation residues (Greer et al., 2007). Treatment of myotube cultures with AICAR, an activator of AMPK, induces protein breakdown and stimulates the ubiquitin ligases MAFbx and MuRF1 expression via FoxO (Tong et al., 2009).

Some of the most serious physiological consequences of ageing relate to its effects on skeletal muscle. Sarcopenia is the term widely used to describe the slow, progressive loss of muscle mass, strength and function with advancing age (Thompson, 2009). Several cellular mechanisms have been involved in the pathogenesis of sarcopenia, including mitochondrial dysfunction (Bua et al., 2002), muscle denervation caused by motoneuron loss, along with altered apoptotic and autophagic signaling (Wohlgemuth et al., 2009). Aging is characterized by a decrease in the capacity to respond to insulin and maintain glucose homeostasis (Gupte et al., 2008) and increased muscle ROS and oxidative damage (Mecocci et al., 1999). The response to exercise and the capacity for muscle hypertrophy in older animals and humans also appears to be limited (Blough and Linderman, 2000; Welle et al., 1996). This impaired muscle adaptive capacity could be due, at least in part, to an age-related

decline in the activation of AKT. Indeed contraction-induced activation of AKT is decreased with age and this finding is associated with reduced protein synthesis (Funai et al., 2006; Haddad and Adams, 2006). It is also possible that the regulation of the AKT substrate, mTOR, may be affected with aging (Paturi et al., 2010).

Sestrins

Oxidative stress and the accumulation of oxidized and modified proteins and protein aggregates are hallmarks of aged tissues often associated with the onset of age-related diseases.

Sestrins are a family of highly conserved proteins ubiquitously expressed in all adult tissues, although at different levels. Mammals express three Sestrins (Sesn1, Sesn2 and Sesn3), whereas *Drosophila melanogaster* and *Caenorhabditis elegans* have single orthologs. Sesn1 is the member of the family most similar to invertebrate Sesn and it is the most representative mammalian ortholog in skeletal muscle (Budanov et al., 2010).

Sestrins were initially identified as stress-responsive proteins (Budanov et al., 2002; Peeters et al., 2003) that could function as antioxidants, controlling the activity of peroxiredoxins, which scavenge reactive oxygen species

(ROS) (Budanov et al., 2004). The only structural similarity identified between Sestrins and other proteins is limited to a conserved N-terminal region that contains a motif found in prokaryotic proteins, including the *Mycobacterium tuberculosis* AhpD protein (Budanov et al., 2004). AhpD is a component of alkyl-hydroperoxidereductase which provides protection against reactive oxygen species (ROS) (Bryk et al., 2002). Given their similarity to AhpD, the Sestrins were examined for effects on ROS accumulation. Inhibition of Sesn1 or Sesn2 in cultured RKO cells results in the accumulation of ROS, conversely overexpression of Sesn1 or Sesn2 prevents ROS accumulation (Budanov et al., 2004).

Recently, Sestrins were also associated with biochemical and physiological functions that do not appear to depend on their redox activity. Studies have shown that Sesn1 and Sesn2 activate the AMP-responsive protein kinase (AMPK) and target it to phosphorylate TSC2 and stimulate its GAP activity, thereby inhibiting mTOR in cultured HEK293 cells (Budanov and Karin, 2008). These studies were further expanded to Drosophila, taking advantage of the single dSesn gene. dSesn was characterized as a negative feedback regulator of TOR, induced in response to the accumulation of ROS that results from persistent TOR activation. In this negative feedback mechanism, dSesn expression is controlled by FoxO and its inhibitory action on TOR is dependent on AMPK activation and the phosphorylation of the TSC1-TSC2 complex (Lee et al., 2010b). Ablation of the single dSesn

gene results in several age-related pathologies, including the accumulation of ROS, mitochondrial dysfunctions, ubiquitinated protein aggregates within muscle fibers, as well accelerated degeneration of skeletal and cardiac muscles.

Aims

Aims

Muscle aging is a physiological process characterized by a significant decline in overall tissue mass and functionality, accompanied by a reduction in satellite cell number and function and consequent loss of regenerative capacity, which is particularly prominent in geriatric individuals undergoing sarcopenia. To understand the processes that contribute to the phenotype associated with skeletal muscle aging, it is necessary to identify molecular regulators of stem cell functional decline in the elderly and novel regulators of adult muscle growth and atrophy. The general aims of this work were:

I - To analyze the function of the Bmi1/p16INK4a axis in muscle stem cell biology during (physiological) aging, particularly during sarcopenia.

II - To analyze the function of Sestrins as new regulators of adult muscle growth and atrophy.

Materials & Methods

Mice

Bmi1$^{-/-}$ FVB were generated from inter-crossing heterozygotes (Bmi1$^{+/-}$ FVB), obtained from Dr. M. van Lohuizen's Lab. Wild-type (WT) littermates were used as controls. SAMP8 and SAMR1 mice were purchased from Harlan Laboratories. Old (28 months) mice were a kind gift from Dr. M. Blasco (CNIO). Rag2$^{-/-}$:γC$^{-/-}$ mice were obtained from Vall d'Hebron All other experiments were performed using C57BL6 WT mice. All animal experiments were approved by the Catalan Government Animal Care.

Induction of muscle regeneration

Young/adult Bmi1-deficient and age matched WT mice were anesthetized with ketamine/xylazine (80/10 mg/kg, intraperitoneally). Regeneration of skeletal muscle was induced by intramuscular injection of 300 µl of 10−5M cardiotoxin (CTX, Latoxan) in the Tibialis anterioris (TA) muscle group of the mice as described (Suelves et al., 2007). The experiments were performed in the right hind-limb muscles of mice. Contralateral uninjured muscles were used as reference. Seven days post-injury animals were sacrificed and TA muscles were dissected, frozen in isopentane, cooled with liquid nitrogen, and stored at −80°C before analysis. TA muscles were subjected to histological analysis in cryosections. Four animals of each genotype were analyzed.
Alternatively, Extensor digitorum longus (EDL) muscle of young/adult Bmi-1$^{-/-}$ or WT mice was grafted onto the TA

muscle of Rag2$^{-/-}$:γC$^{-/-}$ immune-compromised recipient mice. Briefly, the EDL muscles with both tendons attached were removed from the anatomical bed and transplanted onto the surface of the TA muscle of the recipient mouse. The tendons were sutured onto the underlying tissues. The skin was closed and the grafts were allowed to regenerate. The transplanted EDL myofibers degenerate but commence to regenerate and undergo de novo myogenesis at the expense of its own satellite cells (Grounds et al., 2005).

Denervation

Two months old C57BL6 WT mice were anesthetized with ketamine/xylazine (80/10 mg/kg, intraperitoneally). A small incision was made in the right hind limb of the animal and muscles were denervated by removing a 5-mm section of the sciatic nerve. The ends of the nerve were folded back and sutured to prevent nerve re-growth. At the designated times, animals were sacrificed and TA muscles were dissected, frozen in isopentane cooled with liquid nitrogen, and stored at −80°C until before being used in RT-qPCR or histological analysis in cryosections. Cross-sectional area (CSA) and RNA levels were compared to those of the TA muscle of age-matched WT mice not subject to surgery. Four animals of each group were analyzed for each time point studied.

Immobilization

TA muscles of two months old C57BL6 WT mice were infected as described above and three days after mice were submitted the immobilization protocol. Mice were anesthetized with ketamine/xylazine (80/10 mg/kg, intraperitoneally). The right injected hind limb was immobilized with a plastic stick placed over and under the limb and fixed with a medical adhesive bandage. Animals were monitored on a daily basis for chewed plaster, abrasions and problems with ambulation. The animals were free to move and ate and drank ad libitum. The immobilization procedure prevented movement of the immobilized leg alone. After seven days animals were sacrificed and TA muscles were dissected, frozen in isopentane cooled with liquid nitrogen, and stored at −80°C before analysis. TA muscles were subjected to histological analysis in cryosections. At least three animals of each group were analyzed.

Induction of compensatory hypertrophy

Two months old C57BL6 WT mice were anesthetized with ketamine/xylazine (80/10 mg/kg, intraperitoneally), and compensatory hypertrophy (CH) of plantaris muscles was induced by surgical section of the distal tendon of the medial and lateral gastrocnemius muscle, which was folded back and sutured close to its proximal origin. This procedure induces an adaptive growth response to functional overloading in the soleus and plantaris muscles. Two weeks

after surgery, animals were sacrificed and plantaris muscles were dissected, frozen in isopentane cooled with liquid nitrogen, and stored at −80°C until analysis by RT-qPCR. RNA levels were compared to those of the plantaris muscle of age-matched WT mice not subject to surgery. Five animals of each group were analyzed.

Exercise training protocol

Two months old C57BL6 WT mice were trained on a motorized treadmill (Exer 6M Treadmill; Columbus Instruments, Inc., Columbus, Ohio) five times per week (Monday-Friday) for three weeks. The exercise training protocol consisted of 20-min warm-up running at a stepwise increasing speed ranging from 0.08 to 0.2 m/s followed by a 20-min training period at a speed of 0.2 m/s, a 5-min intense training at a speed of 0.25 m/s and a 5-min cool-down at a stepwise decreasing speed ranging from 0.25 to 0.08 m/s. Following the procedure animals were sacrificed and TA muscles were dissected, frozen in isopentane cooled with liquid nitrogen, and stored at −80°C. TA muscle of trained and age-matched WT untrained mice were analyzed by RT-qPCR. Five animals of each group were analyzed.

Muscle infection with Adeno-associated virus (AAV)

Two months old C57BL6 WT mice were used for injection. All AAVs were diluted to $2{,}5 \times 10^{12}$ genome copies (GC)/ml in PBS. A small skin incision was made in the targeted muscle

area after the mouse had been anesthetized, and AAV particles were injected into the TA muscle (40 µl, 1×10^{10} GC/site) using a Hamilton syringe. At the designated times, animals were sacrificed and TA muscles were dissected, frozen in isopentane cooled with liquid nitrogen, and stored at −80°C before analysis. TA muscles were analysed by RT-PCR, RT-qPCR, Western Blot and histology in cryosections.

Human samples

Patients muscle biopsies were a kind gift from Dr. Montse Olivé (Institut de Neuropatologia, IDIBELL, Barcelona, Spain) and contain samples from patients suffering from myotilinopathy, desminopathy, inclusion body myositis and healthy controls.

Satellite cell isolation by FACS

Muscles of neonatal (P8), young/adult (2 months) and old (28 months) WT mice or neonatal and young/adult Bmi1-deficient mice were collected by surgery, mechanically disaggregated and dissociated in Ham's F10 media containing collagenase D 0.1% (Roche) and Trypsin-EDTA 0.1% at 37°C for 20 min twice and then filtered. Cells were incubated in lysing buffer (BD Pharm Lyse™) for 10 min on ice, ressupended in PBS 2,5% Goat Serum and counted. Biotin-conjugated anti-CD45, anti-CD31, anti-CD11b and anti-Sca-1 antibodies and streptavidin microbeads were used to exclude the Lin (-) negative population from the cell suspension using magnetic

columns (Miltenyi Biotech). The remaining population was labeled with APC-conjugated anti-CD34 and PE-conjugated anti-α7-integrin. All antibodies used for FACS sorting are listed in table1. Cells were sorted using a FACS Aria II (BD). Isolated satellite cells were used either for RNA extraction for microarray analysis or cultured in Ham's F10 supplemented with 20% FBS for proliferation assays.

Antibody	Manufacter	Species
PE-anti-α7integrin	Abcam	rat
APC-anti-CD34	BD Pharmingen	rat
Biotin- anti-CD45	BD Pharmingen	rat
Biotin- anti-CD31	eBioscience	rat
Biotin-anti-CD11b	BD Pharmingen	rat
Biotin-anti-Sca-1	BD Pharmingen	rat

Table 1: Antibodies used for FACS sorting of satellite cells.

Proliferation assays

Young/adult and old WT and young/adult Bmi1-defficient FACS isolated satellite cells were cultured in Ham's F-10 medium containing 20% FBS. 100 cells were plated in 6-well plates and after 72 hours in culture, proliferating cells were labeled with BrdU (1.5 µg/ml; Sigma) for 1 hour. BrdU-labeled cells were detected by immunostaining using rat anti-BrdU antibody (Oxford Biotechnology; 1:500) and a specific secondary biotinylated goat anti-rat antibody (Jackson Inmunoresearch; 1:250). Antibody binding was visualized using Vectastain Elite ABC reagent (Vector Laboratories) and

DAB. BrdU positive cells were quantified as percentage of the total number of cells analyzed. In parallel, 25 cells were plated in 24-well plates and total number of cells was counted daily, for four days (n=3 animals analyzed per phenotype in 5 assays per animal).

Microarray: RNA isolation and, cDNA amplification and whole transcriptome analysis of FACS sorted satellite cells

FACS sorted satellite cells were collected in lysis buffer and RNA extraction and cDNA amplification were performed at the Genomic Facility of the IRB (Barcelona, Spain), following a protocol described in (Gonzalez-Roca et al., 2011). cDNA was used on a transcriptome analysis by Agilent SurePrint G3 Mouse GE 8x60K microarray, performed at the microarray Unit of CRG (Barcelona, Spain). Microarray analysis was performed with at least 3 animals/phenotype.

Histology and Immunohistochemistry in muscle cryosections

Tibialis anterior (TA), Plantaris and Extensor digitorum longus (EDL) muscles were frozen in isopentane cooled with liquid nitrogen, and stored at −80°C until analysis. 10 µm sections were collected from the midbelly of muscles and were either stained with hematoxylin/eosin (HE) or immunostained using anti-eMHC antibody (F1.652, Developmental Studies Hybridoma Bank). Labeling was performed using the

peroxidase M.O.M Kit Staining (Vector Laboratories) according to the manufacturer's instructions. Briefly, cryosections were air dried, kept unfixed, washed on PBS, incubated in 3% Hidrogen peroxidase twice for 30 min and blocked for 1 h at room temperature (RT) with MOM blocking solution in PBS. Sections were incubated with primary antibody for 1 hour at room temperature, washed on PBS and incubated with biotinylated secondary antibody for 30 min at RT and ABC solution for 10 min at RT. Peroxidase signal was developed with diaminobenzidine (DAB) (Sigma) and tissue sections were dehydrated in 70%/96%/100% Ethanol and Xilol and mounted with DPX.

Single fiber Isolation and Immunohistochemistry

Single myofibers were isolated from EDL muscles carefully dissected from young/adult WT and Bmi1-deficient mice. Muscles were digested in 0.2 % Collagenase Type I (Sigma) in DMEM for 90 minutes, then individual myofibers were dissociated mechanically as previously described (Rosenblatt et al.). Single myofibers were cultured in growth media (GM) for the designated times, fixed in 4% paraformaldehyde for 10 minutes and then were used for immunostaining. Fibers were permeabilized with 0,5%Triton/PBS for 10 min, unspecific binding was blocked with 10% Goat Serum/10% Fetal Calf Serum/PBS blocking solution for 30 min at RT, and fibers were incubated with primary antibodies diluted in 6% Horse Serum /PBS over night at 4ºC, rocking. Primary antibodies

are listed in table 2. Primary antibody was washed and fibers were incubated with AlexaFluor568 and 488-conjugated secondary antibodies for 1,5 hours at RT, counterstained with DAPI and mounted with Vecta Shield (Vector Laboratories).

Antibody	Manufacter	Species	Dilution
Anti-Bmi1	Abcam	mouse	1/50
Anti-CD34	BD Pharmingen	rat	1/50
Anti-Pax7	Developmental Studies Hybridoma Bank	mouse	1/50
Anti-MyoD	Dako (5.8A)	mouse	1/50
Anti-Myogenin	Santa Cruz Biotecnology, (M-225)	mouse	1/50

Table 2: Antibodies used for immunohistochemistry in single fibers.

Digital image acquisition and processing

Digital images were acquired using an upright microscope DMR6000B (Leica) equipped with a DFC300FX camera for immunohistochemical color pictures or a Hamamatsu ORCA-ER camera for immunofluorescence pictures. HCX PL Fluotar 10X/0.30, 20X/0.50 and 40X/0.75 objectives were used. Acquisition was performed using Leica Application or LAS AF software (Leica). Images were composed and edited in Photoshop CS5 (Adobe), where background was reduced using brightness and contrast adjustments applied to the whole image. To assess myofiber size, individual fibers were outlined and their cross-sectional area (CSA) was determined with the public domain image analysis software ImageJ (NIH,

USA). For satellite cell quantifications in single fibers, number of satellite cells was counted of each phenotype per fiber.

RT-qPCR: RNA extraction, cDNA synthesis and PCR

Total RNA was isolated from FACS sorted satellite cells or muscle tissue of mouse or human origin using Tripure reagent (Roche Diagnostic Corporation) or RNeasy Micro Kit (Qiagen), and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) or quantitative real time PCR (RT-qPCR). For qPCR experiments, DNase digestion of 10 mg of RNA was performed using 2 U DNase (Turbo DNA-free, Ambion). Complementary DNA was synthesized from 2 mg of total RNA using the First-Strand cDNA Synthesis kit (Amersham Biosciences). For quantitative real time PCR, reactions were performed on a LightCycler 480 System using a Light Cycler 480 SYBR Green I Master (Roche Diagnostic Corporation) and specifics primers (table 3). Thermocycling conditions were as follow: initial step of 10 min at 95ºC, then 50 cycles of 15s denaturation at 94ºC, 10s annealing at 60ºC and 15s extension at 72ºC. Reactions were run in triplicate, and automatically detected threshold cycle (Ct) values were compared between samples. Transcripts of the ribosomal protein L7 gene were used as endogenous control, with each unknown sample normalized to L7 content.

Gene	Species	Forward Primer	Reverse Primer
p16INK4a	mouse	CATCTGGAGCAGCATGGAGTC	GGGTACGACCGAAAGAGTTCG
Myostatin	mouse	CCAGGAGAAGATGGGCTGAATC CCT	GCAGCACCGGGATTCCGTGG
Sestrin1	mouse	GGACGAGGAACTTGGAATCA	ATGCATCTGTGCGTCTTCAC
	human	CAGCATTGGAAAACATTAGGCAA	CCGAAGACTCGGTATTTGAAAGC
Sestrin2	mouse	TAGCCTGCAGCCTCACCTAT	TATCTGATGCCAAAGACGCA
	human	AAGGACTACCTGCGGTTCG	CGCCCAGAGGACATCAGTG
Sestrin3	mouse	CATGCGTTTCCTCACTCAGA	GGCAAAGTCTTCGTACCCAA
	human	TTAAGACAGTGACCTGCTATCCT	GCTTGCATTCGTGCTTCCATTA
Glut4	mouse	GTGACTGGAACACTGGTCCTA	CCAGCCACGTTGCATTGTAG
HKII	mouse	CGGAATGGGGAGCCTTTGG	GCCTTCCTTATCCGTTTCAATGG
UCP3	mouse	GGCCCTTGTAAACAACAAAATAC	GGCAACAGAGCTGACAGTAAAT
CytC	mouse	ATTTCAACCCTTACTTTCCCG	CCACTTATGCCGCTTCATGGC
L7	mouse	GAAGCTCATCTATGAGAAGGC	AAGACGAAGGAGCTGCAGAAC

Table 3: Primers used for RT-PCR and RT-qPCR.

Protein extraction and Western Blotting

Preparation of cell lysates and Western blotting were performed as described in (Perdiguero et al., 2007). Briefly, cell lysates were prepared with IP buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EGTA, 5 mM EDTA, 20 mM NaF and 25mM □-glycerophosphate) supplemented with protease and phosphatase inhibitors (Complete Mini, Roche Diagnostic Corporation; phosphatase inhibitor cocktail, Sigma). Cleared lysates containing approximately 50 mg of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and detected with the primary

antibodies listed in table 4. Secondary anti–rabbit, anti-rat and anti-mouse IgG-HRP antibodies (Dako) were used, and HRP was detected using an ECL detection system according to the manufacturer's instructions (GE Healthcare).

Antibody	Manufacter	Species	Dilution
Anti-Phospho-AKT (Ser473)	Cell Signaling Technology	rabbit	1/1000
Anti-AKT total	Cell Signaling Technology	mouse	1/2000
Anti-Phospho-GSK3β	Cell Signaling Technology	rabbit	1/1000
Anti-Phospho-FoxO1 (Thr2a)/FoxO3a(Th32)	Cell Signaling Technology	rabbit	1/1000
Anti-Tubulin	Sigma	mouse	1/4000
Anti-Phospho-S6 (Ser235/236)	Cell Signaling Technology	rabbit	1/1000
Anti-S6 total	Cell Signaling Technology	mouse	1/1000
Anti-Phospho-AMPK (Thr172)	Cell Signaling Technology	rabbit	1/1000
Anti-AMPK total	Cell Signaling Technology	rabbit	1/1000

Table 4: Antibodies used for western blot.

Statistical analysis

Prism software (GraphPad Software) was used for all statistical analyses. Results from corresponding time points of each group were averaged and used to calculate descriptive statistics. One-way analysis of variance and Tukey posthoc test or a Kruskall Wallis and Dunn's posthoc test was used on multiple comparisons and all possible pairwise comparisons among groups at each time point. Data are means ± SEM. Significance was accepted at $P < 0.05$.

Results

The role of the Bmi1/p16INK4a axis in muscle stem cell biology

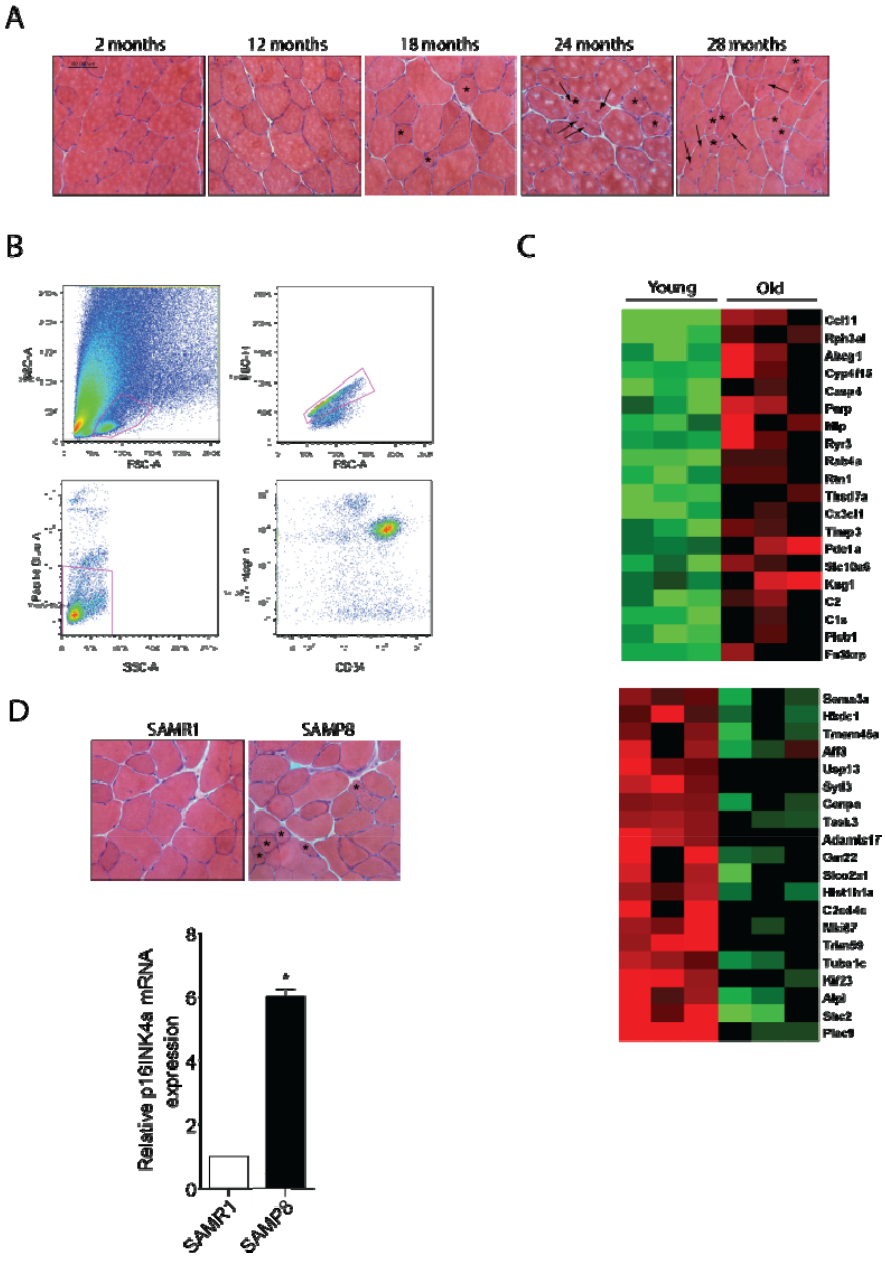
Whole-genome expression analysis of aged satellite cells

Satellite cells sustain skeletal muscle repair and growth throughout life, a function coordinated by cell intrinsic signaling pathways. The functionality of these stem cell-intrinsic pathways likely deteriorates with age thereby compromising tissue repair in response to stress or injury. Here we aim to study the global changes in gene expression taking place in aged satellite cells as an approach to identify the factors responsible for age-associated stem cell loss of functionality. We hypothesize that these deleterious factors will be operating at maximal levels in satellite cells from sarcopenic muscle.

First of all, we analyzed histologically the muscles of mice at different ages, ranging from young/adult, adult/old and aged/sarcopenic (2, 12, 18, 24 and 28 months of age). As expected, signs of deep muscle wasting and atrophy were maximal in the oldest/sarcopenic mice (Figure 4A)

Figure 4. FACS isolation and microarray analysis of satellite cells from aged mice. (A) H/E staining of transverse sections of two, twelve, eighteen, twenty-four and twenty-eight months TA muscles illustrates increased signs of muscle atrophy with age. **(B)** FACS sorting strategy. Representative example of cell sorting strategy and gating scheme. **(C)** Heat maps from global comparative transcriptome analysis indicating the top 20 up-regulated and down-regulated genes in aged satellite cells. **(D)** (Up) H/E staining of transverse sections of SAMR1 and SAMP8 TA, (Down) Validation by RT-qPCR of p16INK4a up-regulation in aged

satellite cells of SAMP8 mice. Data are mean ± SEM of at least three experiments, *P<0.05.



Accordingly, we isolated the quiescent satellite cell population from both young (2 months) and old/sarcopenic (28 months) mice muscles by FACS, gating on integrin α-7 (+)/CD34(+) (positive selection) and Lin- (CD31, CD45, CD11b, Sca1) (negative selection) cells (Figure 4B). RNA isolated from these samples was amplified thought the Whole Transcriptome Amplification (WTA) method (Gonzalez-Roca et al., 2011) and the resulting cDNA was used on a transcriptome analysis by Agilent SurePrint G3 Mouse GE 8x60K microarray, which covers 39,430 Entrez Gene RNAs and 16,251 lincRNAs (long intergenic non-coding RNAs) of the mouse genome.

The whole-genome microarray identified 3181 genes that were found to be consistently (3 replicates per condition), significantly ($P<0.05$) and differentially (>1.3 fold) expressed between old satellite cells and young satellite cells. 1611 and 1570 of these genes were respectively up-regulated and down-regulated in satellite cells extracted from aged muscle. The gene expression data of the first 40 differentially expressed genes according to our criteria of significance was split into up-regulated and down-regulated genes and are listed in Figure 4B.

The microarray data was further analyzed using the gene annotation tool from DAVID (Dennis et al., 2003) to identify enriched gene Ontologies (GO) (Harris et al., 2004). The up-regulated dataset was significantly enriched in transcripts associated with the cell adhesion and adipogenesis biological

processes. In this context, several collagen transcripts (Col4a5, Col4a6, Col4a4, Col1a2, Col18a1, Col8a1, Col15a1) and collagen binding and regulatory transcripts (Timp3, thbs4) were found up-regulated in aged satellite cells, as well as genes typically associated with the adipogenic lineage (Rarres2, Arl4a, Selenbp1) (Table5, Figure 4B). In addition, transcripts associated with apoptosis and cell cycle regulation were also altered in aged satellite cells. Positive regulators of programmed cell death (Casp4, Casp8, Perp) and negative regulators of proliferation (p16INK4a/cdkn2a and p21CIP/cdkn1a) were significantly up-regulated, while transcripts associated with the positive regulation of cell cycle progression (ki67, Kif23), DNA replication and ribosome biogenesis were found down-regulated (Table 5, Figure 4B).

Up-regulation of p16INK4a suggests that cell cycle regulation and proliferation may be compromised in aged satellite cells, particularly in satellite cells of very old sarcopenic muscle. p16INK4a is a negative regulator of the cell cycle, classically associated with cellular senescence (Lowe and Sherr, 2003).

In order to validate p16INK4a up-regulation in aged satellite cells from sarcopenic muscle we used a mouse model of aging: The Senescence-Acelerated Mouse (SAM). SAM was established as a murine model of senescence acceleration and age-associated disorders. These strains show characteristic pathological phenotypes that are similar to age-associated disorders often observed in elder mice (Takeda et al., 1991). SAMP8 strain (Senescence Accelerated Mouse-

Prone 8) has been proposed as a model for skeletal muscle aging studies, as soleus muscle of this mice displays several typical features of muscle senescence (Derave et al., 2005). Indeed, we confirm the atrophic phenotype of SAMP8 mice (Figure 4C). We isolated satellite cells from SAMP8 mice (and of its counterpart SAMR-1 control) and analyzed the expression of p16INK4a by RT-qPCR. Consistently with our microarray data, p16INK4a was significantly induced in SAMP8 derived satellite cells (Figure 4C).

p16INK4a has been previously described as a direct target of the polycomb-group gene Bmi1 and as the main mediator of Bmi1-dependent regulation of cell proliferation and senescence in mouse embryonic fibroblasts (Jacobs et al., 1999). This prompted us to analyze the skeletal muscle phenotype of Bmi1-/- mice as well as to characterize Bmi1-deficient satellite cells, with the idea of having a mouse model with potentially enhanced/advanced expression of p16INK4a.

	Biological process (GO)	Gene Symbol	Description
Up-regulated	Cell adhesion	Col4a5	collagen, type IV, alpha 5
		Col4a6	collagen, type IV, alpha 6
		Col4a4	collagen, type IV, alpha 4
		Col1a2	collagen, type I, alpha 2
		Col18a1	collagen, type XVIII, alpha 1
		Col8a1	collagen, type VIII, alpha 1
		Col15a1	collagen, type XV, alpha 1
		Thbs4	thrombospondin 4
		Timp3	TIMP metallopeptidase inhibitor 3
	Adipogenesis	Rarres2	retinoic acid receptor responder 2
		Arl4a	ADP-ribosylation factor-like 4A
		Selenbp1	selenium binding protein 1
		Aldh9a1	aldehyde dehydrogenase family 6,subfamilyA1
	Apoptosis	Casp4	caspase 4
		Casp8	caspase 8
		Perp	TP53 apoptosis effector
	Cell Proliferation (negative regulation)	Cdkn2a	cyclin-dependent kinase inhibitor 2A (p16INK4a)
		Cdkn1a	cyclin-dependent kinase inhibitor 1A (p21CIP)
Down-regulated	Ribosomal biogenesis	Mphsph10	M-phase phosphoprotein 10
		Sdad1	SDA1 domain containing 1
	DNA replication	Top1mt	DNA topoisomerase 1, mitochondrial
		Dntt	deoxynucleotidyltransferase, terminal
		Pola2	polymerase (DNA directed), alpha 2
	Cell Proliferation (positive regulation)	Ki67	antigen identified by monoclonal antibody Ki-67
		Kif23	Mitotic kinesin-like protein 1

Table 5: List of biological processes gene ontologies (GO) and associated genes affected in aged satellite cells.

Bmi1 loss anticipates the expression of p16INK4a and other age-associated genes in satellite cells of young mice

Mice lacking Bmi1 succumb in early adult life due to defects in their hematopoietic and neuronal tissues (van der Lugt et al., 1994), but whether skeletal muscle tissue development is also affected by Bmi1 loss has never been investigated.

Young adult Bmi1-deficient mice were considerably smaller then the age-matched WT littermates (Figure 5A) and displayed a corresponding reduction in muscle size (Figure 2B). Evaluation of body weight showed that these differences were not significant at early post natal stages (P8) but dramatically increased thereafter (Figure 5C). These results suggest that Bmi1 is not critically required for fetal/early muscle development but is indispensable for efficient growth of the young adult muscle. Accordingly, myofiber cross-sectional area was not significantly altered in early post-natal muscle but considerably reduced in adult mice (Figure 5D). Since the number of Bmi1-/- myofibers in the adult mouse was comparable with that of littermate controls, but the size of each myofiber was reduced at P35, we concluded that Bmi1 loss causes defects of late postnatal/adult muscle growth characterized by reduced muscle mass with smaller muscle fibers, typical of atrophying senescent/sarcopenic muscle.

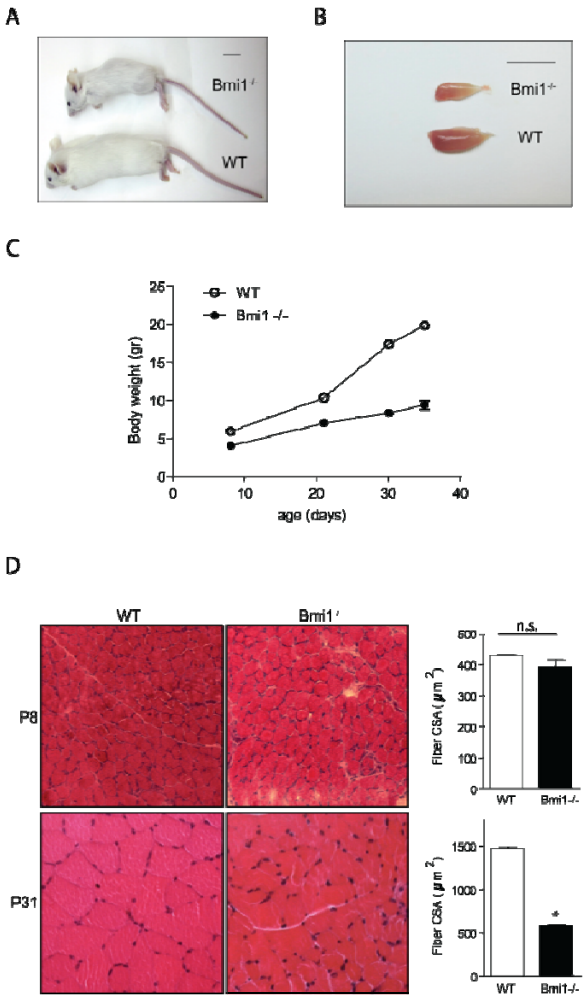


Figure 5. Bmi1 deficiency results in adult skeletal muscle defects. (A, B) Body and muscle mass in P35 Bmi1-/- mice and age-matched WT control littermates. Scale bars: 1cm, A; 0,5 cm, B. **(C)** Body weight of Bmi1-/- mice and age-matched WT control littermates from early post-natal stages (P8) to young/adult stages (P35). Data are mean of measurements for at least four animals. **(D)** H/E staining of transverse sections and myofiber cross-sectional area (CSA) of P8 and P35 Bmi1-/- and age-matched WT control littermates TA muscles. Data are mean ± SEM of at least three experiments, *P<0.05, n.s. not significantly different.

Since postnatal day 21 is regarded as the boundary delimiting postnatal satellite cell proliferation and entry into quiescence (i.e., the normal satellite cell state in resting adult muscle), we aimed to investigate whether Bmi1 would regulate satellite cell proliferation and quiescence before and after P21, respectively. Accordingly, we comparatively examined the proliferating (P8) and quiescent (P35) satellite cell population in muscle of Bmi1-/- and WT mice. Satellite cells were isolated by FACS sorting as described above and whole-genome expression analysis was performed using Agilent SurePrint G3 Mouse GE 8x60K microarray.

We used a linear model approach to compute differentially expressed genes in order to assess the comparisons between WT and Bmi1-/- satellite cells at P8 and P35. From these initial results, we selected those genes having an adjusted p-value lower than 0.05 and a fold change cut-off of at least ±1.5. The total number of the differentially expressed genes, according to the described criteria, is showed in Figure 3A. The gene expression data of the first 8 and 40 differentially expressed genes at P8 and P35 respectively was split into up-regulated and down-regulated genes and is shown in Figure 3B. Due to the fact that we had too few genes regulated at P8, downstream analysis was done only for the P35 dataset.

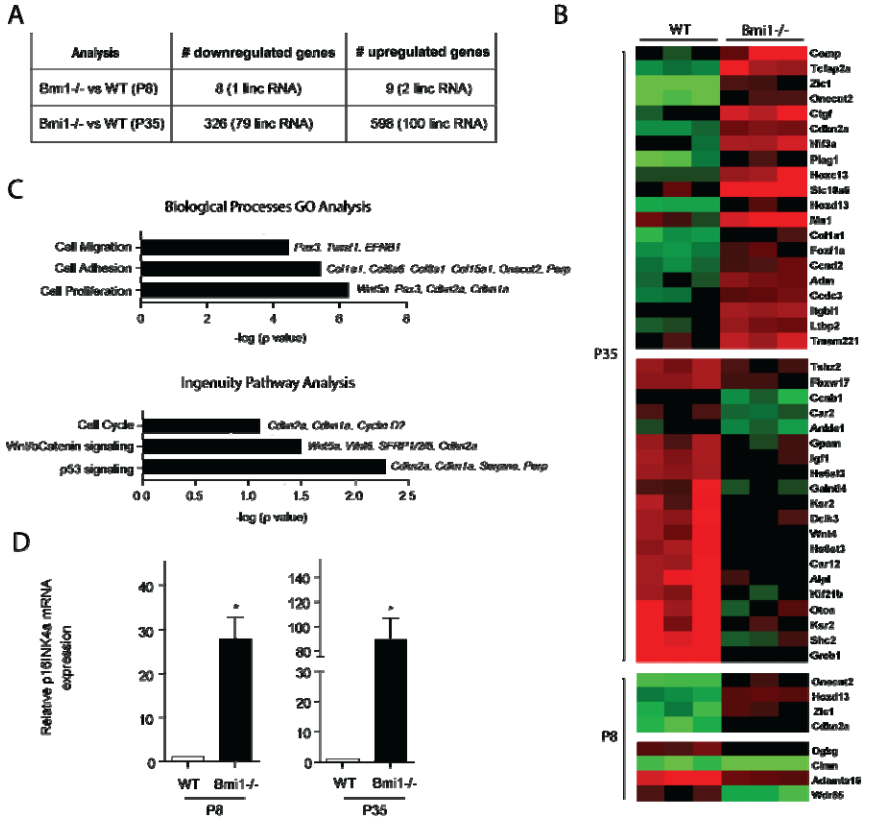


Figure 6. Microarray analysis of satellite cells from Bmi1-deficient mice. (A) Summary table indicating the number of transcripts up-regulated and down-regulated in P8 and P35 Bmi1-/- satellite cells. **(B)** Heat maps from global comparative transcriptome analysis indicating the top 20/top 4 up-regulated and down-regulated genes in P35/P8 Bmi1-deficient satellite cells. **(C)** Gene ontology (GO) analysis of biological processes (upper bar diagram) and Ingenuity Pathway Analysis (IPA) of signaling pathways (lower bar diagram) associated with the dataset of genes up-regulated in P35 Bmi1-deficient satellite cells, using the gene annotation tool from the DAVID database. Black bars represent the log10 of p values of each category. **(D)** Validation by RT-qPCR of p16INK4a up-regulation in satellite cells of P8 and P35 Bmi1-/- mice. Data are mean ± SEM of at least three experiments, *P<0.05.

In order to understand the biological processes underlying the transcriptional changes, the selected sets of up-regulated and down-regulated genes were classified according to the biological process ontology of the Gene Ontology (GO) Consortium (Harris et al., 2004), using the gene annotation tool from DAVID (Dennis et al., 2003). The dataset was further analyzed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Mountain View, CA) to identify the most affected signaling pathways.

The gene ontology analysis of our microarray data revealed that the genes involved in the regulation of cell proliferation were among the most affected in Bmi1-deficient satellite cells (Figure 6C). Negative regulators of cell proliferation (p16INK4a/cdkn2a and p21CIP/cdkn1a) were up-regulate while transcripts associated with the positive regulation of cell cycle progression (ki67) were found down-regulated in Bmi1-/- satellite cells. Furthermore, cell adhesion and cell migration associated transcripts were also found up-regulated in Bmi1-deficient satellite cells (Figure 6C). It is worth noticing that those (cell proliferation and cell adhesion) were among the biological processes we identified as most affected in aged satellite cells (see above). IPA core analysis further identified cell cycle, Wnt signaling and p53 signaling as some of the most affected signaling pathways (Figure 6C).

p16INK4a (cdkn2a) transcripts, which are not expressed in WT satellite cells at P35 (but only in aged satellite cells from

sarcopenic mice) were found up-regulated in Bmi1-deficient satellite cells. Interestingly, although few genes were significantly altered at P8, p16INK4a was found up-regulated in Bmi1-deficient satellite cells already during the post-natal proliferative phase, suggesting that cell cycle progression is already compromised in Bmi1-/- satellite cells at this stage. This up-regulation was further increased at P35, with p16INK4a being one of the most differentially expressed genes in adult Bmi1-deficient mice. These observations were validated by RT-qPCR in isolated satellite cells, confirming the microarray results (Figure 6D).

In the adult Bmi1-/- mice, p16INK4a up-regulation was accompanied by the activation of other molecules that have been reported to be involved in senescent cell states. Perp, described as p53 target gene and apoptosis effector, was up-regulated in Bmi1-/- satellite cells and its function has also been associated with p53 mediated senescent state (Ota et al., 2011). Perp gene transcripts were also up-regulated in aged satellite cells.

Interestingly, many other transcripts induced in Bmi1-deficient mice were also up-regulated in aged satellite cells from sarcopenic mice. Those include cell adhesion molecules associated with the extracellular matrix (Timp3, thbs4, Col8a1, Col15a1) and genes involved in adipogenesis (Rarres2, Arl4a, Selenbp1). In addition Bmi1-defficient mice also up-regulate CTGF (connective tissue growth factor) and FN1 (Fibronectin 1), both associated with a fibrogenic

phenotype (Morales et al., 2011; Vial et al., 2008). These observations suggest that Bmi1-defficient satellite cells have an increased tendency to enter alternative differentiation programs by adopting an adipogenic and fibroblastic fate.
This data supports the idea that Bmi1 loss, in parallel to a senescence promoting role through the regulation of cell proliferation, may also be contribute to an aged-like phenotype by compromising satellite cell transcriptional identity.

Bmi1 loss impairs satellite cell number and self-renewal

The similarities between Bmi1-deficient and aged satellite cells at the level of gene expression prompted us to further characterize muscle stem cell functionality in Bmi1-deficient mice in comparison to previously described phenotypes of aged satellite cells.
Isolated myofibers provide an accessible means to study the activation, proliferation, differentiation and self-renewal of satellite cells in their native position beneath the basal lamina that surrounds each muscle fiber. This model preserves potentially important interactions between satellite cells and myofibers. Myofibers were isolated from the extensor digitorum longus (EDL) of P35 WT and Bmi1-deficient mice and analyzed in culture for stem cell function. First we started by confirming the expression of Bmi1 in satellite cells of the WT muscle. For that purpose we have co-labeled WT

muscles with antibodies against Bmi1 and CD34, used here as a marker of adult muscle progenitors (Beauchamp et al., 2000). Indeed, Bmi1 staining was detected in CD34 positive satellite cells of WT mice immediately after myofiber isolation (Figure 7A).

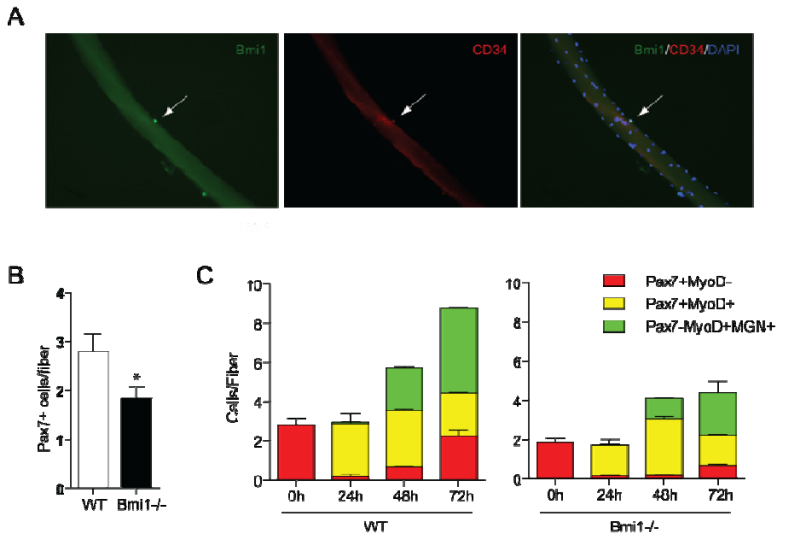


Figure 7. Reduced number and impaired self-renewal of satellite cells in Bmi1-defficient mice. (A) Bmi1 expression in satellite cells (arrow) shown by co-immunostaining of EDL WT myofibers with antibodies against Bmi1 (green) and CD34 (Red). Counterstaining with DAPI is shown in blue. **(B)** Total number of Pax7 positive, MyoD negative satellite cells in single myofibers isolated from EDL muscle of young/adult Bmi1-defficient and age-matched WT control littermates. (C) As in B, total number of Pax7+/MyoD- (Red), Pax7+/MyoD+ (Yellow) and Pax7-/MyoD+/Mgn+ (green) quantified after culture in growth medium for twenty-four, forty-eight and seventy-two hours. Data are mean ± SEM of at least three experiments, *P<0.05.

Since reported analysis of EDL isolated myofibers indicate a decline in satellite cell numbers with age (Shefer et al., 2006), we decided to investigate whether the satellite cell population of Bmi1-deficient skeletal muscle was also reduced. Since Pax7 positive MyoD negative staining, we have co-labeled EDL isolated myofibers with Pax7 and MyoD antibodies and quantified the number of quiescent satellite cells in WT and Bmi1-deficient muscles. The number of resident satellite cells per unit of EDL fibers was significantly reduced in Bmi1-deficient mice (Figure 7B), indicating that, like in aged muscle, the satellite cell population is depleted in Bmi1 -/- skeletal muscles.

Upon stimulation by mitogen-rich medium (growth medium), isolated quiescent satellite cells on the myofibers are activated and up-regulate MyoD. Satellite cells then proliferate, and either down-regulate Pax7, up-regulate myogenin and proceed to differentiate, or down-regulate MyoD and return to a quiescent-like state, modeling self-renewal. In order to access which muscle stem cell functions were affected in Bmi1-deficient mice, we cultured the isolated myofibers for 24, 48 and 72 hours in growth medium and then analyzed the fate of the associated satellite cells by immunofluorescence, using antibodies against Pax7, MyoD and MGN. Satellite cell activation was not impaired in Bmi1-deficient mice since after 24 hours in growth medium, the majority of the satellite cells co-stained for Pax7 and MyoD in

both genotypes (Figure 7C, 24h, yellow). Similarly, satellite cells myogenic potential was also not affected in Bmi1 -/- mice since a population of satellite cells down-regulating Pax7 and still expressing MyoD and MGN appears in both WT and Bmi1-deficient cells (Figure 7C, 48h and 72h, green). However, beyond 48 hours the total number of WT satellite cells was progressively increased (up to three times the initial number of resident cells at 72 hours), while Bmi1-deficient satellite cell population only doubled their initial number by 72 hours, suggesting a decline in proliferative capacity. Furthermore, at 72 hours the number of Pax+MyoD- satellite cells in the WT is back to the original level of a basal state while in Bmi1-/- the initial population of quiescent cells was not re-established (Figure 7C, 72h, red), indicating that Bmi1 is necessary for the efficient self-renewal of satellite cells.

Considering the above described phenotype, the increased expression of p16INK4a in Bmi1-deficient satellite cells and the identification of cell cycle associated pathways altered in the Bmi1-/- mice, we decided to further characterize the proliferative capacity of Bmi1-deficient satellite cells in vitro.

For that purpose, we have analyzed the capacity of satellite cells to generate progeny in culture. An equivalent population of Bmi1-deficient or WT FACS-isolated satellite cells was plated and total number of cells quantified daily. After 48h in culture the total number of myoblasts derived from Bmi1-deficient satellite cells was already significantly reduced when compared to WT cultures. The differences in total number of

cells considerably increased thereafter and at 96h Bmi1-deficient progeny was almost half that of age-matched WT satellite cells (Figure 8A, left). An equivalent experiment performed with aged satellite cells showed a similar growth profile (Figure 8A, right). We confirmed that this phenotype is, at least in part, due to a reduced proliferative capacity of Bmi1-deficient myoblasts using 5-bromo-2'-deoxyuridine (BrdU) incorporation as a measurement of proliferation rate. The percentage of BrdU positive cells in 72h cultures exposed to one hour pulse of BrdU was significantly lower for Bmi1-deficient derived cultures (17,5%) when compared to WT (41,5%), suggesting that Bmi1-deficient satellite cells indeed proliferate at a lower rate (Figure 8B).

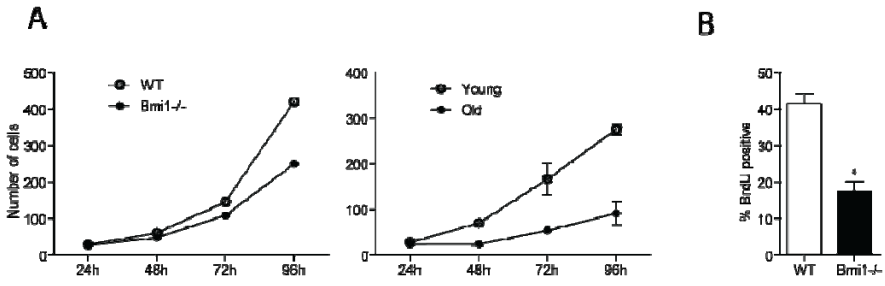


Figure 8. Bmi1 loss and age impairs the proliferative capacity of satellite cells. (A) Total number of cells derived from an equal number of satellite cells isolated by FACS from either Bmi1-defficient mice and age-matched WT control littermates (left) or young and old mice (right), after twenty-four, forty-eight, seventy-two and ninety-six hours of culture in proliferative conditions. **(B)** Fraction of satellite cells isolated by FACS from Bmi1-defficient mice and age-matched WT control littermates that incorporated BrdU when cultured in proliferative conditions for seventy-two hours and subjected to one hour pulse with BrdU. Data are mean ± SEM of at least three experiments, *P<0.05.

Bmi1 loss impairs myofiber regeneration

With age, there is a gradual decline in the effectiveness of the regenerative response of skeletal muscle to damage, a phenotype which has been associated to the age-related decline in satellite cell functionality.

To confirm the importance of Bmi1 in satellite cells in vivo, Bmi1-/- and WT muscles were challenged to regenerate by intramuscular injection of cardiotoxin that causes acute injury by rapidly destroying muscle fibers while preserving the satellite cell pool.

One week after injury, regenerating myofibers of WT mice exhibited obvious signs of advanced regeneration, as indicated by the presence of large central-nucleated fibers with low expression of embryonic myosin heavy chain (eMHC), a marker of early muscle regeneration, while Bmi1-/- central nucleated myofibers had a reduced size and still exhibited intense eMHC staining, indicating that satellite cell-dependent muscle repair is delayed/defective in the absence of Bmi1 (Figure 9A).

Since Bmi1 loss might affect other cell types involved in the muscle regeneration process, we aimed to specifically confirm the satellite cell intrinsic functions of Bmi1 during this repair process. To this end, we transplanted the EDL muscle of Bmi1-/- or WT mice onto the tibialis anterior (TA) muscle of Rag2-/-:γC-/- immunocompromised recipient mice (Colucci et al., 1999). In this quimeric mouse, the transplanted EDL

myofibers degenerate but commence to regenerate and undergo de novo myogenesis at the expense of its own satellite cells, thus constituting an optimal model to assess specifically the capacity of WT and Bmi1-deficient satellite cells in supporting the regeneration of transplanted WT and Bmi1-/- EDL muscles, respectively, within a common heterologous host environment.

One week after transplantation, the newly formed Bmi1-/- central nucleated myofibers had a reduced size, indicating that the delayed/defective repair in the absence of Bmi1 was caused by satellite cell intrinsic factors and not by the influence of other cell types potentially involved in the regeneration process (Figure 9B).

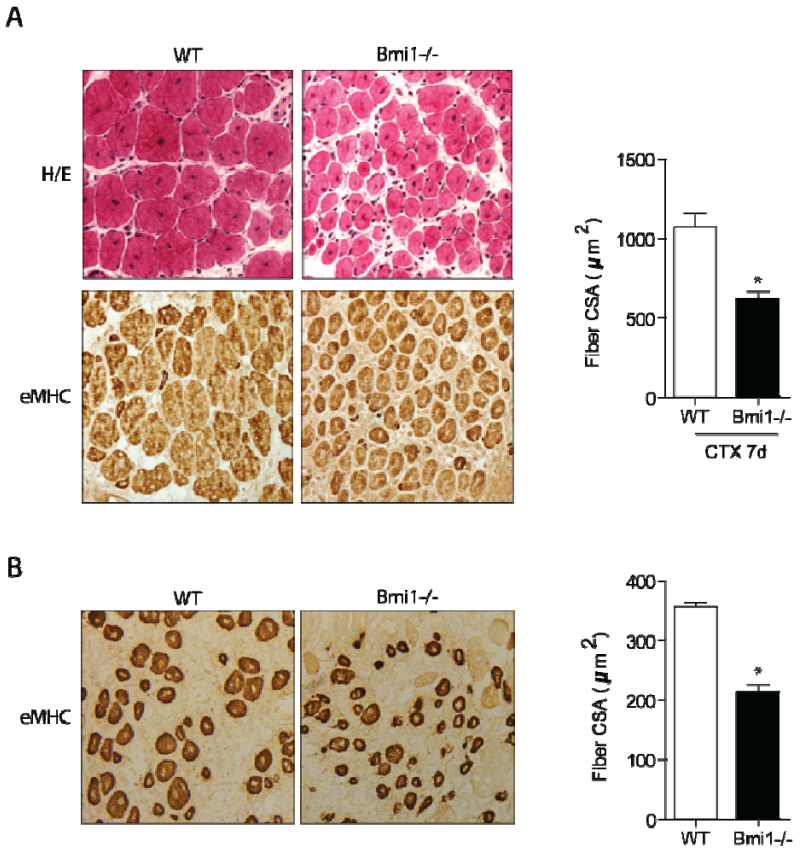


Figure 9. Bmi1 loss impairs satellite cell intrinsic regenerative capability. (A) Cryosections of regenerating CTX-injured muscles from Bmi1-deficient mice and age-matched WT control littermates stained with H/E (top left) and antibodies against eMHC (bottom left) seven days after injury and mean cross-sectional area (CSA) of regenerating myofibers (right). **(B)** Cryosections of regenerating grafted EDL muscles from Bmi1-deficient mice and age-matched WT control littermates stained with antibodies against eMHC (left) seven days after graft and mean cross-sectional area (CSA) of regenerating myofibers (right). Data are mean ± SEM of at least three experiments, *P<0.05.

Microarray analysis of Bmi1 deficient skeletal muscles reveals an overlapping transcriptome with aged skeletal muscle

We further characterized the senescent/sarcopenic phenotype of Bmi1 null muscle through a microarray analysis of the transcriptome of adult muscle fibers. For that purpose we used RNA isolated from TA muscle and performed a whole-transcriptome analysis with Agilent SurePrint G3 Mouse GE 8x60K microarray. Using a similar linear model approach and selection criteria described for the Bmi1-deficient satellite cells array (adjusted p-value lower than 0.05 and a fold change cut-off of at least ±1.5) we identified 1490 genes differentially expressed. 906 and 584 of these genes were respectively up-regulated and down-regulated in Bmi1-deficient muscle. The gene expression data of the first 40 differentially expressed genes according to our criteria of significance was split into up-regulated and down-regulated genes and are listed in Figure 10A. Among the most up-regulated genes (top heatmap block, Figure 10A) we found, as in adult satellite cells, p16INK4a (cdkn2a) and zic1.

The up-regulation of p16INK4a in Bmi1-/- was validated by RT-qPCR. Also in this case we used samples from SAMP8 mice, a model for skeletal muscle aging which develops anticipated atrophy, and from physiologically aged mice (28 months) to evaluate whether this induction was found in aged/sarcopenic muscle. RT-qPCR confirmed that indeed

p16INK4a was up-regulated in SAMP8 and aged skeletal muscle (Figure 10B). Considering that muscle is largely a post-mitotic tissue, it is interesting to see that this up-regulation of p16INK4a is also significantly found in muscles of geriatric mice.

The gene ontology analysis for biological processes revealed that the genes up-regulated in our dataset were associated with biosynthetic processes, insulin receptor signaling pathway and tissue morphogenesis (Figure 10C).

Insulin receptor signaling and biosynthetic processes are biological processes associated with the regulation of muscle growth and atrophy. Genes assigned to these categories include several members of the FoxC, FoxD and FoxO family, the later being directly implicated in the regulation of adult muscle mass. FoxO genes up-regulation is associated with atrophy-like phenotypes through their function as direct positive regulators of muscle atrophy F-box (MAFbx), a muscle-specific ubiquitin ligases which we also find up-regulated in Bmi1-deficient muscle fibers. Finally, Myostatin (Mstn), another negative regulator of muscle mass, acting through the phospho-Smad2/3 pathway, is also significantly up-regulated in Bmi1-/- mice skeletal muscle. Mstn up-regulation was validated by RT-qPCR (Figure 10D), which confirmed the results of the microarray data.

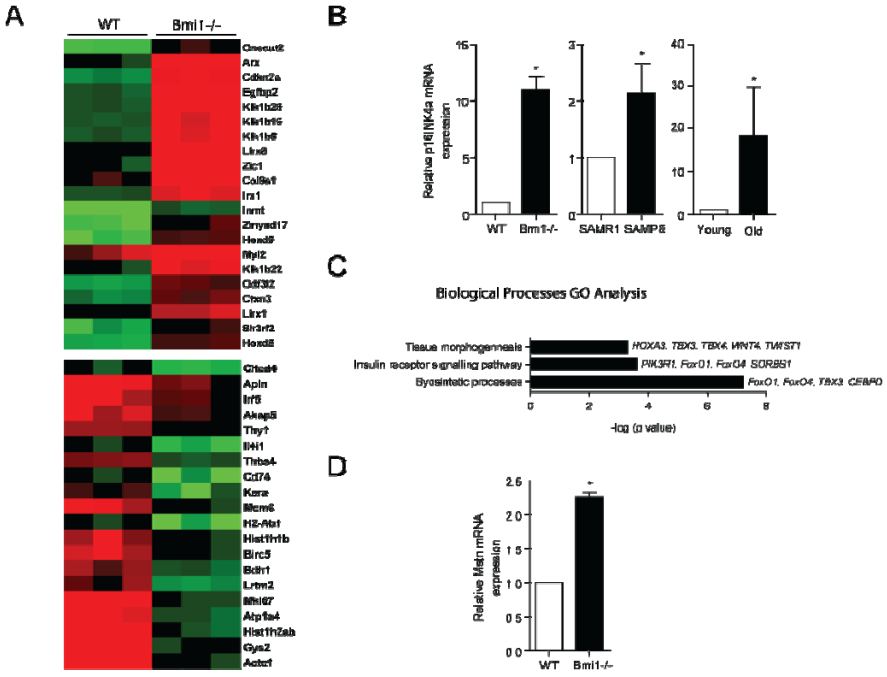


Figure 10. Microarray analysis of TA muscle of Bmi1-deficient mice. **(A)** Heat maps from global comparative transcriptome analysis indicating the top 20 up-regulated and down-regulated genes in Bmi1-deficeint TA muscle. **(B)** Validation by RT-qPCR of p16INK4a up-regulation in TA muscle of young/adult Bmi1-/-, SAMP8 and old (28month) mice. **(C)** Gene ontology (GO) analysis of biological processes associated with the dataset of genes up-regulated in Bmi1-deficient TA muscle, using the gene annotation tool from the DAVID database. Black bars represent the log10 of p values of each category. **(D)** Validation by RT-qPCR of Myostatin up-regulation in TA muscle of Bmi1-/- mice. Data are mean ± SEM of at least three experiments, *P<0.05.

Interestingly, there was an extensive overlap between the alteration we found in Bmi1-deficient skeletal muscles and the ones previously described in very old skeletal muscles

(Edwards et al., 2007). Some of the common genes are represented in Table 6.

Gene Symbol	Fold change	Gene description
p16INK4a	+12,1	cyclin-dependent kinase inhibitor 2A (cdkn2a)
Sesn1	+3,1	Sestrin1
Ddit4	+2,7	DNA-damage-inducible transcript 4
p21CIP	+1,8	cyclin-dependent kinase inhibitor 1A (cdkn1a)
Igfbp3	+1,7	IGF binding protein 3
Mcl1	+1,5	myeloid cell leukemia sequence 1 (BCL2-related)
GADD45a	+1,5	growth arrest and DNA-damage-inducible, alpha

Table 6: List of genes commonly affected in Bmi1-deficient and old skeletal muscle, as described by Edwards et al. (2007).

Overall, our data supports the idea that Bmi1-deficient atrophic/sarcopenic phenotype is associated with gene expression alterations in both the satellite cell and muscle fiber that resemble those found in physiologically aged skeletal muscle and in the SAMP8 mice. These results potentially implicate Bmi1-dependent gene regulation in the aging process, particularly during sarcopenia.

The role of Sestrins in muscle growth and atrophy

Sestrins1-3 are expressed in skeletal muscle and are down-regulated in human myopathies

Muscle homeostasis is a dynamic process between anabolism and catabolism essential for the maintenance of muscle mass and function throughout adult life. Some of the most serious physiological consequences of ageing relate to the loss of the proper balance between mechanisms of growth and atrophy that results in a slow, progressive decline of muscle mass, strength and function. Despite the impact and relevance of age-associated muscle phenotypes, the underlying biochemical and molecular mechanism remain poorly understood. Several factors have been identified to contribute to this loss of muscle homeostasis, including a decline in the state of activation of growth pathways, such as the PI3K/AKT signaling pathway, and an increase in muscle ROS and oxidative damage.

Sestrins were initially identified as stress-responsive proteins (Peeters et al., 2003) that could function as antioxidants. Although Sestrins are ubiquitously expressed in the adult organism, the relative levels of each isoform are tissue dependent. In mammalian skeletal muscle, Sestrin 1 (Sesn1) is the isoform with the highest expression levels (Figure 11A) and this is the tissue where it is most abundantly found (Velasco-Miguel et al., 1999), suggesting a possible role for Sesn1 as an important regulator of muscle physiology.

Given that the ablation of drosophila Sestrin results in several age-related pathologies, including accumulation of ROS within muscle fibers and accelerated muscle degeneration (Lee et al., 2010b), we asked whether Sesn expression was altered in human myopathies. Analysis of muscle biopsies of patients suffering from myotilinopathy, desminopathy and inclusion body myositis, myopathies characterized by progressive muscle weakness and decreased muscle mass, showed that in all three cases hSesn1 mRNA expression was reduced by an average of 75% with respect to a control group of healthy individuals (Figure 11B). These observations led us to further investigate a possible involvement of Sesn1 in the regulation of adult skeletal muscle homeostasis.

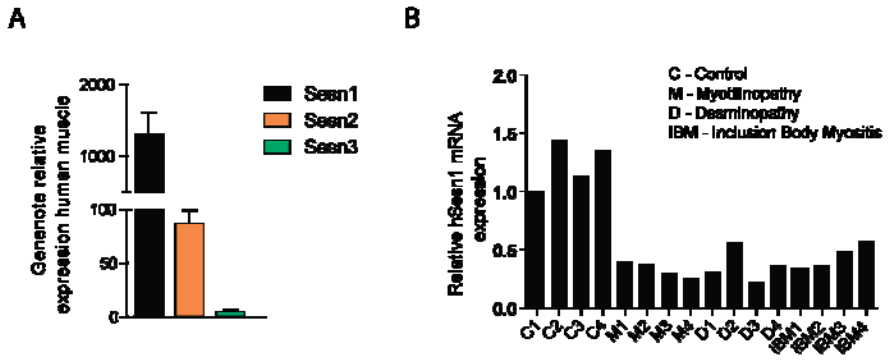


Figure 11. Sestrins1-3 are expressed in human skeletal muscle and are down-regulated in several human myopathies. (A) Relative expression levels of Sesn1-3 in human skeletal muscle according to data obtained from genenote. **(B)** RT-qPCR analysis of Sesn1 expression in skeletal muscle of healthy individuals (CNTRL) and patients suffering from Myotilinopathy, Desminopathy and Inclusion Body Myositis.

Sestrin1-3 expression levels are modulated in mouse models of adult muscle growth and atrophy

We used well-established models for the study of skeletal muscle atrophy and growth/hypertrophy in mice to determine the regulation of Sestrins in these processes. First, we examined the effects of experimentally induced atrophy on the expression levels of the three Sestrin isoforms. For that purpose the sciatic nerve was cut, resulting in denervation and atrophy of the Tibialis Anterior (TA) muscle. The levels of Sesn1, Sesn2 and Sesn3 mRNA, as measured by RT-qPCR, diminished in TA muscles undergoing rapid atrophy caused by denervation (Figure 12A). This marked reduction in expression occurred early, within the first day after the surgery, and remained low thereafter for Sesn1 and Sesn2, while Sesn3 progressively recovered its basal levels (Figure 12A).

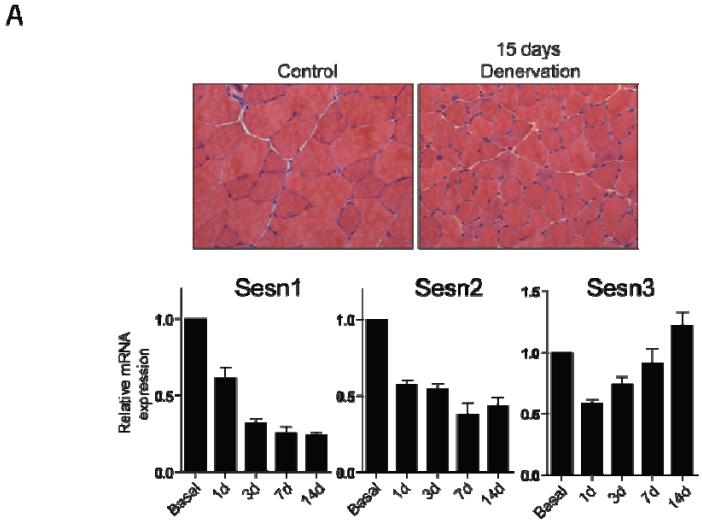
Next, we examined the regulation of Sesn1-3 expression during physiological adult muscle growth. For that purpose, we first subjected 12 week-old mice to a 3 week intensive exercise (treadmill running protocol), known to increase muscle mass, and found an up-regulation of Sesn1 and Sesn3 (Figure 12B). To confirm the association of muscle growth with increased Sesn expression, we next subjected the mice to an extreme procedure to induce hypertrophy, by inhabilitating the gastrocnemius muscle and forcing the

plantaris muscle to grow in a compensatory process (compensatory hypertrophy, CH). We found an induction of Sesn1/2/3 expression in the overloaded plantaris muscle correlating with increased myofiber size (Figure 12C).

These results suggest a previously unidentified function of Sestrins as potential new regulators of physiological adult muscle growth. Since the mechanisms regulating muscle growth are distinct from those involved in muscle atrophy we decided to characterize the molecular mechanisms downstream of Sestrin through an in vivo gain of function approach, focusing our analysis on Sesn1 and Sesn2.

Figure 12. Sestrin1-3 expression levels are regulated in mouse models of muscle hypertrophy and atrophy. (A) H/E staining of transverse sections (top) and comparative RT-qPCR analysis of Sesn1-3 expression (bottom) in the TA muscle one, three, seven and fourteen days after denervation**. (B)** Comparative RT-qPCR analysis of Sesn1-3 expression (right) in mice subjected to a three week intensive training protocol (left) in respect to sedentary mice. **(C)** H/E staining of transverse sections (top left), fiber size evaluated by CSA (top right) and comparative RT-qPCR analysis of Sesn1-3 expression (bottom) in the plantaris muscle of mice with induced compensatory hypertrophy (CH) fifteen days after gastrocnemius tenotomy. Data are mean ± SEM of at least three experiments, *P<0.05.

Results


A
Control
15 days
Denervation
Sesn1
Sesn2
Sesn3
Relative mRNA expression
Basal
1d
3d
7d
14d
B
Intensive treadmill running
everyday for 5 days/wk
during 3 consecutive weeks
Running speed (m·s⁻¹)
Time (min)
Sesn1
Sesn2
Sesn3
Sedentary
Trained
Relative mRNA expression
C
Control
15 days CH
Fiber size (μm²)
Basal
15 days CH
Sesn1
Sesn2
Sesn3
Basal
CH
Relative mRNA expression

Over-expression of Sesntrin1-2 in murine skeletal muscle by Adeno-associated viral transduction

Adeno-associated viruses (AAV) have emerged as reliable tools to achieve stable transgene expression following in vivo delivery in skeletal muscle. AAV2/1 is considered the serotype that confers the highest level of transduction to date in skeletal muscle (Xiao et al., 1999).

We developed, in collaboration with the Center for Animal Biotechnology and Gene Therapy (CBATEG), two AAVs constructs expressing the cDNA of hSesn1 and hSesn2 (AAV2/1-hSesn1 and AAV2/1-hSesn2) (Figure 13A).

AAV2/1-hSesn1 and AAV2/1-hSesn2 were injected into the right TA muscle and the expression levels of hSesn1 and hSesn2 accessed by RT-PCR, using the contra lateral limb injected with AAV2/1(-) as a control. Viral transduction of muscle with the AAV2/1-hSesn1 and AAV2/1-hSesn2 led to a stable expression of both Sestrin isoforms as soon as 6 days and at least for 28 days post-infection (Figure 13B). Furthermore, infection with an AAV2/1 expressing GFP allowed us to confirm that this method achieves a homogenous transduction of most myofibers (Figure 13C).

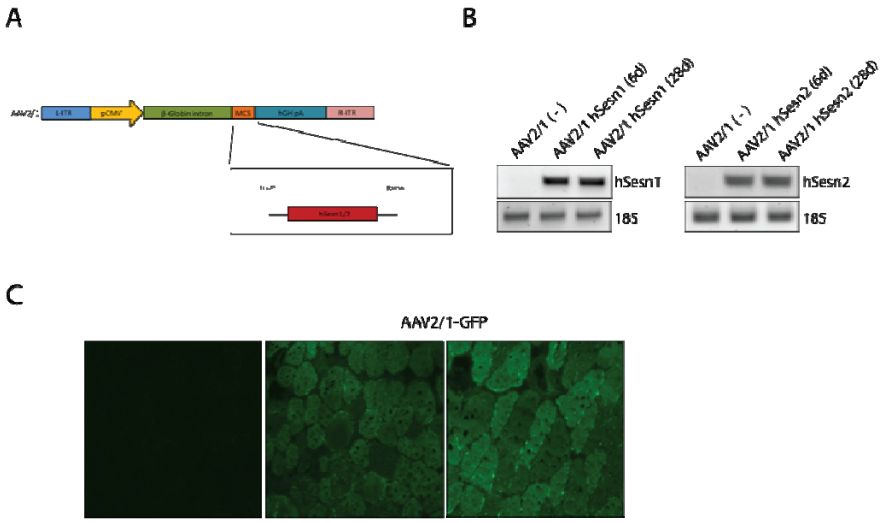


Figure 13. AAV1/2-hSesn1/2 transduction in the murine skeletal muscle. **(A)** Schematic representation of the AAV1/2 constructs generated through the insertion of the cDNA sequence of hSesn1/2. **(B)** RT-PCR analysis of hSesn1 and hSesn2 expression in TA muscle transduced with AAV2/1-hSesn1 and AAV2/1-hSesn2, six and twenty-eight days after injection. **(C)** GFP fluorescence detected in cryosections of TA muscle transduced with AAV2/1-GFP six days after injection.

hSesn1/2 over-expression induces hypertrophy and prevents immobilization-induced atrophy

To directly examine the growth promoting function of Sestrins in intact muscle (in the absence of additional hypertrophic stimuli), we over-expressed hSesn1/2 in TA muscles of 12 week-old mice via AAV2/1 delivery.

Comparison of individual myofiber size between AAV2/1-hSesn1/2 and AAV (-) transduced TA muscles revealed that

Sestrin over-expression induced a significant myofiber growth 28 days after infection, as indicated by H/E staining and average fiber size (Figure 14A). Frequency distribution of myofiber size also shows an over-representation of very large myofibers (CSA>5000 μm^2) in TA muscle over-expressing hSesn1/2 (Figure 14B).

Since Sestrin expression is decreased in atrophying muscle (Figure 12C) we examined whether exogenous delivery of Sestrin will preserve myofiber size in immobilized muscle. For that purpose the right limb was immobilized for one week following an established procedure, resulting in a significant reduction in myofiber size. We found that over-expression of hSesn1 via AAV delivery partially prevented the immobilization-induced atrophy. (Figure 14C)

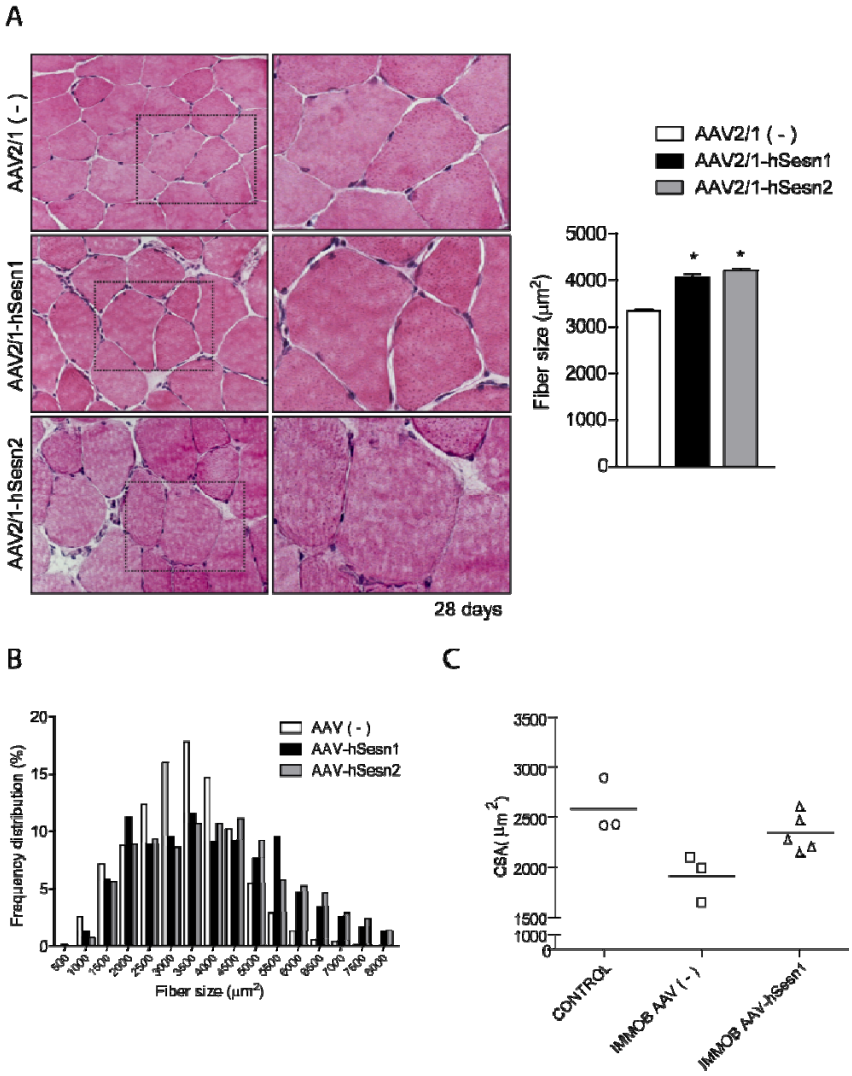


Figure 14. hSesn1/2 overexpression induces hypertrophy. (A) H/E staining of transverse sections (left) and fiber size evaluated by CSA (right) of TA muscle transduced with AAV2/1 (-) or AAV2/1-hSesn1/2, twenty-eight days after injection. **(B)** Frequency distribution of myofiber size in muscle transduced with AAV2/1 (-) or AAV2/1-hSesn1/2. **(C)** Fiber size evaluated by CSA of TA muscle transduced with AAV2/1 (-) or AAV2/1-hSesn1, extracted from mice subjected to a seven day immobilization protocol, three days after injection. Data are mean ± SEM of at least three experiments, *P<0.05.

AKT is activated in response hSesn1/2 over-expression

Since IGF1/PI3K/AKT is one of the most relevant signaling pathways involved in the regulation of muscle mass, we asked whether the main components of this signaling cascade were altered during hSesn1/2 induced muscle growth. Indeed, analysis of protein extracts from muscles infected with AAV2/1-hSesn1/2 or AAV2/1 (-) showed a significant increase of phospho-AKT levels 28 days after infection (Figure 15A). Likewise, overexpression of hSesn2 resulted in increased levels of phospho-AKT (Figure 15B), indicating the activation of the pathway in response to increased levels of Sestrin. Interestingly, although no changes in fiber size were found at 6 days, the activation of AKT was already detected at this time (Figure 15C).

Physiological muscle growth is regulated by the balance between protein synthesis and degradation. AKT promotes protein synthesis through the direct phosphorylation and consequent inhibition of the glycogen synthase kinase 3b (GSK3β). In addition, AKT prevents protein degradation by phosphorylating and repressing the function of the transcription factors of the FoxO family, required for the transcriptional regulation of two muscle-specific ubiquitin ligases, muscle ring finger1 (MuRF1) and muscle atrophy F-box (MAFbx). Consistently, the levels of phospho-GSK3β and phospho-FoxO1/3a were significantly increased in muscles transduced with AAV2/1-hSesn1 in respect to control

infections (Figure 15A), suggesting a possible involvement of these two branches of the AKT pathway in Sestrin-induced muscle growth.

A third component downstream of AKT activation is the mTOR signaling pathway, associated with the positive regulation of protein synthesis. Interestingly, our data suggest that mTORC1 may not be implicated in Sestrin-induced muscle growth, based on the lack of induction (or even downregulation) of phospho-S6 in muscles infected with AAV2/1-hSesn1 (Figure 15D). Sestrin function in drosophila has been previously associated with mTOR inhibition through AMPK dependent mechanisms. Using anti phospho-AMPK antibody as an indirect way to examine activated AMPK, we did not observe a significant increase in the activation of this molecule when hSesn1 was over-expressed in intact muscle (Figure 15E).

Since AKT activity is the major hypertrophic pathway activated during physiological muscle growth (i.e. exercise or compensatory hypertrophy) these results suggest that Sestrin(s) is a novel regulator of this growth-promoting signaling cascade, acting through downstream pathways independent of mTORC1. In addition, this Sestrin growth-promoting effect might proceed independently of AMPK activation.

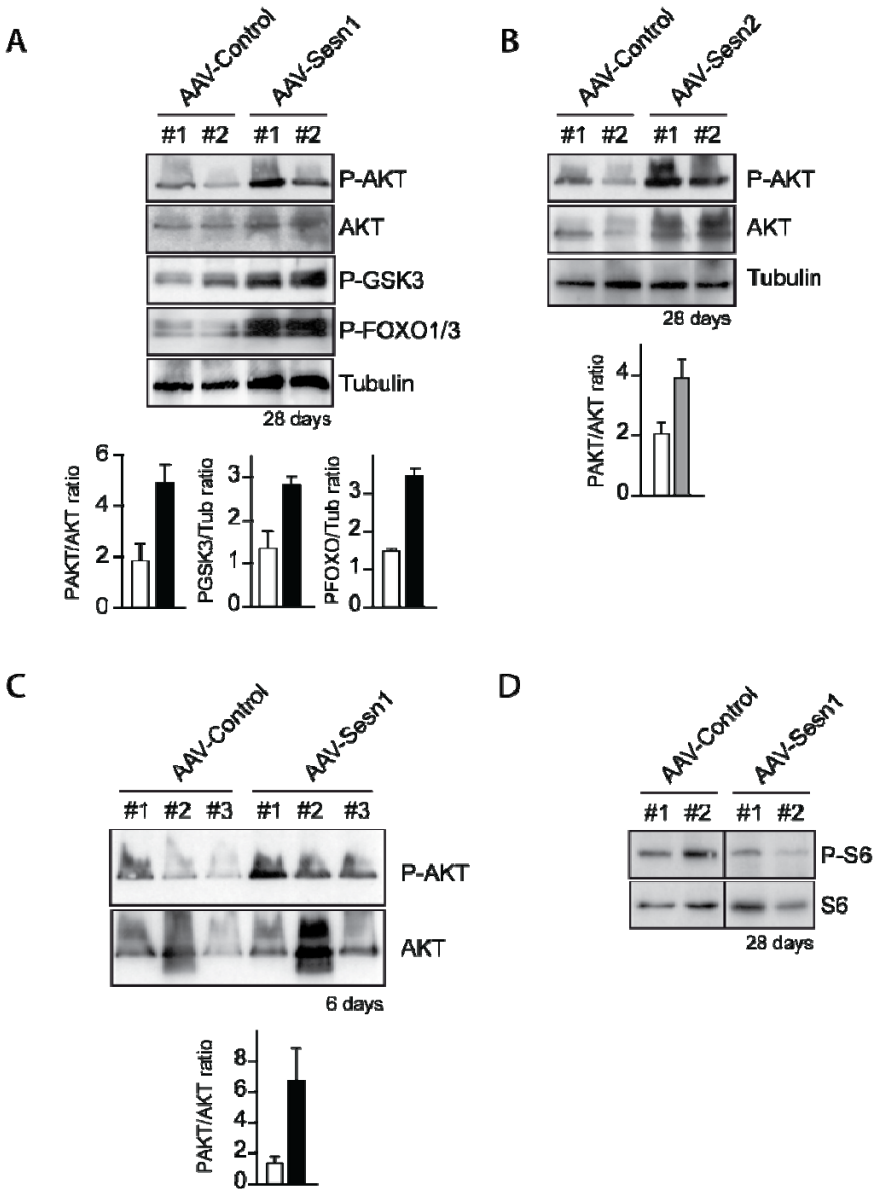


Figure 15. AKT is activated in response hSesn1/2 over-expression. Phospho-AKT (A, C and D), Phospho-GSK3 (A), Phospho-Foxo1/3a (A), Pospho-S6 (B) and Phospho-AMPK (E) levels evaluated by western blot and normalized to the levels of the corresponding loading controls (total AKT, Tubulin for Phospho-GSK3 and Phospho-Foxo1/3a, total S6 and total AMPK) twenty-eight days (A, B, C and E) or six days (D) after AAV1/2-hSesn1(A, B, D and E) or AAV1/2-hSesn2 (C) infection

hSesn1/2 promotes the expression of genes typically up-regulated during exercise

Physiological muscle growth occurs in response to exercise and it involves the up-regulation of several metabolic and mitochondrial biogenesis associated genes. Interestingly, after hSesn1 overexpression, the levels of Glucose transporter type 4 (Glut4) and Hexokinase II (HKII), two major components of skeletal muscle carbohydrate metabolism that have been found also up-regulated in response to exercise (Kraniou et al.; O'Doherty et al.), were significantly increased. In addition, the mitochondrial uncoupling protein UCP3 and Cytochrome C (CytC) were induced after 28 days of hSesn1over-expression (Figure 16), similarly to what was previously reported for mice submitted to bouts of intensive exercise (Beyer et al.; Tsuboyama-Kasaoka et al.). Together, this data further supports the idea that Sestrins promote growth associated pathways in the skeletal muscle.

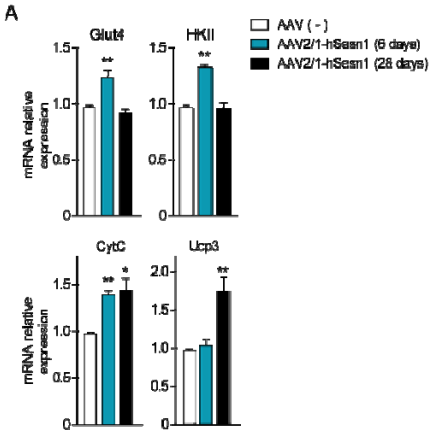


Figure 16. hSesn1/2 promote the expression of genes typically up-regulated during exercise-induced hypertrophy RT-qPCR analysis of Glut4, HKII, CytC and UCP3 in the TA muscle transduced with AAV2/1-hSesn1 six and twenty-eight days after injection. Data are mean ± SEM of at least three experiments, *P<0.05.

Discussion

Aging is characterized by a progressive decline in body function and physiology at the level of several organs and it is associated with a number of degenerative diseases, Alzheimer, cancer and other pathological conditions. There are some pieces of evidence that aging is accompanied by a decline in the homeostatic and regenerative capacity of all tissues and organs (Kirkwood, 2005; Rando, 2006), characterized by slower wound healing in the skin, decreased skeletal muscle mass and strength, skewed ratio of cellular constituents in the blood and a decline in neurogenesis (Sharpless and DePinho, 2007). The homeostatic and regenerative activities of these tissues rely on the resident stem cells and it is thought that age-associated changes in tissue structure and function result, to a great extent, from a decline in stem cell function (Bell and Van Zant, 2004; Dorshkind et al., 2009; Jones and Rando, 2011). Aged stem cells loss of functionality can emerge as either a defect in self-renewal ability that results in a depletion of the stem cell pool with age, or as a loss of activation/differentiation potential, which results in impairment in the generation of the appropriate progeny (Liu and Rando, 2011).

The age-associated wasting of the skeletal muscle is accompanied by a decline in regenerative ability. In this work, we have analyzed muscle stem cells (satellite cells) isolated from physiologically aged geriatric WT mice and Bmi1-deficient mice (displaying anticipated aging traits) and

identified p16INK4a as a common molecular marker associated with defects in satellite cell proliferation and self-renewal. In addition, we have identified a number of overlapping biological processes dysregulated in aged and Bmi1-deficient satellite cells, suggesting that Bmi1-dependent epigenetic regulation may underlie many of the intrinsic changes taking place in chronologically aged satellite cells, particularly in those from very old, geriatric mice. Finally, we have uncovered a regulated expression of Sestrins in mouse models of skeletal muscle atrophy and hypertrophy, and in human myopathies, and identified a novel function for Sestrins as skeletal muscle growth promoting factors, acting through the IGF1/PI3K/AKT pathway.

Aging and the regulation of satellite cell function and regenerative ability

In aged skeletal muscles, the impairment in the regenerative response correlates with a decrease in satellite cell functionality and numbers (Schultz and Lipton, 1982; Shefer et al., 2006). This age-related decline in muscle stem cell function could be due to an intrinsic aging of the satellite cell, to an alteration of the extrinsic signals in the aged niche/environment or to a combination of the two. Among the cell-intrinsic changes mediating the age-related decline in

satellite cell function are alterations at the level of the genome and epigenome, which converge in specific patterns of gene expression regulation. Here, we have, for the first time, characterized the transcriptome of physiologically aged satellite cells from sarcopenic muscle_and described the main changes in gene expression associated with the aging process of muscle stem cells. Our study revealed many differences in the transcriptional patterns between young and old satellite cells, providing evidence that stem cell intrinsic changes do in fact take place during aging and may account for the associated loss of functionality. Gene Ontology (GO) and Ingenuity core Pathway (IPA) analysis are commonly used to extract relevant information from microarray data, revealing the most relevant processes and pathways associated with the gene expression changes between two conditions and we have used them here to identify the biological processes and signaling pathways intrinsically affected in aged muscle stem cells.

The GO database has a hierarchical nature such that genes map onto one or more GO functional categories, which may be interrelated. This may result in the representation of one gene in more than one biological process, reflecting the complexity of the biological systems (Harris et al., 2004). IPA core analysis delivers a rapid assessment of the signaling and metabolic pathways, molecular networks, and biological processes that are most significantly perturbed in the dataset of interest (IPA, Ingenuity Systems, Mountain

View, CA). Our analysis confirmed, at the gene expression level, that cell cycle progression is compromised in aged satellite cells. Genes associated with the positive regulation of cell proliferation, DNA synthesis and ribosomal biogenesis were found down-regulated, while genes involved in the apoptotic pathway and in the irreversible exit of cell cycle were found up-regulated. These changes are in line with studies indicating that satellite cells from aged muscles showed a delayed response to activation cues and an initially reduced proliferative expansion in vitro (Schultz and Lipton, 1982; Shefer et al., 2006). Satellite cells also seem to be more prone to undergo senescence or apoptosis with aging (Jejurikar et al., 2006).

Among the cell-cycle associated genes up-regulated in aged satellite cells, we found p16INK4a, which has been characterized in other tissues as a robust biomarker and possible effector of cellular aging (Krishnamurthy et al., 2004). Given that the expression of p16INK4a significantly accumulates with normal aging in a variety of tissues in rodents and humans, and that p16INK4a and ARF appear to be among the main mediators of stem cell aging in vivo (Janzen et al., 2006), we sought to further explore the effects of p16INK4a expression in satellite cells.

Epigenetic transcriptional regulators of the INK4-ARF locus play a crucial role in cellular senescence. Polycomb Group (PcG) proteins are direct regulators of p16INK4a expression (Bracken et al., 2007) and loss of Bmi1-dependent

repression of p16INK4a has been consistently associated with cell proliferation impairment and senescence (Jacobs et al., 1999). But whether the Bmi1/p16INK4a axis is operative in aging satellite cells remained uninvestigated. Since p16INK4a is not expressed in young/adult satellite cells, we used Bmi1-loss-of function as an approach to anticipate p16INK4a expression in muscle stem cells.

Bmi1 deficiency had no effect on the embryonic and early postnatal development of skeletal muscle. These results are in line with other studies that have suggested the partial redundancy and functional overlap of certain PcG proteins in early embryonic stages, in particular between Bmi1 and Mel18 (Akasaka et al., 2001). However, in the young/adult muscle we observed a significant reduction in myofiber size accompanied by a decreased number of satellite cells and an impaired regenerative capacity, suggesting that Bmi1 function is essential for post-natal muscle homeostasis. The phenotype of Bmi1-deficient mice resembles that of physiologically aged mice, characterized by an atrophic skeletal muscle with a reduced number of resident satellite cells.

Many of the affected pathways identified by IPA analysis in Bmi1-deficient satellite cells are associated with cellular senescence. Cellular senescence is one of the biological consequences of aging and likely simultaneously one of the cellular programs responsible for many of the key features of the aging process. p16INK4a is a key regulator of both

oncogene-induced and aging-induced cell senescence (Gil and Peters, 2006) and it is thought that it may have a pivotal role in promoting aging by limiting stem cell proliferation and self-renewal in several tissues. An increase in p16INK4a expression with age has been shown to contribute to age-dependent decline in pancreatic islet regenerative capacity (Krishnamurthy et al., 2006), decreases in forebrain progenitors and neurogenesis (Molofsky et al., 2006) and hematopoietic stem cell repopulation defects (Janzen et al., 2006). We found that Bmi1 loss anticipated p16INK4a expression in young/adult satellite cells, which otherwise is exclusively expressed in aged geriatric muscle tissue. More interestingly, similarities between Bmi1-deficient and aged skeletal muscle were also found at the level of satellite cell functionality. As previously suggested for aged satellite cells, we found that Bmi1-deficient satellite cells proliferate at a lower rate and fail to self-renew, resulting in a depletion of the muscle stem cell population. It is likely that the up-regulation of p16INK4a is, at least in part, responsible for the self-renewal defects in Bmi1-/- and aged satellite cells. Thus, it is tempting to propose that p16INK4a up-regulation, by promoting cell senescence, may accelerate the satellite cell decline in sarcopenic muscle.

Most of the signaling pathways in senescence converge on the activation of the tumor suppressors p53 and the retinoblastoma protein (pRb). The INK4-ARF locus plays a crucial regulatory role in the status of both proteins. p19ARF

prevents p53 degradation by interacting with MDM2, the p53-associated E3 ubiquitin-ligase (Zhang et al., 1998). In parallel, p16INK4a engages the pRb pathway by inhibiting cyclin D-dependent kinases that would otherwise phosphorylate and inactivate pRb (Serrano et al., 1993). In a hypophosphorylated active state, pRb prevents E2F and histone acetyltransferases (HATs) to open chromatin structure and trans-activate E2F-responsive genes important for G1 to S phase transition (Chicas et al., 2010).

The p53 pathway is important to establish and maintain growth arrest during senescence and, in MEFs, the loss p19ARF-p53 axis leads to spontaneous escape from senescence (Kamijo et al., 1997). p21CIP expression is induced by p53 and can also inhibit cyclin-dependent kinases upstream of pRb (El-Deiry et al., 1993; Harper et al., 1993). Indeed, our results indicate that both p19ARF and p21CIP are up-regulated in aged satellite cells from sarcopenic muscle and IPA analysis of the mRNA data of Bmi1-deficient satellite cells points to p53 signaling as one of the pathways most significantly affected.

It is still unclear what determines whether a cell enters a transient/reversible (quiescence) or permanent/irreversible (senescence) cell-cycle arrest. This is a critical question since maintenance of the stem cell pool in a given tissue (i.e. muscle) throughout adulthood will require reversibility of the quiescence state, while this process would be dysregulated at advanced ages because cells might engage an irreversible

senescence pathway (Figure 17). Although many factors may be involved in this decision some studies have implied that pRb is crucial to sustain the senescence signal and regulate secondary events that lock the arrest into an irreversible state (Narita et al., 2003). A distinct heterochromatic structure designated senescence-associated heterochromatic foci (SAHF) was described in senescent human fibroblasts and p16INK4a was shown to have a causative role in SAHF formation. Importantly, these heterochromatic foci are not present in quiescent cells (Narita et al., 2006; Narita et al., 2003). Interestingly, it was also shown that pRb family protein members assume distinct roles in reversible and irreversible cell cycle exit. p107 and p130 are the predominant pRb members bound to E2F responsive promoters in quiescent cells, while pRb protein is recruited in senescent cells (Rayman et al., 2002; Takahashi et al., 2000). It would be interesting to explore whether a predominance of p107/p130 is found in young/adult quiescent satellite cells versus the expected pRb prevalence in aged/Bmi1-null satellite cells with increased p16INK4a expression.

One other hypothesis worth considering is that p53 could have a role in determining the decision between cell senescence and quiescence based upon the relative levels of this molecule and its action on mTOR. Blagosklonny et al. demonstrated that the same p53-activating treatment can induce senescence or quiescence depending on the strength

of the p53 response. Moderate doses of the p53-stabilizing compound nutlin induce a moderate p53 response that does not inhibit mTOR and, therefore, results in senescence. In contrast, high doses of the same compound produce a strong p53 response, inhibiting mTOR and resulting in quiescence (Leontieva et al., 2010). Given that Sestrins are p53 targets and their function has been involved in the negative regulation of mTOR (Budanov and Karin, 2008), it would be interesting to further explore if Sestrins could have a role in this decision point. Results from our lab indicate that Sestrins are in fact up-regulated in quiescent satellite cells (Laura García-Prat, unpublished data), suggesting a possible role for Sestrins in providing a link between p53 activation and mTOR inhibition, which is essential to establish a state of reversible cell-cycle exit.

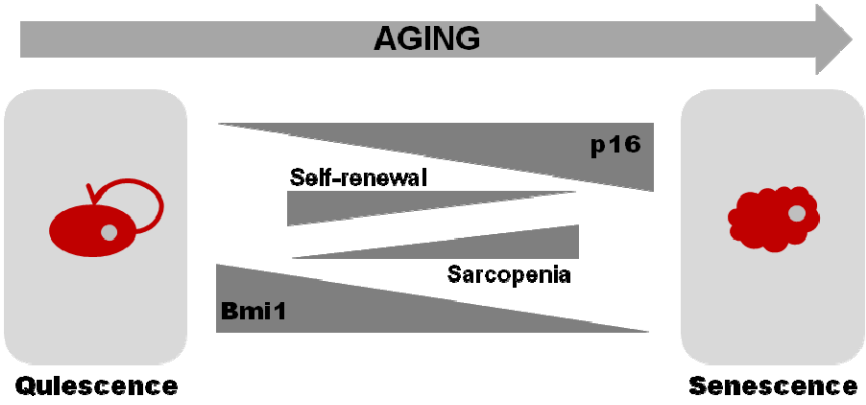


Figure 17: Proposed model for the role of p16INK4a/Bmi1 axis regulating the transition from quiescence to senescence in aging.

Another feature associated with muscle aging is an increased tendency of old satellite cells to enter alternative differentiation programs by adopting adipogenic and fibroblastic fates (Asakura et al., 2001; Brack et al., 2007; Shefer et al., 2004). Interestingly, we found several genes commonly associated with adipogenic and fibrogenic lineages up-regulated in aged as well as in Bmi1-/- satellite cells, further supporting the similarities in the phenotype of aged and Bmi1-dificient muscle stem cells.

The parallel up-regulation of p16INK4a and impairment of proliferation and self-renewal in aged and Bmi1-/- satellite cells suggests that epigenetic regulation of the INK4a locus by polycomb proteins could be at least part of the molecular mechanism underlying the aging-induced senescent-like phenotype of satellite cells in skeletal muscle. Furthermore, it suggests that cell intrinsic alterations driven by gene expression regulatory mechanism are indeed sufficient to alter satellite cell functionality, particularly in geriatric muscle (Figure 18). In addition to our in vitro and ex vivo studies, we performed in vivo regeneration assays, using a heterochimeric grafting model, trying to further support this idea. Transplanting a Bmi1-/- EDL muscle onto the TA of immunocompromised Bmi1+/+ mice allowed us to confirm that the defective/delayed regenerative response in the absence of Bmi1 is caused by dysregulation of satellite cell intrinsic factors rather than by the influence of other cell types involved in muscle regeneration.

At variance with our results, elegant studies from Matheu et al. using transgenic mice with increased INK4/ARF gene dosage have a global anti-aging effect and extended median longevity. The authors suggest that the INK4/ARF locus might favor quiescence and prevent unnecessary proliferation. We believe that these results may apply to tissues with continuous proliferation, which will be protected by excess p16INK4a. However, skeletal muscle is a tissue with very little turnover is resting conditions and its stem cells remain relatively quiescent throughout life. Hence, we hypothesis that no p16INK4a is required to promote satellite cell quiescence. It will be interesting to study in the INK4/ARF-tg/tg the satellite cell behaviour in aging mice. Although further experiments need to be performed we favor the hypothesis that in the skeletal muscle compartment an excess of p16INK4a will provoke a transition of the satellite cell from a quiescent to a senescent state. In line with this, results from studies using mutant mouse strains that devolop age-related phenotypes at an early age, such as the BubR1 hypomorphic mice, suggest that p16INK4a promotes aging only in certain tissues, since deleting p16INK4a results in the attenuation of both cellular senescence and premature ageing in these tissues.

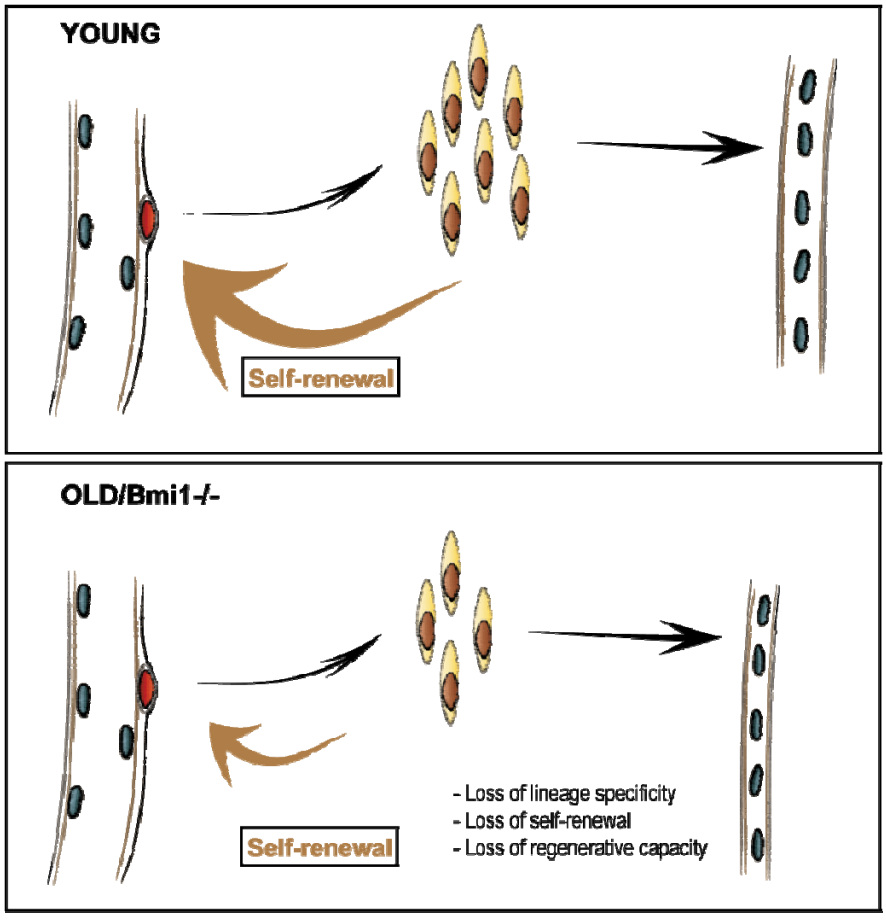


Figure 18: Satellite cell intrinsic changes in young and old/Bmi1-/- skeletal muscle.

Aging and the regulation of skeletal muscle growth and atrophy

One feature of the Bmi1-deficient muscle is the atrophic/sarcopenic phenotype observed at the level of the adult myofiber, which resembles that found in aged skeletal muscle. Adult muscle growth and atrophy processes are mostly dependent on the fine-tuned regulation of the IGF1/PI3K/AKT pathway, which ensures the equilibrium of skeletal muscle homeostasis (Schiaffino and Mammucari). Our results suggest that components of the IGF1/PI3K/AKT might be altered in Bmi1-/- skeletal muscle. Atrogin-1 (MAFbx), a gene usually found up-regulated in different models of muscle atrophy and responsible for muscle wasting by increasing protein degradation through the ubiquitin-proteasome system (Gomes et al., 2001), is also found up-regulated in Bmi1-deficient skeletal muscle and may be partially responsible for the observed phenotype. Since atrogin-1 is a direct target FoxO transcription factors (Sandri et al., 2004), it is likely that FoxO up-regulation in Bmi1-deficient mice is, at least in part, responsible for the induction of atrogin-1. Furthermore, we find Myostatin expression up-regulated in Bmi1-deficient skeletal muscle. Myostatin is a negative regulator of muscle mass acting through the activin receptor IIB (ActRIIB) on Smad2 and Smad3 and capable of activating FoxO1 (McFarlane et al.,

2006). This suggests that, in addition to an increase in FoxO expression, there may also be a Mstn-dependent increase in FoxO activation which can contribute to the induction of Atrogin-1 and the appearance of atrophy-like phenotypes.

Our analysis revealed an interesting overlap between gene expression changes in Bmi1-deficient (reported here) and aged skeletal muscle tissue (Edwards et al., 2007). Stress-response genes such as GADD45a (Growth arrest and DNA-damage-inducible, alpha) and Ddit4 (DNA-damage-inducible transcript 4, also known as REDD1, Regulated in Development and DNA Damage responses) were among the commonly affected genes. REDD1 has been previously described as a hypoxia-induced inhibitor of mTOR (Vadysirisack and Ellisen) also associated with skeletal muscle atrophy (Hulmi et al., 2011). GADD45 function has been previously associated with cell cycle arrest, DNA repair, cell survival, senescence and apoptosis (Liebermann et al.).

Given that skeletal muscle is largely a post-mitotic tissue, we were surprised to find that a large number of transcripts involved in the regulation of the cell cycle (such as p16INK4a) were differentially expressed in WT and Bmi1 null muscles. In general, transcripts involved in the positive regulation of cell cycle progression were down-regulated, while genes involved in the negative regulation of cell proliferation were found up-regulated. Although intriguing, we have found such alterations in skeletal muscle of geriatric mice, in agreement with two previous reports (Edwards et al.,

2007) (Baker et al.), further supporting the idea that Bmi1-deficient skeletal muscle phenotype resembles that of aging.

A general feature of aged tissues is oxidative stress and accumulation of oxidized and modified proteins and protein aggregates, which can be the driving forces behind age associated diseases. Sarcopenia, the progressive loss of muscle mass with age, is characterized by a decrease in the capacity to respond to insulin and maintain glucose homeostasis (Gupte et al., 2008) and increased muscle ROS and oxidative damage (Mecocci et al., 1999). Considering the age-related increase in oxidative stress in skeletal muscle, it is conceivable that a stress response is activated during aging to impact this process.

In drosophila, the inactivation of dSesn (the only Sesn gene) leads to accelerated tissue aging, characterized by accumulation of triglycerides, muscle degeneration, cardiac malfunction and oxidative stress (Lee et al., 2010b). In agreement, we find that human patients with pathologies characterized by progressive muscle weakness and decreased muscle mass exhibit reduced levels of Sestrins, which might reflect the anticipation of muscle wasting in the absence of protective factors.

Interestingly, we found Sestrin1 up-regulated in muscle of Bmi1-/- mice, similarly to what had been previously reported for aged mice (Edwards et al., 2007). Although in apparent contradiction with the association of Sestrin loss with muscle

wasting, it is conceivable to think that, in the course of the normal aging process, the sarcopenic skeletal muscle initiates a stress response aimed at counteracting the progression of muscle wasting. In this case, Sestrin up-regulation is part of a stress response and not the underlying cause of the sarcopenic phenotype. This hypothesis is in line with previous suggestions regarding Sestrins function in aging. As proposed by Lee JH et al (2010), in addition to a natural protective role under normal unstressed conditions, evidenced by the accelerated aging phenotypes in dSesn null flies, Sestrins may also act as suppressors of aging that are responsive to stressful stimuli. In this regard, Sestrins may reprogram cells to adapt to stressful conditions by attenuating anabolism and enhancing autophagic catabolism, through the activation of AMPK and inhibition of mTOR (Budanov et al., 2010; Lee et al., 2010a)

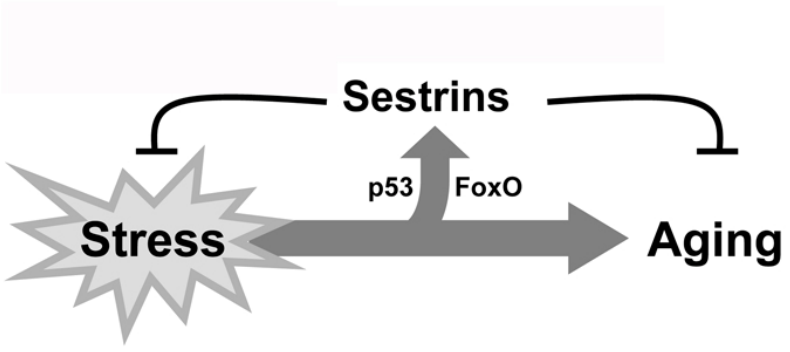


Figure 19: Sestrins function as stress-induced proteins in aging

Thus, in the skeletal muscle, Sestrins may also function as stress-induced proteins acting to counteract muscle degeneration in aging and disease, opening the questions on how Sestrins' expression is regulated under these conditions and which mechanisms underlie Sestrin function in preventing muscle degeneration and/or promoting muscle growth.

p53 is known to activate anti-oxidant responses and Sesn1/Sesn2 are regulated by p53 in response to DNA-damaging agents. Gene expression profiling of aged skeletal muscle revealed an induction in the p53-mediated transcriptional program and identified Sesn1 among the up-regulated target genes (Edwards et al., 2007). Sesn1 and Sesn3 are also transcriptionally regulated by the FoxO family of transcription factors (Nogueira et al., 2008). We find Sesn1 up-regulated in Bmi1-deficient skeletal muscle, which might be a consequence of the increased FoxO expression and activity. Moreover, in silico analysis of Sestrins promoter regions reveals the presence of FoxO binding sites, suggesting a possible direct regulation. FoxO-induced Sestrin expression was proposed to mediate their function in the inhibition of ROS, as part of a feedback loop mechanism that involves the activation of AMPK, inhibition of mTOR and finally an increase in autophagy in drosophila. Our results propose yet another possible mechanism by which Sestrins might engage a feedback loop with FoxO proteins in mice. We show here that over-expression of hSesn1 in the

mammalian skeletal muscle activates AKT and increases the level of the inhibitory phosphorylated form of FoxO1 and FoxO3, independently of AMPK activation. This raises the possibility of a negative feedback loop mechanism whereby FoxOs induce Sestrin expression and the high level of Sestrin inhibits FoxO activity, through the activation of AKT. Uncoupling the antioxidant and metabolic functions of Sestrin during aging will be the goal of future studies.

Our results describe for the first time a novel function of Sestrin proteins as promoters of adult skeletal muscle growth. This is evidenced by the induction of myofiber growth upon Sesn1/2 over-expression in the skeletal muscle. In addition, we find changes in gene expression in Sestrin transcripts under conditions of rapidly induced muscle atrophy (in which Sestrins are down-regulated) and physiological growth (in which Sestrins are up-regulated). The growth promoting function of Sestrins seems to proceed through a different molecular mechanism than the one described for the dSesn function as a feedback inhibitor of TOR that prevents age-related pathologies in drosophila. Lee et al (2010) described an induction of dSesn in response to chronic TOR activation and the consequent accumulation of ROS that cause activation of JNK and FoxO. Once induced, dSesn activates AMPK and inhibits TOR, allowing for autophagic clearance of damaged mitochondria and protein aggregates (Lee et al., 2010b). Our results suggest that

Sestrins exert a growth promoting action via activation of AKT using downstream pathways independent of mTORC1, such as the inhibition of the glycogen synthase kinase 3β (GSK3β) and the inhibitory phosphorylation of FoxO family transcription factors. In addition, this Sestrin growth promoting effect might proceed independently of AMPK activation.

Muscle growth occurs in physiological conditions such as the response to exercise and we show here that Sestrin expression is up-regulated during exercise and that several metabolic and mitochondrial biogenesis associated genes, usually induced in the skeletal muscle by exercise (Beyer et al., 1984; Kraniou et al.; O'Doherty et al.; Tsuboyama-Kasaoka et al., 1998) are up-regulated upon Sesn1/2 over-expression. Physical activity can be a valuable countermeasure to sarcopenia in its treatment and prevention (Pillard et al., 2011). Although the molecular mechanisms associated with skeletal muscle hypertrophy are aging sensitive (Haddad and Adams), it would be interesting to explore whether Sestrins may also be used therapeutically to counteract the effect of sarcopenia.

Conclusions

Conclusions

Conclusions

1. Cell intrinsic alterations driven by epigenetic regulatory mechanisms are sufficient to alter satellite cell functionality during aging.

2. p16INK4a expression in satellite cells from physiologically aged, sarcopenic mice and from Bmi1-deficient mice correlates with defects in satellite cell number, proliferation and self renewal capacity, suggesting a link between p16INK4A-mediated cell senescence and satellite cell decline in sarcopenic muscle.

3. Sestrins expression is regulated during physiological skeletal muscle growth and atrophy in mice and humans.

4. Sestrins are novel regulators of adult skeletal muscle growth as evidenced by their capacity to directly increase muscle mass.

5. Sestrins act through the IGF1/PI3K/AKT pathway.

Conclusions

References

References

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References

Appendix

Appendix

Appendix

In the course of my PhD Thesis I have collaborated in other projects from the laboratory which resulted in several publications listed below:

p38/MKP-1-regulated AKT coordinates macrophage transitions and resolution of inflammation during tissue repair.

Perdiguero E, **Sousa-Victor P**, Ruiz-Bonilla V, Jardí M, Caelles C, Serrano AL, Muñoz-Cánoves P.

J Cell Biol. 2011 Oct 17;195(2):307-22.

Regulation of skeletal muscle stem cells through epigenetic mechanisms.

Sousa-Victor P, Muñoz-Cánoves P, Perdiguero E.

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Epigenetic regulation of myogenesis.

Perdiguero E, **Sousa-Victor P**, Ballestar E, Muñoz-Cánoves P.

Epigenetics. 2009; 4(8):541-50

Efficient adult skeletal muscle regeneration in mice deficient in p38beta, p38gamma and p38delta MAP kinases.

Ruiz-Bonilla V, Perdiguero E, Gresh L, Serrano AL, Zamora M, **Sousa-Victor P**, Jardí M, Wagner EF, Muñoz-Cánoves P.

Cell Cycle. 2008; 7(14):2208-14

Genetic analysis of p38 MAP kinases in myogenesis: fundamental role of p38alpha in abrogating myoblast proliferation.

Perdiguero E, Ruiz-Bonilla V, Gresh L, Hui L, Ballestar E, **Sousa-Victor P**, Baeza-Raja B, Jardí M, Bosch-Comas A, Esteller M, Caelles C, Serrano AL, Wagner EF, Muñoz-Cánoves P.

EMBO J. 2007; 26(5):1245-56