Regulation of cell proliferation and differentiation by p38 MAPK in distinct physiopathological processes

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A mi familia y amigos,

pero sobre todo a mi hermanito.

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SUMMARY

SUMMARY

The p38 mitogen activated protein kinase (MAPK) has a crucial role in cells' stress adaptation and survival. It also plays a role in physiological processes such as differentiation of skeletal muscle or in pathologies such as cancer.

In response to environmental stresses, a delay in cell cycle progression is required for cell adaptation. In the present study, we demonstrate that p38 MAPK maximizes cell survival upon stress by downregulating the E2F transcriptional program through the direct targeting of Retinoblastoma (RB) protein, a key regulator of the G1/S transition. p38 phosphorylation of RB at specific sites in the N terminus increases its affinity towards E2F, represses E2F-driven gene expression, and delays cell-cycle progression. These phosphorylation events render RB insensitive to CDK regulation, converting it into a strong suppressor of cell proliferation. Remarkably, the expression of a phosphomimetic RB mutant in cancer cell lines reduces colony formation and decreases their proliferative and tumorigenic potential in a mouse xenograft model.

The p38 MAPK signaling pathway is also a major regulator of myogenesis (the process whereby quiescent muscle stem cells -satellite cells- expand and differentiate to form muscle fibers), by inducing cell cycle withdrawal and the expression of the muscle-specific transcriptional program. RB is also a regulator of the myogenic differentiation process. We explored the possible role of p38 in muscle cell differentiation using normal myoblasts and

rhabdomyosarcoma (RMS) cells (which are defective in p38 MAPK activation), via RB phosphorylation. Our results strongly suggest that, despite the important role of p38 for differentiation of both myogenic models, this function does not depend on p38-phosphorylated RB.

p38 MAPK has also been associated to aging of muscle stem cells. Aged satellite cells have a cell-autonomous increase in the activity of p38, thus contributing to defective control of quiescence, expansion and self-renewal capacities. In this study we show that p38α-deficient aged satellite cells display an increased proliferative capacity and reduced levels of senescence-associated markers. Moreover, we demonstrate that attenuation of p38 signaling enhanced the regenerative capacity of aged satellite cells. These findings have potential biomedical implications for regeneration of old skeletal muscle.

Keywords: p38 MAPK, RB, stress, cell cycle, proliferation, skeletal muscle differentiation, muscle stem cells, satellite cells, aging, muscle regeneration, senescence.

RESUMEN

p38 MAPK tiene un papel fundamental en la adaptación al estrés y la supervivencia celular. Además, también regula otros procesos fisiológicos como la diferenciación del músculo esquelético o patologías como el cáncer.

En respuesta a estrés, la célula requiere un retraso en la progresión del ciclo celular para adaptarse. En el presente estudio, demostramos que p38 MAPK maximiza la supervivencia celular en repuesta a estrés mediante la regulación negativa del programa E2F actuando directamente sobre la proteína Retinoblastoma (RB), un regulador clave de la transición G1/S. p38 fosforila a RB en sitios específicos en el extremo N terminal, aumenta su afinidad hacia E2F, reprime la expresión génica dirigida por E2F y retrasa la progresión del ciclo celular. Estas fosforilaciones hacen que RB sea insensible a la regulación por CDKs, convirtiéndolo en un fuerte supresor de la proliferación celular. Sorprendentemente, la expresión de un mutante RB fosfomimético en líneas celulares de cáncer reduce la formación de colonias y disminuye su potencial proliferativo y tumorogénico en un modelo de xenoinjerto en ratón.

La vía de señalización de p38 MAPK es también un importante regulador de la miogénesis (el proceso por el cual las células madre musculares -células satélite- en quiescencia se expanden y diferencian para formar fibras musculares), mediante la inducción de una parada del ciclo celular y la expresión del programa transcripcional específico del músculo. Hemos estudiado el posible papel de p38 en la diferenciación miogénica, empleando

modelos de mioblastos normales y de células de rhabdomyosarcoma (RMS) (los cuales tienen un defecto en la activación de p38), a través de la fosforilación de RB. Nuestros resultados sugieren que, a pesar de la importancia de p38 en la diferenciación de ambos modelos miogénicos, dicha function no depende de la fosforilación de RB por p38.

p38 MAPK también se ha asociado al envejecimiento de las células madre musculares. Las células satélite envejecidas sufren un aumento en la actividad de p38, lo que contribuye a una deficiente capacidad para mantener la quiescencia, expansión y autorrenovación. En este estudio, mostramos que las células satélite envejecidas deficientes en p38α muestran una capacidad proliferativa aumentada y niveles reducidos de marcadores asociados a la senescencia. Además, demostramos que la atenuación de la señalización por p38 aumenta la capacidad regenerativa de las células satélite envejecidas. Estos hallazgos tienen potenciales implicaciones biomédicas para la regeneración del músculo esquelético durante la vejez.

Palabras clave: p38 MAPK, RB, estrés, ciclo celular, proliferación, diferenciación del músculo esquelético, células madre musculares, células satélite, envejecimiento, regeneración muscular, senescencia.

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I. INTRODUCTION

I. INTRODUCTION

1. Signaling pathways and cell adaptation.

During lifetime, cells have to adapt to different situations in their microenvironment to maintain homeostasis. Eukaryotic cells can sense extracellular stimuli to generate a quick response through signaling cascades in order to survive to a stress situation or, in the case of progenitor cells, to start a process to become a mature differentiated cell. Cell proliferation, differentiation, tissue repair, and even cell death, are often regulated by signaling pathways that respond and control different aspects of cell physiology according to its needs.

Cells interact with molecules from their microenvironment or neighboring cells as ligands binding to cell surface receptors. These ligands include mitogens, hormones, neurotransmitters, cytokines or growth factors, among others, which activate cellular receptors, resulting generally in the activation of second messengers. This activation takes place by enzymatic modifications of the messengers, often by the addition of a phosphate group by kinases (phosphorylation) or the removal of it by phosphatases (dephosphorylation). This signal can be recognized by the next component downstream of the cascade, and ultimately, leads to an intracellular response, such as alteration in the expression of a gene or even the induction or repression of a whole process.

Diverse signaling cascades have been described to orchestrate physiological processes. Among them, mitogen-activated protein kinase (MAPK) signaling pathways are critical in situations of environmental stress, sensing extracellular stimuli that are converted into an intracellular response for cellular adaptation (Cuenda and Rosseau, 2007). These pathways are crucial for cell fate, and their deficient regulation can lead to loss of homeostasis and diseases, thus impairing cell functions, and resulting in the cell's inability to survive or in an abnormal or uncontrolled cell proliferation that can result in cancer (Cuenda and Rosseau, 2007).

1.1. Mitogen-activated protein kinase (MAPK) modules.

MAPK pathways mediate signal transduction from cell surface receptors to downstream effectors (as transcription factors) that lead to cellular responses like cell proliferation, growth, motility, survival and apoptosis, and play a role in physiopathological processes such as cancer, immune disorders and neurodegenerative diseases (Cuenda and Rosseau, 2007).

MAPK pathways are organized in modules (**Figure i1**), and exert their functions through the sequential activation of three distinct layers of kinases: MAPKK kinases (MAPKKK, MAP3K, or MEKK), MAPK kinases (MAPKK, MAP2K, MKK or MEK) and MAPK. MAPKKKs usually get activated by its interaction with small G-proteins or phosphorylation by protein kinases downstream from cell surface receptors (Cuevas et al., 2007). Once activated, they directly phosphorylate MAPKKs, which in turn activate MAPKs by dual phosphorylation of conserved threonine and tyrosine residues

in the TXY motif at the activation loop (Canagarajah et al., 1997; English et al., 1999). The existence of docking sites allows the selective activation of MAPK by MAPKKs, providing them with high signaling specificity (Bardwell and Thorner 1996). Additionally, the association with specific multi-domain scaffolding proteins, which interact simultaneously with several components, contributes to further substrate specificity and regulates its spatio-temporal activation (Meister et al., 2013; Cuenda and Rousseau, 2007).

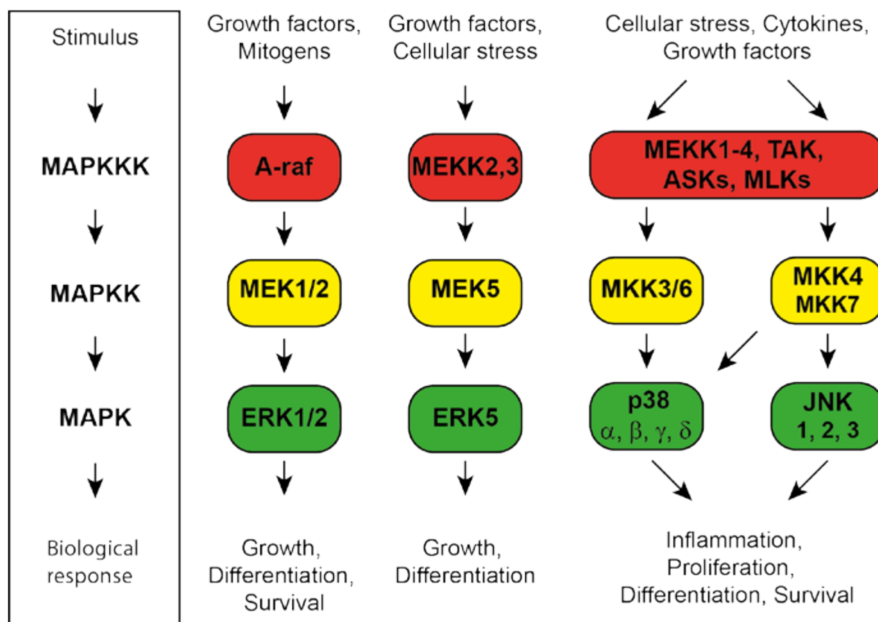

Figure i1: MAPK pathways. ERKs, JNKs, and p38 signaling pathways respond to different types of stimuli and trigger diverse cellular responses. Main signaling molecules are represented.

1.2. MAPK families.

Three main MAPK families have been described in multicellular organisms (Chang and Karin, 2001): the extracellular

signal-regulated kinases (ERK), the jun-amino-terminal kinases (JNK) and p38 kinases (**Figure i1**).

The ERK module is mainly involved in cell growth and differentiation and it can be divided into two main subfamilies: ERK 1/2 and ERK5. The ERK 1/2 pathway is activated by phosphorylation on a TEY motif in its activation segment by subsequent activation or A-raf MAPKKK and MEK1/2 MAPKKs, in response to mitogens and growth factors (McKay and Morrison 2007; Shaul and Seger 2007). In the case of ERK 5, signaling through MEKK2,3 and MEK5 are described to be responsible of their activation, and it takes place upon stimulation by growth factors, but also by cellular stress (Wang and Tournier, 2006).

The JNK stress-activated protein kinase (SAPK) proteins are encoded by three genes, *MAPK8* (which encodes JNK1), *MAPK9* (JNK2) and *MAPK10* (JNK3), which are alternatively spliced to give at least ten isoforms (Gupta et al., 1996). JNK1 and JNK2 are expressed in almost every tissue, whereas JNK3 is mainly found in the brain (Cuevas et al., 2007; Bode and Dong, 2007). JNKs can be activated by the upstream MKK4 and MKK7 kinases, which in turn are described to be regulated by the MAPKKKs LZK, MEKK1 and 4, MLK2 and 3, TAK1, ASK1 and TPL2. A key JNK target is the transcription factor AP1, which regulate the transcription of a plethora of target genes that contain AP1-binding sites (Wagner and Nebreda, 2009).

2. p38 MAPK signaling pathway.

The mammalian p38 MAPK family includes four different isoforms: p38α (MAPK14 or SAPK2a), β (MAPK11 or SAPK2b), γ (MAPK12, ERK6 or SAPK3) and δ (MAPK13 or SAPK4) (Nebreda and Porras, 2000). These four isoforms are encoded by different genes (*MAPK11-14*) and are differently represented in mammalian tissues, being p38α and p38β ubiquitously expressed in the vast majority of cell types. Remarkably, while p38α is highly abundant, p38β appears to be expressed at very low levels and its contribution to p38 MAPK signaling is not clear. The other isoforms are expressed in a more restrictive and tissue-specific manner: p38γ is mainly expressed in skeletal muscle and the δ isoform is expressed in testis, pancreas, kidney and small intestine (Ono and Han, 2000). Despite they have a 60-70% sequence homology (Goedert et al., 1997) and overlapping specificity for their substrates, some differences between them have been reported (Ono and Han, 2000; Goedert et al., 1997). Some studies have described the functional redundancy between the p38 family members when they were genetically deleted, observing that the lack of one isoform can usually be complemented by the function of the other isoforms (Sabio et al. 2005).

2.1. Mechanisms of activation.

The p38 MAPK pathway becomes activated by environmental stresses such as high osmolarity, UV irradiation, heat shock, hypoxia, oxidative stress, and inflammatory cytokines (Cuadrado and Nebreda, 2010). p38 MAPK activation requires a

dual phosphorylation at Thr-Gly-Tyr (TGY motif) in the activation loop sequence by upstream MAPKKs. These phosphorylations,that take place in Thr180 and Tyr182 residues and induce conformational changes that stabilize this flexible loop in an open and extended conformation, thus facilitating the binding of different substrates (Raingeaud et al., 1995) (**Figure i2**). Three dual-specificity MAPKKs are capable to activate p38 MAPK family members: while MKK6 is able to phosphorylate all family members, MKK3 activates α, γ, and δ, but not β isoform. Both MAPKKs are highly specific for p38 MAPKs (Enslen et al., 1998; Alonso et al., 2000). Moreover, MKK4, an activator of the JNK pathway, has been also described to do this function (Doza et al., 1995; Brancho et al., 2003). The relative contribution of these MAPKKs to p38 activation depends on the stimulus and the cell type, due to variations in its expression levels depending on the cell type.

Pyrimidyl-imidazole drugs, such as SB203580, are specific inhibitors of p38α and β isoforms through competitive binding at the ATP-binding pocket (Goedert et al., 1997) (**Figure i2**). Other inhibitors such as BIRB796 lead to a conformational kinase reorganization that prevents ATP binding and activation of all p38 MAPK isoforms (Pargellis et al., 2002).

A wide variety of MAPKKKs have been proved to orchestrate p38 activation. This is the case for MEKK (MAPK/ERK kinase kinase) 3 and MEKK4, TAK1 (TGF*β*-activated kinase 1), ASK1 (apoptosis signal-regulating kinase 1), DLK1 (dual-leucine-zipper-bearing kinase 1), MLK3 (mixed-lineage kinase 3), TPL2 (tumour progression loci 2), TAO (thousand-and-one amino acid) 1 and 2, and ZAK1 (leucine zipper and sterile-*α* motif kinase 1).

Moreover, some MAPKKKs that trigger p38 MAPK activation can also activate the JNK pathway. There is also a high degree of complexity upstream of the cascade, where the activation of MAPKKKs involves the phosphorylation by STE20 family kinases and association with small GTP-binding proteins of the Rho family together with ubiquitination-based mechanisms. Through these high diverse regulatory mechanisms, cells have the ability to respond to many different stimuli, facilitating both signal fine-tuning and cross-talk with other pathways (Cuevas et al., 2007).

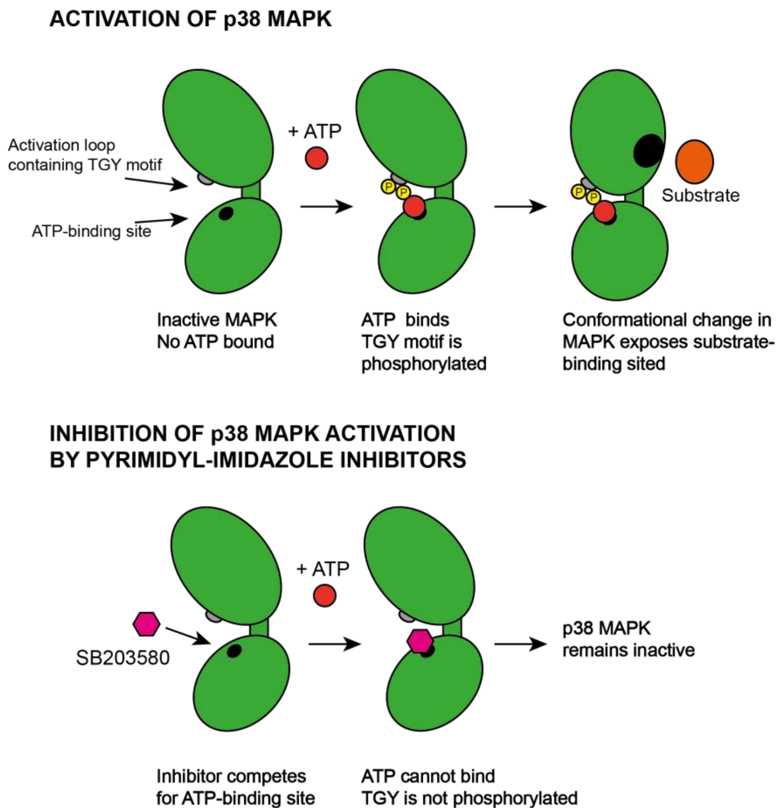

Figure i2: p38 MAPK activation, and inhibition by specific inhibitors. p38 is activated through ATP-dependent dual phosphorylation at the activation loop. A conformational change allows the binding to its substrates. Pyrimidyl-imidazole drugs as SB203580, an ATP analog, competes for the ATP binding site and the kinase remains inactive. Adapted from Coulthard et al., 2009.

Following activation and induced by the conformational change upon phosphorylation on the activation loop, p38 translocates from the cytoplasm to the nucleus, where is able to activate specific substrates through docking-mediated interactions (Raingeaud et al. 1996). p38 MAPKs are able to phosphorylate specific substrates on serine or threonine residues followed by a proline (S/TP sites), leading to modulation of diverse physiological processes. Nevertheless, occasional reports have shown the phosphorylation of non-proline-directed sites by p38 MAPKs (Cheung et al., 2003; Reynolds et al., 2000).

p38 MAPKs also have kinase-independent functions, attributed to direct binding to targets in the absence of phosphorylation. In *Saccharomyces cerevisiae*, the p38 MAPK analog Hog1 is directly recruited to chromatin as a component of transcription complexes in response to osmotic stress, and some of its gene-expression regulatory functions might not require Hog1 kinase activity (Alepuz et al., 2003; de Nadal and Posas, 2010). This chromatin association has been also reported in mammalian cells in response to stress (Ferreiro et al., 2012b) and during muscle differentiation (Segales et al., 2016a).

p38 targets in the nucleus include transcription factors, chromatin remodeling enzymes, protein kinases and RNA binding proteins. p38 can also target and phosphorylate cytoplasmic proteins involved in apoptosis, endocytosis, mRNA stability, protein degradation and localization, cell migration or cytoskeleton dynamics (Trempolec et al., 2013).

2.2. Regulation of the p38 MAPK pathway.

Inactivation of the p38 MAPK pathway is mainly achieved by the activity of several phosphatases that target the Thr and Tyr residues in the activation loop and removes the phosphate groups responsible for the activation of the pathway. Generic phosphatases that dephosphorylate Serine/Threonine residues, including PP (protein phosphatase) 2A and PP2C (as Wip1), or tyrosine residues, such as STEP (striatal enriched tyrosine phosphatase), and HePTP (haematopoietic protein tyrosine phosphatase) lead to monophosphorylated MAPK forms (Askari et al, 2009; Lindqvist et al, 2009; McAlees et al., 2009). These variants have been proved to be 10-20-fold less active (phosphoresidue Thr180 alone) or even inactive (Tyr182 residue alone), albeit its biological significance remains to be unraveled (Zhang et al., 2008).

The DUSPs (dual-specificity phosphatases)/MKPs (MAPK phosphatases) family members, which dephosphorylate both phosphotyrosine and phosphothreonine residues, have also been described to regulate MAPK activity. Most MKPs include a dual-specific phosphatase domain and a docking domain that mediates the interaction with the MAPK substrate. Furthermore, the association of MAPK to the MKP docking domain increases its phosphatase activity. MKPs 1, 4, 5 and 7 are able to dephosphorylate p38*α* and p38*β* in addition to JNK MAPKs. Remarkably, different stimuli known to activate MAPK signaling lead to a transcriptional up-regulation of some MKPs, and are thought to play an outstanding role limiting the extent of MAPK activation (Owens and Keyse, 2007).

Although p38 activity is generally controlled by phosphorylation by upstream kinases and dephosphorylation by specific phosphatases, additional regulatory mechanisms are involved in the regulation of the pathway. Among them, scaffolding proteins and compartmentalization of the MAPK components, alterations in protein expression and post-translational modifications have been reported (reviewed in Cuadrado and Nebreda 2010).

2.3. Physiological functions regulated by p38 signaling pathway.

Besides controlling cellular responses to different environmental and genotoxic stresses, p38 is able to regulate a wide range of biological processes going from immune responses and inflammation, to proliferation, differentiation and cell migration (reviewed in Cuadrado and Nebreda, 2010; Kyriakis and Avruch, 2012) (**Figure i3**). Deregulation of the p38 MAPK pathway has been involved in the development of different pathologies, such as cancer, inflammatory states, cardiovascular dysfunctions or Alzheimer's disease (Cuenda and Rousseau, 2007).

2.3.1. Cell survival.

Cells are continuously exposed to different kinds of insults to which they have to adapt. p38 MAPKs participate in the cell's decision to survive or die depending on the cell type and the duration and strength of the insult.

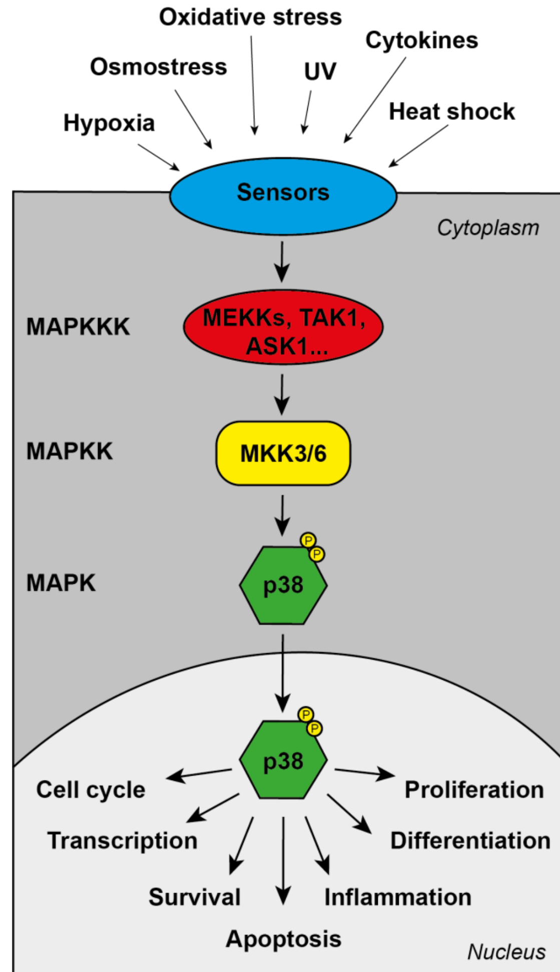

Figure i3: Physiological processes regulated by the p38 MAPK signaling pathway. p38 is activated by extracellular stimuli and regulates several physiological processes.

On one hand, p38 can induce apoptosis through a number of transcriptional and post-transcriptional mechanisms. For example, p38 targets and directly phosphorylates BimEL protein, a member of the Bcl-2 family considered as key components of the cell death machinery (Cai et al., 2006). At a transcriptional level, p38 activation of FOXO3a transcription factor and later induction of

BimEL expression has also been defined as a novel apoptotic mechanism (Cai and Xia, 2008). p38 also plays a major role in an integrated regulation of N-terminal phosphorylation that regulates p53-mediated apoptosis following UV radiation (Bulavin et al., 1999). p38α also sensitizes cells to apoptosis via both up-regulation of proapoptotic proteins (as Bax and Fas) and down-regulation of survival pathways in mouse cardiomyocytes and fibroblasts (Porras et al., 2004).

On the other hand, the p38 signaling pathway promotes cell growth and survival mediating the induction of antiapoptotic inflammatory cues, autophagy programs, or by indirect regulation of genes associated with cell survival (reviewed in Thornton and Rincon, 2009).

2.3.2. Regulation of gene expression.

When cells are challenged by different kinds of stress, extensive changes in gene expression have to be made in order to adapt, and p38 tightly regulates this process. Whole genome analysis in mouse embryonic fibroblasts (MEFs) upon different insults (as osmostress, TNFα and the protein synthesis inhibitor anisomycin) revealed that the expression of more than 60% of early-induced genes was mediated by p38 (Ferreiro et al., 2010a). Further transcriptome analysis showed that, among the p38-induced genes, there was a clear enrichment in transcription factors. This suggests that p38 is able to coordinate a comprehensive transcriptional program involved in long-term adaptation to stress (Ferreiro et al., 2010a).

The regulation of mRNA biogenesis is one of the key processes in the induction of p38-regulated gene expression in response to stress. Gene expression is triggered following p38 recruitment to chromatin by association with specific transcription factors (Chow and Davis, 2006; Edmunds and Mahadevan, 2004), which allows the subsequent recruitment of the RNA Pol II machin ery and transcription initiation (Ferreiro et al., 2010b). Furthermore, p38 has also been described to target transcription factors and chromatin-remodeling enzymes, modulating its stability, localization and its interaction with DNA and other regulatory proteins. As an example in the context of muscle differentiation, the SWI/SNF complex subunit BAF60c is phosphorylated by p38, allowing chromatin remodeling and activation of muscle-specific promoters (Forcales et al., 2011).

A direct phosphorylation by p38 has been reported in a high number of transcription factors including ATFs (1, 2, and 6), SAP1 and 2, CHOP, Elk1, p53, or members of the muscle transcriptional coactivators of the myocyte enhancer factor 2 (MEF2) family (MEF2A, MEF2C, and MEF2D) (Cuadrado and Nebreda, 2010; Edmunds and Mahadevan, 2004; de Nadal et al., 2011). In addition, other kinases downstream of p38 such as mitogen and stress-activated kinase 1 (MSK1) and MSK2 are able to phosphorylate other transcription factors as CREB, ATF1, NFκB, STAT1 and STAT3 (Wiggin et al., 2002; Zhang et al., 2001; Zhang et al., 2004), and nucleosomal proteins as histone H3 and highmobility- group 14 (HMG-14) (Arthur, 2008) (**Figure i4**).

Besides modulating mRNA biogenesis, p38 is also implicated in the regulation of mRNA stability and translation

(Cargnello and Roux, 2011; Tiedje et al., 2014). Additionally, p38 acts on some mRNAs through regulation of the RNA binding protein HuR (Farooq et al., 2009; Lafarga et al., 2009; Tiedje et al., 2012). Furthermore, p38 is able to target the MAPK signal-integrating kinase Mnk, which phosphorylates the eukaryotic initiation factor 4E (eIF4E), leading to rapid adjustment of protein synthesis upon stress (Lawson et al., 2013; Shveygert et al., 2010).

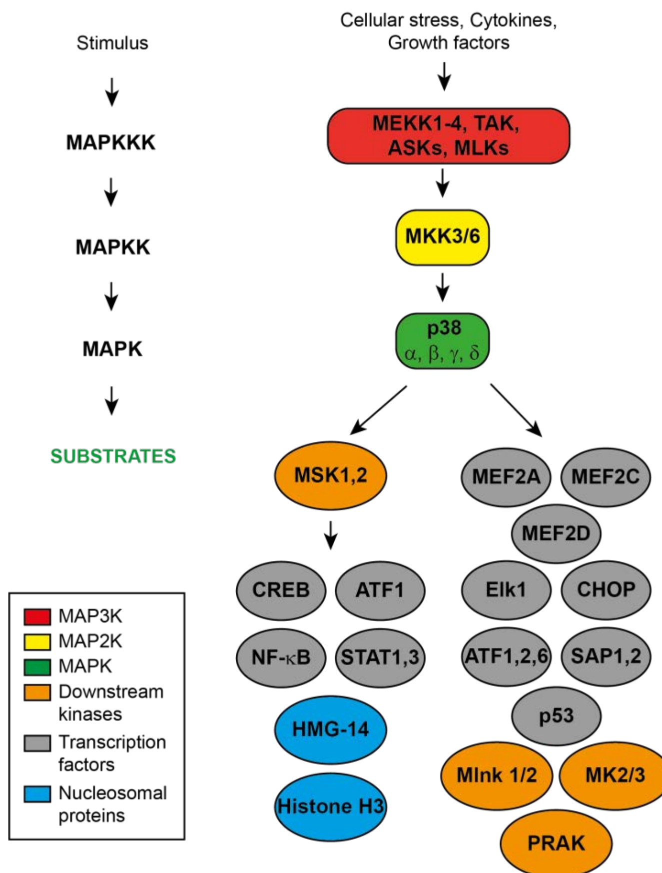

Figure i4: Regulation of gene expression by p38 MAPKs. Upon its activation, p38 targets a high variety of substrates including downstream kinases, transcription factors and chromatin remodeling-enzymes, in order to exert its functions.

2.3.3. Cell cycle.

p38 MAPKs can control cell growth through the activation of different cell cycle checkpoints, and accordingly, acting as tumor suppressors (Ambrosino and Nebreda, 2001; Bulavin and Fornace, 2004). Upon stress, p38 controls cell cycle arrest at G1/S and G2/M phase transitions, dependent on different mechanisms (Reinhardt et al., 2007; Cuadrado et al., 2009; Lafarga et al., 2009).

In G1 phase, p38 is able to phosphorylate the CDC25A phosphatase upon osmostress, which promotes its degradation and in turn leads to a delayed G1/S transition (Goloudina et al., 2003). The same outcome is achieved through direct activation of p53, which results in the accumulation of the cyclin-dependent kinase (CDK) inhibitor $p21^{CIP1}$, one of its direct targets (Kim et al., 2002; Kishi et al., 2001). Moreover, the activation of the p38 MAPK cascade in CCL39 fibroblast cell line significantly decreases cyclin D1 transcription and translation and, conversely, its inhibition by SB203580 has an opposite enhancing effect (Lavoie et al., 1997). In agreement with this, p38α has been involved in the inhibition of the proliferation-promoting JNK pathway, inducing the upregulation of the JNK phosphatase MKP-1, and leading to downregulation of cyclin D1 expression and induction of cell cycle withdrawal in the context of muscle differentiation (explained in detail in following chapters) (Perdiguero et al., 2007b). Interestingly, p38 (and JNK) was also proposed to inactivate retinoblastoma (RB) protein, a key regulator of the restriction point transition in G1, upon Fas stimulation (Wang et al., 1999); nonetheless, the direct targeting of RB by these kinases and the underlying mechanism was never demonstrated. More recently, Joaquin and collaborators showed

how p38 directly targets the p57^{KIP2} CDK inhibitor, which leads to a reduced cyclin A/CDK2 activity, delaying cell cycle progression and increasing cell survival upon stress (Joaquin et al., 2012).

p38 has also been implicated in the control of G2 to M phase progression by delaying G2/M transition in response to DNA damage. The activation and stabilization of p53 (Bulavin et al., 1999; Huang et al., 1999; She et al., 2001; She et al., 2000) and the inhibition of the CDC25B phosphatase (Bulavin et al., 2001; Lemaire et al., 2006) have been proposed as the possible mechanisms. Moreover, the p38 downstream kinase MAPKAP kinase (MK) 2 can also phosphorylate CDC25B (Lemaire et al., 2006). Additionally, this G2/M delay has also been observed in cells subjected to osmostress, but the exact contribution of p38 has not been clarified (Dmitrieva et al., 2002; Mavrogonatou and Kletsas, 2009).

3. Retinoblastoma (RB) protein and cell cycle regulation.

The retinoblastoma (RB) tumor suppressor protein and its closely-related family members are found in organisms as distantly related as mammals, plants, and insects (reviewed in van den Heuvel and Dyson, 2008). Among them, these proteins are essential regulators of cell cycle progression from G1 to S phase through the negative regulation of E2F transcription factors. During cell cycle, RB is inactivated by cyclin-dependent kinase (CDK) phosphorylation, allowing E2F-mediated gene expression (Henley and Dick, 2012). In this context, RB exerts their functions through direct association with chromatin remodelers and modifiers that contribute to the repression of genes important for cell cycle progression (Brehm and Kouzarides, 1999).

RB has emerged as a multi-functional adaptor protein that is able to bind to a high variety of protein partners. RB function is mainly related to its ability to regulate cell cycle progression, which has a direct impact on cell proliferation, differentiation and senescence. Recent studies have found additional tumour-suppressor functions of RB, including alternative roles in cell cycle, maintenance of genome stability and apoptosis (Dick and Rubin, 2013; Vélez-Cruz and Johnson, 2017). Remarkably, inactivation of the RB pathway is one of the most fundamental events in the inhibition of cell proliferation that might lead to cancer (Henley and Dick, 2012).

3.1. The pocket protein family.

RB is the most studied member of the pocket protein family, which is composed of RB (p105, *RB1* gene), p107 (*RBL1* gene), and p130 (*RBL2* gene). The RB gene (*RB1*) was first identified based on its mutation in a rare malignancy of the eye (Friend et al., 1986; Lee et al., 1987).

Many structural properties shared by the RB family members were unraveled form crystallographic data (**Figure i5A**). The most common sequence homology is located in the well-conserved small pocket region, which contains the A and B domains that are separated by a flexible spacer region (Classon and Dyson, 2001). The small pocket is considered the minimal fragment able to interact with viral oncoproteins, like E1A and Tag, which induce aberrant proliferation (Hu et al., 1990). Inside this region, a LXCXE motif contacts a shallow groove on RB, being one of the most conserved structures among pocket family, and among pocket proteins across species (Lee et al., 1998). Moreover, a high number of cellular proteins have been reported to contain an LXCXE-like motif, allowing them them to interact with RB, p107 and p130 (Dick, 2007). Many of these LXCXE containing proteins have chromatin regulatory activity, or are constituents of complexes that have this function.

On the other hand, the large pocket fragment has been recognized as the combination of the small pocket and the C-terminal region (**Figure i5A**), and it is the minimal growth suppressing domain found in the pocket protein family (Bremner et

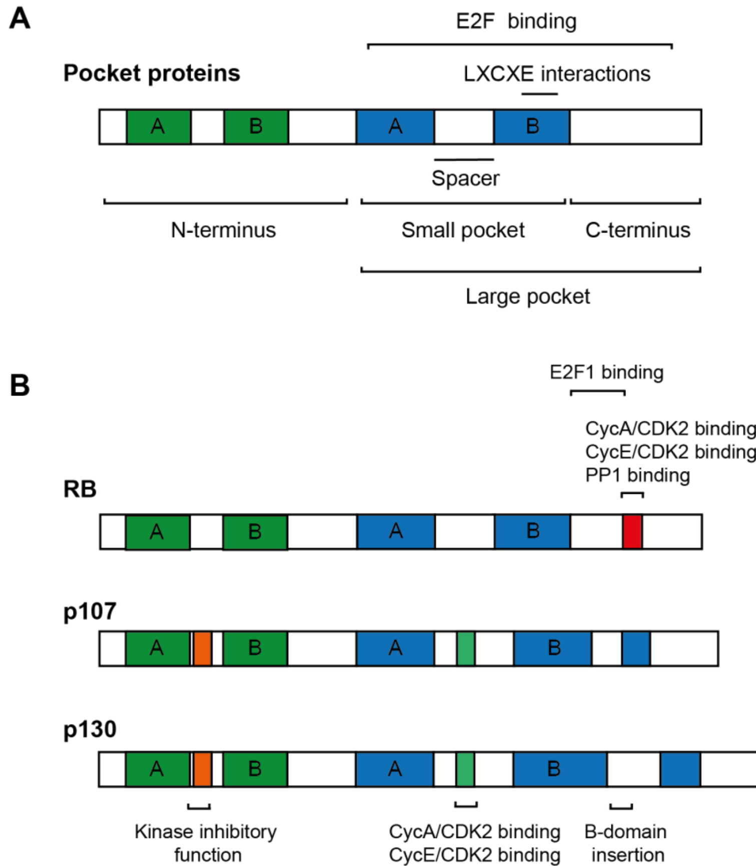

Figure i5: Schematic representation of the pocket protein family structure. A, The small pocket is the minimal region required to bind to oncoviral proteins through their LXCXE motif. The large pocket is the minimal growth suppressing domain and able to interact with E2F transcription factors. **B**, Comparison of pocket proteins ORF structures. We highlight the main structural differences between RB and its relatives p107 and p130. Adapted from Henley and Dick, 2012.

al., 1995). This region is sufficient to associate with E2Fs and suppress their transcription (Hiebert et al., 1992; Qin et al., 1992), and plays an essential role in their ability to control proliferation. Finally, the N-terminal domain is formed by two rigidly connected cyclin-like folds, highly similar to the A and B boxes of the RB pocket, and this architecture is shared by the other pocket proteins

(**Figure i5A**) (Hassler et al., 2007). Due to its highly disordered nature, this region has been crystallized only in part, which has hindered its further study (Burke et al., 2010, 2012).

Two exclusive features of RB that have emerged recently are a docking site for the E2F1 transcription factor, and a C-terminal short peptide region that is competitively occupied by CDKs or protein phosphatase 1 (PP1) (**Figure i5B**). A number of structures in p107 and p130 are absent in RB, as the characteristic insertions in the B domain at their small pockets. In p130, this region is subjected to regulatory phosphorylation to maintain protein stability (Litovchick et al., 2004). Moreover, p107 and p130 contain longer spacer regions than RB, allowing them to bind stably with CDK complexes (Lacy and Whyte, 1997; Zhu et al., 1995; Woo et al., 1997), and a region in the N-terminus that has been related to CDK function inhibition (Woo et al., 1997). Altogether, these structural features allow pocket proteins to interact with numerous binding partners, and is intimately related with their function controlling cell cycle progression.

3.2. Cell cycle regulation by the RB protein family.

Cell cycle advancement harbors a high degree of complexity and its regulation involves the participation of a number of players, including the pocket protein family, E2F transcription factors, cyclin-CDK complexes, CDK inhibitor (CKI) proteins or chromatin-remodeling and modifying complexes.

3.2.1. The E2F family of transcription factors.

The RB transcriptional repressor plays a key role in cell cycle progression (Dick and Rubin, 2013; Chinnam and Goodrich, 2011; van den Heuvel and Dyson, 2008). RB function in cell cycle control is mediated through its association with E2Fs at the promoters of genes important for S phase progression and cell proliferation. Their association either blocks the recruitment of transcriptional co-activators or recruits transcriptional co-repressors to these promoters, thus inhibiting the expression of these genes and repressing G1/S cell cycle transition.

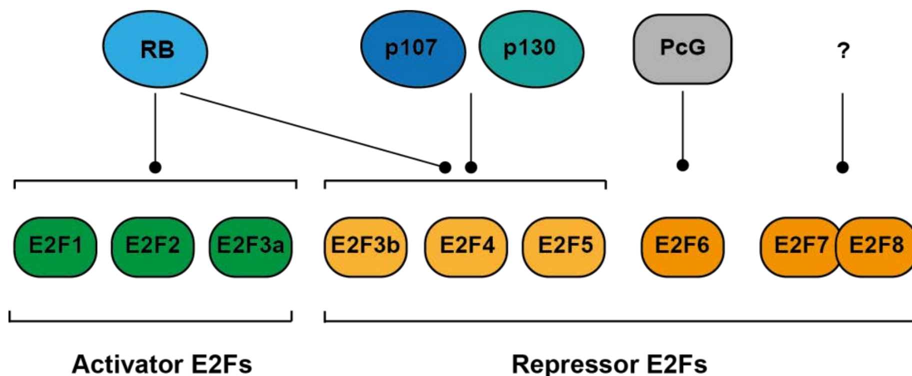

Figure i6: Interactions among pocket proteins and E2F transcription factors. RB preferentially binds to activator E2Fs (E2F1-3a), as well as to E2F3b, which mainly functions as a repressor. p107 and p130 preferentially associate with repressor E2Fs (E2F4-5). E2F6-8 are transcriptional repressor complexes without binding pocket proteins. E2F6 functions as a repressor, and has been purified in complexes with polycomb-group (PcG) proteins. E2F1-6 form heterodimers with DP1-4 proteins to allow DNA binding (not shown), while E2F7 and E2F8 associate as homo- or heterodimers and are thought to recruit repressor complexes to DNA. Adapted from van den Heuvel and Dyson, 2008.

The E2F family of transcription factors (E2F1-8, identified in mouse cells) are the main target of the pocket protein family of transcriptional repressors (Trimarchi and Lees, 2002; Degregori and Johnson, 2006). Biochemical studies subdivide the E2F proteins into different classes with distinct modes of action (**Figure i6**). E2F1-3a are associated with transcriptional activation and preferentially targeted by RB. E2F3b (encoded in the same locus as E2F3a), E2F4 and E2F5 are transcriptional repressors and are generally associated to p107 and p130. Besides, E2F6-8 function as transcriptional repressors independently of pocket proteins association. Interestingly, E2F6 has been purified in complexes with polycomb-group (PcG) proteins (Attwooll et al., 2004; Ogawa et al., 2002; Trimarchi et al., 2001). Of note, E2F1–E2F6 harbour conserved dimerization domains and form DNA-binding heterodimers with proteins of the differentiation-regulated transcription factor-1 polypeptide (DP) family. E2F7 and E2F8 lack a DP-binding domain but contain the tandem repeats of an E2F DNA-binding domain, and are able to form homodimers and heterodimers (van den Heuvel and Dyson, 2008).

The best-characterized member of the E2F family is E2F1, which is frequently amplified in human cancers (cBioPortal for Cancer Genomics). E2F1 has important roles not only in the transcriptional regulation of cell cycle-related genes, but also in the induction of apoptosis (Degregori and Johnson, 2006; Polager and Ginsberg, 2008), and has also been involved in guarding genome stability after the induction of DNA double strand breaks and UV damage (Biswas and Johnson, 2012; Vélez-Cruz and Johnson, 2012; Vélez-Cruz et al., 2016; Biswas et al., 2014).

3.2.2. Pocket proteins in advancing cell cycle

RB is the major negative regulator of the G1/S phase transition in mammalian cells (Friend et al., 1986; Cobrinik, 2005; Henley and Dick, 2012), but also participates in other cell cycle stages. Here, we will summarize the main events involved in cell cycle progression through the G1 and S phase.

3.2.2.1. G0 and G1 phase.

Among the three pocket proteins, p130 is the most expressed pocket protein in quiescence, also referred to as G0 (**Figure i7A**), and the majority of E2F-containing complexes include p130 and E2F4 (Moberg et al., 1996; Hurford et al., 1997). In this stage, RB is found in complex with E2Fs and its expression is low but detectable, while p107 is nearly undetectable (Moberg et al., 1996; Hurford et al., 1997).

In addition to E2F targets, pocket proteins also participate in transcriptional repression of rRNA and tRNA genes during quiescence (Cobrinik, 2005). While both RB and p130 have been described to repress rRNA transcription (Ciarmatori et al., 2001; Cavanaugh et al., 1995; Hannan et al., 2000), the regulation of tRNA levels appears to be RB-specific (Scott et al., 2001, White et al., 1996). The increase in rRNA and tRNA levels at the onset of G1 phase involves pocket proteins phosphorylation to relieve this transcriptional repression (Cobrinik, 2005).

During the G1 phase, all three pocket proteins are able to interact with E2Fs (**Figure i7B**). In early G1, p130-E2F4 is the most abundant complex on the E2F-responsive promoters mediating the transcriptional silencing of these genes (Wells et al., 2000; Takahashi et al., 2000). In mid- to late G1, a decrease in p130 levels goes together with an increase in p107 expression, replacing it at E2F-responsive promoters (Takahashi et al., 2000). By late G1, RB-E2F complexes become more abundant (Wells et al., 2000), and RB associates at this point with activator E2Fs in a conformation that masks the E2F activation region and prevents transcriptional activation (Hiebert et al., 1992; Helin et al., 1993; Flemington et al., 1993; Zamanian and Thange, 1993). Whether RB-E2F complexes are present at the E2F-regulated promoters remains as an open question (Wells et al., 2000; Takahashi et al., 2000). What is clear is that the pocket family cooperate to inhibit E2F-regulated transcription both in G0 and G1 phase.

Among the E2F target genes subjected to this regulation there are activator E2Fs, cell cycle regulators such as cyclins A and E, and RB and p107 themselves (Hurford et al., 1999; Wells et al., 2000; Takahashi et al., 2000; Mulligan et al., 1998). Others include constituents of the replication machinery such as DNA pol α and the proliferating cell nuclear antigen (PCNA), along with enzymes involved in nucleotide biosynthesis (Dagnino et al., 1995; Lavia and Jansen-Durr, 1999). The mechanism that leads individual E2F-pocket protein complexes to be selective for their target promoters beyond the E2F recognition sequence is unknown. While some promoters are specifically regulated by p130 and p107, like b-Myb (Hurford et al., 1999), and others are exclusively regulated by RB, such as p107 itself (Hurford et al.,

1999; Takahashi et al., 2000; Williams et al., 2006), some have been reported to be occupied unselectively by all pocket family members (Wells et al., 2000).

E2F regulation is often associated with the recruitment of transcriptional repressors, which in many cases bear chromatin regulating enzymatic activity, to E2F- pocket protein complexes (Classon and Harlow, 2002). This allows directed specific enzymatic activities to localized chromatin domains at these promoters. Pocket proteins associate to chromatin remodeling factors (CRFs) through its LXCXE binding cleft and recruit these complexes to E2F-responsive promoters, where they contribute to chromatin condensation and transcriptional repression. Chromatin remodeling enzymes as SWI/SNF (BRG and BRM), histone deacetylases (HDAC1, 2 and 3), and histone methyltransferases (Suv39h1 and 2) have been reported to interact with RB, among others (Robertson et al., 2000; Brehm et al., 1998; Dunaief et al., 1994; Luo et al., 1998; Magnaghi et al., 1998; Vandel et al., 2001; Nielsen et al., 2001; Morrison et al., 2002). These interacting proteins diversity allows pocket proteins to exert widespread effects on chromatin structure. The relevance of repressive modes of chromatin along G1 progression remains unclear. Some works have reported that mutations in the LXCXE binding cleft of RB, disrupting its association with chromatin regulators, is not accompanied with a defect in regulation of G1 progression (Isaac et al., 2006), but a deficiency for this association does compromise cell cycle exit (Francis et al., 2009).

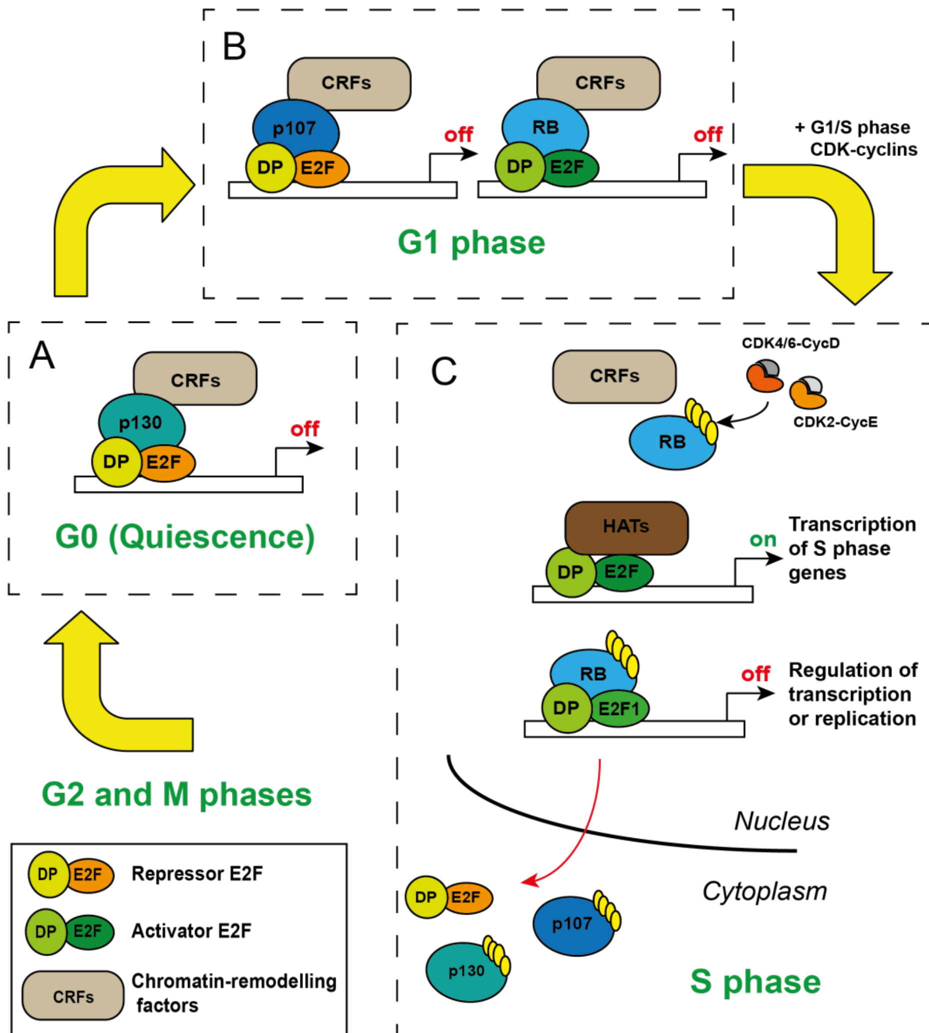

Figure i7: Model of pocket protein regulation in advancing cell cycle. A, In G0, quiescent cells repress E2F target transcription mainly through the actions of p130. **B,** In G1, p107-repressor E2Fs complexes start to replace p130, and RB-activator E2Fs complexes become more abundant. Chromatin remodeling factors (CRFs), as histone deacetylases (HDACs), are recruited to at the E2F promoters and mediate alterations at the chromatin, repressing E2F-mediated gene expression. **C,** At the G1/S transition, cyclin/CDKs phosphorylate pocket proteins, forcing its dissociation from E2F/DP heterodimers and allowing transcription of E2F target genes through the S phase. The repressive heterochromatin changes present in G1 are reversed by the recruitment of histone acetyltransferases (HATs). p130 and 107 proteins and repressor E2F-DP complexes are exported to the cytoplasm. Adapted from Henley and Dick, 2012.

Pocket proteins ability to bind to E2Fs is dependent on its phosphorylation status, which is mainly regulated by CDKs (Mittnacht, 1998). While slow-migrating hyperphosphorylated forms are extensively modified and do not associate to E2Fs, the fastest migrating hypophosphorylated forms are modified at very few positions and are capable to associate with E2F transcription factors. It is critical for cells to maintain low CDK activity until the end of G1. Accordingly, the mechanisms regulating CDK activity are essential for understanding pocket protein function during the G1 phase (Mittnacht, 1998).

CDK regulation has several layers of control, from the association with cyclin subunits into complexes to regulatory phosphorylation that modulates its catalytic activity. Nevertheless, the most relevant mechanism controlling pocket proteins activity is at the level of CDK inhibitor (CKI) proteins, since they act directly upstream of CDKs to block its catalytic activity. There are two main families of CKIs: the CIP/KIP family (which is composed by $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) and the INK4 family (that consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) (Besson et al., 2008). CIP/KIP inhibitors can bind both CDKs and cyclins and inhibit its kinase activity, and are capable to inhibit any G1 cyclin/CDK complex. Conversely, INK4 members can bind only CDK4 and CDK6 in association with D-type cyclins (Sherr and Roberts, 1999). $p27^{KIP1}$ has a critical function in determining the onset of the S-phase. In G1, $p27^{KIP1}$ expression levels are moderately high and cyclin E/CDK2 kinase activity remains low, thus preventing the initiation of DNA synthesis (Sherr and Roberts, 1999). Cyclin D/CDK4 and cyclin D/CDK6 complexes are the first to become active in G1 and they start to phosphorylate the pocket proteins (Mittnacht, 1998).

When cells approach the end of G1, the pocket proteins are partially phosphorylated and allow limited transcription by activator E2Fs, which start to stimulate cyclin E production and in turn generate more cyclin E/CDK2 kinase activity (Mittnacht, 1998; Sherr and Roberts, 1999). The most important barrier to full activation of this kinase complex is the interaction with and inhibition by $p27^{KIP1}$ (Sherr and Roberts, 1999). This is eventually overcome by $p27^{KIP1}$ phosphorylation by cyclin E/CDK2, which results in its targeting for degradation and allowing full kinase activation and entry into S-phase. Thus, $p27^{KIP1}$ has an important role modulating kinase activity to keep pocket proteins active in G1.

In addition to CKI families, p107 and p130 also act as CDK inhibitors by its direct binding to cyclin/CDK complexes (Zhu et al., 1995; Howe and Bayley, 1992; Faha et al., 1992; Ewen et al., 1992; Hauser et al., 1997), and this function is not shared by RB (Castano et al., 1998). In fact, p107 has demonstrated to be as effective as $p21^{CIP1}$ (Castano et al., 1998). Two independent regions in p107 and p130 demonstrated to mediate their ability of to inhibit cyclin E/CDK2 and cyclin A/CDK2 complexes (**Figure i5B**): the spacer region between the A and B pockets, and the highly conserved region in the N-terminal domain (Lacy and Whyte, 1997; Woo et al., 1997). The combination of these dual domains allows p107 and p130 to inhibit kinase activity, interacting with high affinity to cyclin/CDK complexes through the spacer region, and efficiently inhibiting its activity through its N-terminal region (Castano et al., 1998). Indeed, some studies showed that deletion of the spacer region in p130 was sufficient to inhibit cyclin E and A binding, albeit this mutant could still suppress cell growth,

confirming that this function is modulated by different regions of the protein (Lacy and Whyte, 1997).

Despite that RB does not display an analogous kinase inhibitory domain as the other family members (**Figure i5B**), it regulates cyclin/CDK activity through inhibitor proteins. RB interferes in the targeted degradation of $p27^{KIP1}$ to maintain G1 kinase activity at low levels at a number of steps in the degradation pathway (Ji et al., 2004; Binne et al., 2007). To maintain $p27^{KIP1}$ expression, RB functions as a scaffold that associates with APC-Cdh1 and Skp2, simultaneously targeting Skp2 for ubiquitin-mediated degradation (Binne et al., 2007). Since Skp2 is an adaptor that is required for $p27^{KIP1}$ degradation, this results in a net reduction of G1 CDK activity. Moreover, RB interaction with Skp2 competes for its binding with phosphorylated $p27^{KIP1}$ (Ji et al., 2004). Hence, RB preserves CDK activity inhibition during G1 by directly inhibiting $p27^{KIP1}$ ubiquitination. Other studies have also suggested that p107 participates in Skp2 degradation, resulting in a delay in S-phase entry (Rodier et al., 2005; Sangwan et al., 2012). Compared to E2F regulation, how pocket proteins regulate $p27^{KIP1}$ expression remains poorly understood, but some studies have suggested that it may be as critical as E2F regulation in the control through G1 progression (Ji et al., 2004; Binne et al., 2007).

3.2.2.2. G1/S phase transition and progression through S-phase.

The transition into S-phase is crucial for mammalian cells given that, once DNA replication starts, the cell cycle must advance

to complete cell division. The commitment step to initiate DNA replication (often been termed as the 'restriction point') has many features that guarantee cells progression from G1 to S-phase and not in the reverse direction, which can lead to genome instability and conceivably cancer (Henley and Dick, 2012). It has been suggested that the release of E2F from RB control by phosphorylation corresponds specifically with the commitment point (Yao et al., 2008).

The irreversible advancement to S-phase is ensured by a feed forward loop between G1 cyclin/CDKs and RB. RB phosphorylation by cyclin E/CDK2 leads to its dissociation from activator E2Fs, which ensure additional cyclin E transcription and generate more kinase activity towards RB, leading to an increase in free E2Fs and even more cyclin E transcription. Concomitantly, cyclin E/CDK2 become fully active as a consequence of $p27^{KIP1}$ phosphorylation leading to its targetting for degradation (Sherr and Roberts, 1999). Once cells progress to S-phase DNA synthesis is initiated, cyclin E/CDK2 complexes phosphorylate other cyclin E subunits, leading to its degradation, and cyclin E/CDK2 activity comes to an end (Classon and Dyson, 2001). The other members of the pocket family are also phosphorylated in an analogous manner, releasing their associated E2F transcription factors (Classon and Dyson, 2001). In addition, a fraction of these pocket proteins are exported to the cytoplasm, where they form complexes with repressor E2Fs (Verona et al., 1997; Chestukhin et al., 2002) (**Figure i7C**). Once released from RB regulation, activator E2Fs interact with histone acetyltransferase (HAT) enzymes like p300 (**Figure i7C**), which mediate histone H3 and H4 acetylation,

allowing E2F target genes transcription required to complete the S phase (Frolov and Dyson, 2003).

Finally, RB and the other pocket proteins are inactivated at the beggining of the S-phase, remaining functionless until its dephosphorylation at the end of mitosis (Henley and Dick, 2012). Several studies indicated that RB-E2F1 complexes exist in S-phase and are resistant to cyclin/CDK phosphorylation (Calbo et al., 2002: Ianari et al., 2009) (**Figure i7C**). One explanation is that RB has different mechanisms to interact with E2Fs, and one is exclusive to E2F1 (Dick and Dyson, 2003; Cecchini and Dick, 2011) (**Figure i5B**). RB bound to E2F1 in its CDK resistant configuration has altered DNA binding specificity compared with other E2F complexes (Dick and Dyson, 2003). One suggested function is that hyperphosphorylated RB-E2F1 complexes participate in the transcriptional repression of proapoptotic E2F target genes while its ability to drive proliferation is active (Dick and Dyson, 2003). This mechanism has been reported as an S-phase checkpoint as a consequence of disrupted DNA replication following DNA damage (Karantza et al., 1993; Sever-Chroneos et al., 2001). Thus, RB can remain active in a background of high CDK activity (Markham et al., 2006; Chan et al., 2001).

3.2.2.3. Post-translational modifications modulate RB function.

RB phosphorylation by CDKs is the canonical mechanism leading to its inactivation during cell-cycle progression (Hassler et al., 2007; Burke et al., 2010, 2012). The global conformational

changes that occur upon RB phosphorylation are site specific and remarkably diverse, providing RB with different functional outputs (Rubin et al., 2013). Nevertheless, other post-translational modifications in specific RB residues, such as acetylation or methylation among others, have an impact on RB activity (reviewed in MacDonald and Dick, 2013).

3.2.2.3.1. The RB phosphorylation code.

The relative contribution of specific RB domains and phosphorylation sites in the regulation of cell cycle, E2F association, and E2F transcription have been investigated (Burke et al., 2010; Harbour et al., 1999; Brown et al., 1999; Knudsen and Wang, 1997; Chew et al., 1998; Lents et al., 2006). Supported by structural analysis, these studies revealed that diverse phosphorylation events at the pocket loop, the interdomain loop, and the C-terminal domain can induce unique conformational changes that contribute to RB inactivation by inhibiting E2F binding (Rubin et al, 2005; Burke et al., 2010, 2012) (**Figure i8**). CDK phosphorylation usually stimulates protein-protein interactions by creating a phosphoepitope that becomes structured upon association with its target (Hao et al., 2007; Orlicky et al., 2003). Conversely, RB phosphorylation disrupts the association with other proteins by promoting interdomain interactions within RB, rendering its structure incompatible with its binding partners (Rubin et al., 2005; Hassler et al., 2007; Burke et al., 2010, 2012). Thus, the disordered-to-ordered transitions in RB are completely intramolecular and result in the inhibition of intermolecular association (Rubin et al., 2013).

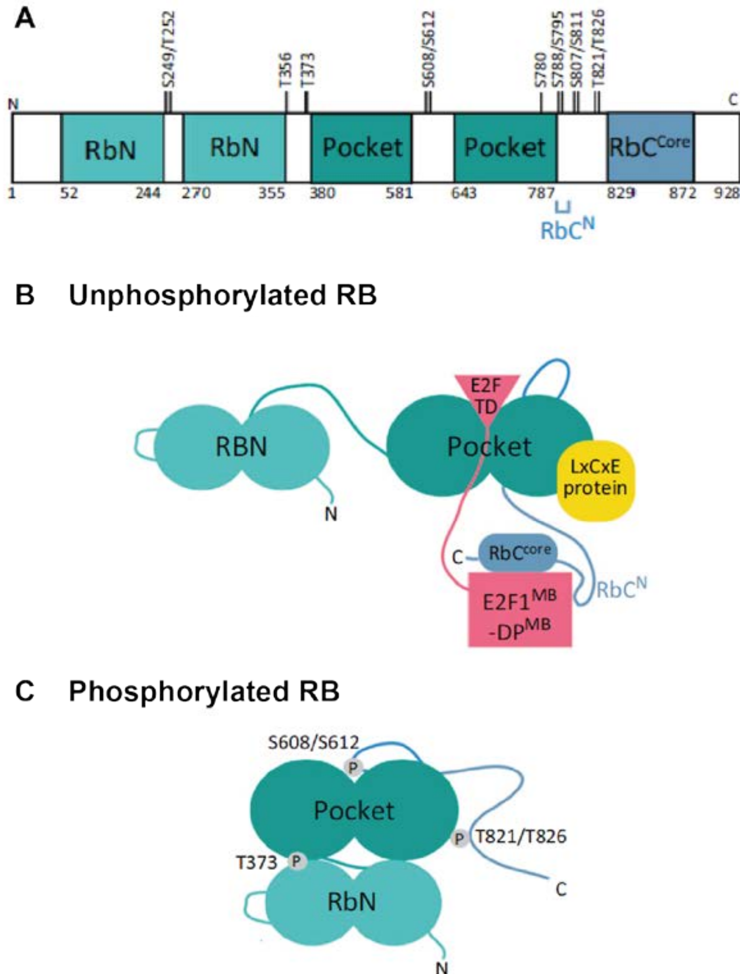

Figure i8: Model of unphosphorylated and phosphorylated RB and its putative partners obtained from crystal structures. A, RB contains 16 consensus S/TP phosphorylation sites distributed along its sequence, but largely excluded from the small pocket. **B,** In the unphosphorylated state, the pocket domain binds the E2F transactivation domain (E2FTD) and a second binding interface exists between RB C-terminus (RbC) and the 'marked box' domains of E2F and its heterodimer partner DP (E2FMB–DPMB). The pocket domain contains a cleft within its second helical subdomain, which binds a linear LXCXE sequence. **C,** Phosphorylation-induced conformational changes result in RB–E2FTD complex inhibition. The association between the pocket domain and E2FTD is inhibited by S608/S612 and T373 phosphorylation. S608/S612 phosphorylation induces RB pocket loop (RbPL) binding to the pocket domain. Adapted from Rubin, 2013.

RB contains 16 consensus CDK phosphorylation sites (serine and threonine residues) in disordered regions of the protein that are distributed along the spacer region and the N- and C-terminal regions, being largely excluded from the small pocket (Lees et al., 1991; Adams et al., 1999; Knudsen and Wang, 1997; Brown et al., 1999). Phosphoprotein analysis by mass spectrometry confirmed the phosphorylation of 13 of the 16 consensus CDK sites in human RB (Dephoure et al., 2008; Olsen et al., 2010) (**Figure i8A**). In the non-phosphorylated state, the pocket domain interacts with the E2F transactivation domain (E2FTD) in a cleft flanked by the two helical subdomains (**Figure i8B**) (Lee et al., 2002; Xiao et al., 2003). A second binding surface exists between the C-terminal domain and the 'marked box' domains of E2F and DP (E2FMB–DPMB) (Rubin et al., 2005). The RBCN and RBCcore regions at the C-terminus are responsible for this secondary interaction (**Figure i8B**). While RBC marked box interaction has been observed between RB and all E2Fs *in vitro* (Rubin et al., 2005), this association appears exclusive for E2F1 *in vivo*, and mediates the specific function of E2F1 inducing apoptosis (Julian et al., 2008; Dick and Dyson, 2003).

During cell cycle, the initial RB phosphorylation events in early G1 phase are catalyzed by CDK4 or CDK6 activated by cyclin D (Ezhevsky et al., 1997; Zarkowska and Mittnacht, 1997) and *in vitro* phosphorylation studies showed to occur on S249, T252, T356, S608, S788, S807, S811, and S826 sites (Zarkowska and Mittnacht, 1997). Later, phosphorylation by cyclin D/CDK4 or 6 and/or cyclin E/CDK2 phosphorylates T5, T373, and S795, and in late G1, cyclin E/CDK2 complexes mediate the phosphorylation of S612 and T821 (Zarkowska and Mittnacht, 1997). RB ability to

block cell proliferation cannot be disrupted by mutation of individual phosphorylation sites, demonstrating that no single site modulates RB interaction with E2F (Knudsen and Wang, 1997; Brown et al., 1999). Instead, several works demonstrated that the modification of the majority of RB phosphorylation sites are needed to abrogate E2F binding, and the phosphorylation of multiple regions is required for E2F-RB dissociation (Knudsen and Wang, 1997) (**Figure i8C**). The current understanding is that the initial phosphorylations of the C-terminal domain initiate structural changes to RB resulting in the establishment of intramolecular interactions, leading to the exposure of secondary CDK phosphorylation sites. As the cells enter late G1, the newly exposed CDK sites are phosphorylated, leading to conformational changes within RB and resulting in a disruption of the E2F binding cleft within the pocket domain. Thus, different combinations of phosphorylation events catalyzed by CDKs inactivate RB by changing the binding affinity for E2Fs, and possibly other interacting proteins as well (reviewed in MacDonald and Dick, 2013). Remarkably, phosphorylation at T373 site at the N-terminal domain proved to be the only event sufficient and necessary for complete RB inactivation (Brown et al., 1999; Knudsen and Wang, 1997; Lents et al., 2006), pointing to allosteric RB N terminus-pocket docking as a potential mechanism for E2F release.

RB pocket affinity for E2F is strong, and redundancy is needed for collective inhibition. Indeed, several studies have reported that multiple phosphorylation events additively inhibit RB-E2F interaction and E2F transcriptional repression (Burke et al., 2010; Brown et al., 1999; Knudsen and Wang, 1997). More in detail, RB phosphorylation at T821/T826 sites induce RB C-

terminal binding to the pocket domain (Rubin et al., 2005), thus inhibiting protein partners association, including viral oncoproteins or deacetylases, to the LXCXE cleft. Moreover, T821/T826 sites immediately precede the RB C-terminal domain sequence required for E2F1–DP binding, and its phosphorylation is also incompatible with RB C-terminal domain binding to the marked-box domains (**Figures i8B and i8C)**. S788/S795 phosphorylation in the C-terminal region also inhibits RB interaction with E2F1–DP (Rubin et al., 2005). Other phosphorylations at the pocket domain and the N-terminal region, such as S608/S612 and T373, have also shown to be relevant for E2F binding inhibition (Burke et al., 2010, 2012). S608/S612 phosphorylation mediates RB pocket loop interaction with the pocket domain in a manner that mimics and competes with E2F (Burke et al., 2010, 2012). T373 phosphorylation generates a hydrophobic surface that binds a cleft in the N terminus, thus allosterically disrupting E2F binding (Burke et al., 2012). Interestingly, there are structural differences in how T373 (allosteric) and S608/S612 phosphorylation (competitive) inhibit E2F. The allosteric change is an efficient mechanism to release bound E2Fs, while the competitive binding of RB prevents free E2F binding. The interdomain interface promoted by T373 phosphorylation, also inhibits other proteins interaction at the LXCXE cleft (Burke et al., 2012).

The structural effects of phosphorylation on other CDK sites remain poorly understood. As an example, several studies reported that RB phosphorylation at S259/T252 sites does not have a clear effect on E2F inhibition, and does not influence the mechanism induced by T373 phosphorylation (Burke et al., 2010, 2012). S259/T252 phosphorylation disrupts E1A-like inhibitor of

differentiation (EID1) binding to the N terminus, a factor known to inhibit RB interactions with transcription-repressing chromatin factors and replication origin complex proteins (Hassler et al., 2007; Ahlander et al., 2008), being these interactions of an electrostatic nature (Hassler et al., 2007).

The analysis of the phosphorylation events by CDKs in other pocket proteins remains poorly characterized. However, phosphoproteomic databases as PhosphositePlus suggest that p107 and p130 are also phosphorylated in similar regions surrounding the pocket region (PhosphositePlus).

3.2.2.3.2. Additional post-translational modifications that modulate RB activity.

Another means of post-translational regulation of RB function involves acetylation and methylation of lysine and arginine residues. Recruitment of RB into the p300/CPB transcriptional co-activator complex by binding the viral oncoprotein E1A leads to the acetylation of RB on K873/K874 in the C-terminus by the histone acetyltransferase activity inherent to this complex (Chan et al., 2001). Acetylation at these sites increases the affinity of RB for Mdm2 and exerts a negative influence on RB cyclin/CDK dependent phosphorylation, thus maintaining RB in an active state (Chan and La Thangue, 2001). Such a state of activation is desirable in cells that are terminally differentiated. Indeed, it was noted that acetylation of RB increased in differentiating U927 cells (Chan and La Thangue, 2001). In another study, RB acetylation was observed in differentiating myocytes (Nguyen et al., 2004).

PCAF, the p300/CBP associated factor, was found to associate with and acetylate RB on K873/874 in these cells. In addition, acetylation of RB was necessary for proper transactivation of MyoD (Nguyen et al., 2004).

In contrast to acetylation, which occurs only on lysine residues, methylation occurs on both lysine and arginine residues (Ng et al., 2012), and has been implicated in transcriptional regulation. Recently, the mono-methyltransferase Set7/9 was shown to actively methylate RB at K873 and at K810. When RB is methylated at K873, a docking site for the heterochromatin binding protein HP1 is created (Munro et al., 2010). HP1 binds methylated histones and functions to repress transcriptional activity by regulating the structure of chromatin (Hediger and Gasser, 2006).

3.2.3. Other physiological processes regulated by RB.

Several studies highlighted other cell cycle-independent functions of RB in mediating apoptosis or maintaining genomic stability. The role of RB in other processes, such as irreversible cell cycle arrest in differentiating muscle cells, will be assessed in the next chapter.

As we mentioned above, in response to genotoxic stress, E2F1 mediates the upregulation of proapoptotic genes in proliferating cells, and RB inhibits E2F1-induced apoptosis by repressing E2F transcription (Hallstrom and Nevins, 2009; Iaquinta and Lees, 2007). More consistent with its role as a tumor suppressor, RB also proved to stimulate proapoptotic gene

expression (Ianari et al., 2009). Phosphorylated RB is found in complex with E2F1 at the promoters of actively transcribed genes following DNA damage, and it is dependent on E2F1-specific association with C-Terminal RB (Julian et al., 2008; Dick and Dyson, 2003). Remarkably, these complexes have been observed during S phase even in the absence of DNA damage, however, the specific RB phosphorylation sites remain unknown (Cecchini and Dick, 2011; Wells et al., 2003) (**Figure i7C**).

RB has also been related with the maintenance of chromosome structure during mitosis, which explains the chromosomal instability (CIN) and aneuploidy reported upon RB loss (Manning and Dyson, 2011). Chromosome instability by the lack of a functional RB has been linked to a lack of regulation of checkpoint and replication genes early in the cell cycle, leading to chromosome errors later in division, and to a deficient recruitment of factors involved in chromatin condensation and histone modifications (Longworth et al., 2008; Coschi et al., 2010; Manning et al., 2010). Interestingly, cells expressing a mutant with specific defects in the RB LXCXE binding cleft (RBΔL) recapitulated the mitotic chromosomal defects, while properly regulated E2F-dependent transcription (Isaac et al., 2006; Coschi et al., 2010). All these data would confirm that protection against CIN is, at least partially, an E2F-independent activity, and phosphorylation events inactivating RB functions during mitosis would be related with the inhibition of both interactions at the LXCXE cleft and E2F repression.

4. Skeletal muscle differentiation, growth and regeneration.

The regulation of skeletal muscle formation (myogenesis) is essential for normal development as well as in pathological conditions such as muscular dystrophies and inflammatory myopathies. Adult skeletal muscle is a postmitotic tissue composed of muscle fibers (myofibers), whose primary function is contraction, which allows organisms to generate force and facilitates voluntary movement. This tissue is also essential for the regulation of whole-body metabolism. Myogenesis is a multi-step and dynamic process by which mature myofibers are formed from satellite cells, the skeletal muscle stem cells (Cornelison and Perdiguero, 2017). These cells are normally in quiescence, and in response to injury, they activate and proliferate into myoblasts, which differentiate into postmitotic mononuclear myocytes that fuse into multinucleated myofibers. All these steps are controlled by myogenic regulatory factors (MRFs), which regulate the subsequent activation of muscle-specific genes (Cornelison and Perdiguero, 2017). Myogenesis is tightly regulated at the transcriptional and translational levels, being these processes in turn modulated by complex epigenetic mechanisms. Many signaling pathways regulate myogenesis, including the PI3K/AKT and p38 MAPK cascades, and cell cycle regulators as RB (Segalés et al., 2016b).

Aging of skeletal muscle is associated with reduction of muscle mass and performance that is clinically described as sarcopenia (Alway et al., 2014; Glass and Roubenoff, 2010; Mitchell et al., 2012). Sarcopenia is known to increase the

predisposition to muscle injury (Faulkner et al., 1995) and, concomitantly, aging is associated with a progressive decline in satellite cell number and function resulting in defective tissue regeneration in rodents and humans (Brack et al., 2005; Chakkalakal et al., 2012; Sousa-Victor et al., 2014a; Verdijk et al., 2012, 2014; Zwetsloot et al., 2013; Conboy et al., 2003, 2005; Garcia-Prat et al., 2016; Collins et al., 2007; Cosgrove et al., 2014).

4.1. Skeletal muscle function.

Skeletal muscle has evolved to allow precise movement in animals, and also allows other vital functions, such as breathing and metabolism. The functional unit of skeletal muscle is the myofiber, able to generate force by contraction. Each myofiber is composed by myofibrils, which in turn contain thousands of sarcomeres, where the actin and myosin filaments are organized and interact to produce the force (**Figure i9**). As indicated above, myofibers are multinucleated syncitial cells generated by the fusion of myoblasts during embryonic and fetal development (Mintz and Baker, 1967).

Skeletal muscle has an outstanding regenerative capacity, even after severe damage that causes extensive myofiber necrosis (Rosenblatt, 1992). As myonuclei are postmitotic, muscle repair and regeneration replies on its muscle stem cells (satellite cells) (**Figure i9**), which are located in a niche on the surface of the myofibre between the sarcolemma and the basal lamina (Katz, 1961; Mauro, 1961).

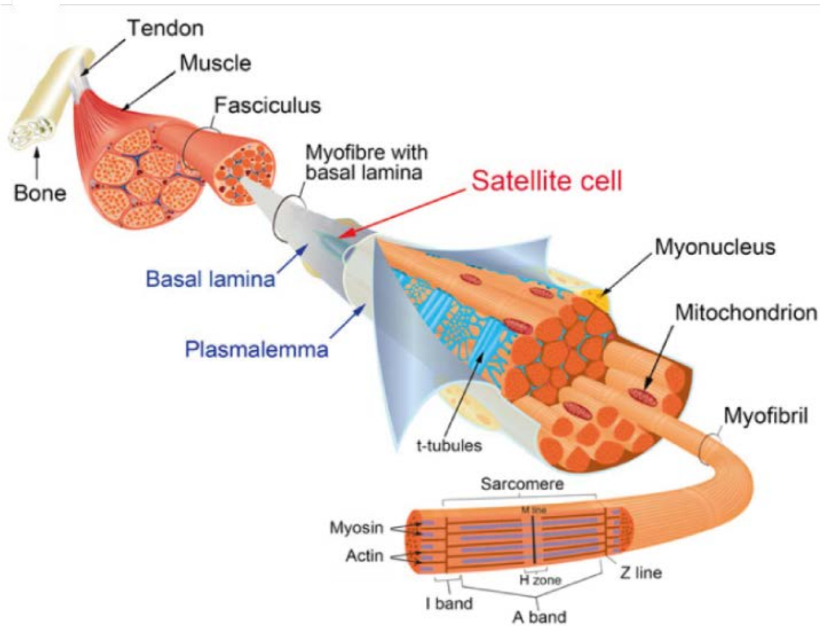

Figure i9: Muscle structure and the satellite cell niche.
Relaix and Zammit, 2012.

4.2. Skeletal muscle differentiation.

In resting adult muscles, satellite cells remain in a dormant state known as quiescence (a reversible G0 state). Different stimuli, associated with injury or disease, induce satellite cell activation and expansion as myoblasts. This process generally results in an asymmetric division, where one daughter cell commits to differentiate (committed progenitor cell) and is able to proliferate, differentiate, and fuse to generate mature myofibers, and the other daughter cell is capable to self-renew and return to quiescence to replenish the stem cell pool (Brack and Rando, 2012; Wang et al., 2014) (**Figure i10**).

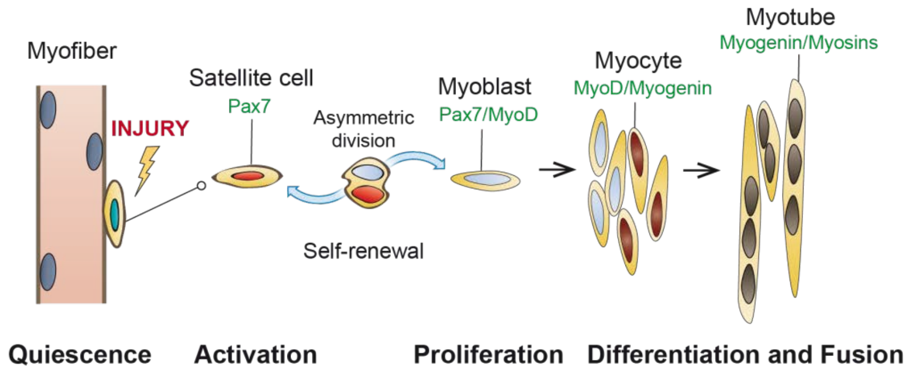

Figure i10: Schematic overview of satellite cell-driven myogenesis. Satellite cells remain in a quiescent state in adult muscles. Upon injury or disease, this population becomes activated and undergoes asymmetric division to generate a self-renewing daughter cell and a committed progenitor or myoblast, which proliferates, differentiates into myocytes, and fuses to form myotubes and new myofibers during adult muscle regeneration. In green, characteristic markers at the different myogenic stages. Adapted from Segalés et al., 2016.

Satellite cell quiescence is characterized by the high expression of the paired-box transcription factor Pax7, considered to be the definitive marker for satellite cells, although Pax3 is also expressed in some satellite cell subpopulations located in certain anatomical locations (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2006; Seale et al., 2000, 2004). Pax7 and Pax3 are thought to be the main regulators of muscle cell specification and tissue formation during the embryonic development (Chang and Rudnicki, 2014; Comai and Tajbakhsh, 2014; Soleimani et al., 2012a). Pax7 has an essential role in adult muscle stem cells maintenance and expansion, and this was demonstrated in several studies where long-term Pax7 ablation resulted in adult satellite cells loss, which results in an impaired muscle regeneration after injury, confirming its requirement for adult muscle repair (Lepper et al., 2011; Sambasivan et al., 2011; von Maltzahn et al., 2013;

Brack et al., 2014). Furthermore, Pax7 inactivation also leads to diminished heterochromatin condensation in adult satellite cells (Gunther et al., 2013), suggesting a role for Pax7 in chromatin organization, a function also suggested for Pax3 (Bulut-Karslioglu et al., 2012).

Many signals from the regenerative microenvironment mediate satellite cells' activation, including adhesion molecules, necrotic cues released from the damaged fibers, and/or growth factors and cytokines produced by neighboring cells, such as interstitial cells, resident macrophages, fibroblasts, and microvasculature-related cells (Giordani and Puri, 2013; Judson et al., 2013; Pannerec et al., 2013; Brancaccio and Palacios, 2015). These extracellular signals are transmitted to the myogenic cell nucleus through signaling cascades, including the p38 MAPK and the PI3K/AKT pathways (Cuenda and Cohen, 1999; Wu et al., 2000; Keren et al., 2006; Serra et al., 2007), and regulate the expression and activity of the bHLH family of muscle-specific regulatory factors (MRFs). The four MRF members (Myf5, MyoD, Myogenin and MRF4) are expressed at different stages of myogenesis, and cooperate with ubiquitously-expressed E proteins (the E2A gene products, E12 and E47, and HEB) and myocyte enhancer factor 2 (MEF2) transcriptional regulators to bind to E- and MEF2-boxes at muscle promoters. As a result, muscle-specific gene transcription is induced and structural and enzymatic muscle proteins such as α-actin, myosin heavy chain (MHC) or muscle creatine kinase (MCK) are transcribed (Lluis et al., 2006; Singh and Dilworth, 2013; Segales et al., 2015) (**Figures i10 and i11**). Several epigenetic mechanisms (including DNA methylation, chromatin remodeling and covalent modification of histones and

transcription factors) are also needed to coordinately regulate the myogenic gene expression program in order to progress through myogenesis (Dilworth and Blais, 2011; Segales et al., 2015).

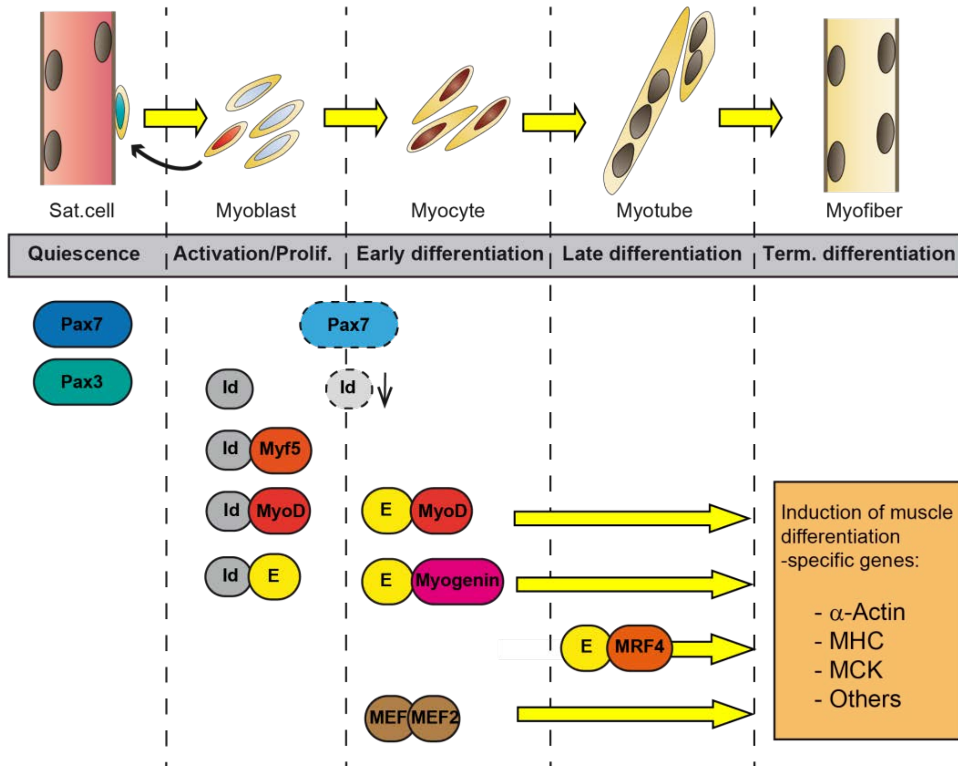

Figure i11: Schematic view of the transcriptional regulation of myogenesis. Satellite cell quiescence is characterized by the expression of Pax7 (and Pax3 at certain subpopulations). Upon different stimuli, satellite cells are activated and commit to proliferate and differentiate or self-renew to replenish the satellite cell pool. In proliferating myoblasts, early MRFs (Myf5 and MyoD) are transcriptionally induced, but they remain bound to Id proteins. At the onset of differentiation Pax7 and Id are downregulated, and MyoD (and later on Myogenin) form heterodimers with E proteins to, in cooperation con MEF2 transcriptional regulators, activate myogenic transcription and differentiate into mononucleated myocytes. At late differentiation, MRF4 is also expressed, and cooperates with the other MRFs in the induction of muscle-specific transcription, to differentiate into multinucleated myotubes and finally fuse into the mature myofiber. Adapted from Lluis et al., 2006.

4.2.1. Transcriptional and translational regulation of myogenesis.

Satellite cell-dependent myogenesis is a well-defined dynamic and multi-step process characterized by the sequential activation of MRFs. Myf5 and MyoD are induced in undifferentiated proliferating myoblasts, while Myogenin and MRF4 are expressed at the early and late phases of differentiation, respectively (Sartorelli and Caretti, 2005; Singh and Dilworth, 2013) (**Figure i11)**. Pax7 is expressed in quiescent muscle stem cells and is also essential for their cell cycle progression by regulating genes involved in cell proliferation, while preventing differentiation (Olguin et al., 2007; Soleimani et al., 2012a; von Maltzahn et al., 2013). Interestingly, some studies uncovered a quiescence signature, revealing that more than 500 genes are highly upregulated in quiescent satellite cells compared with cycling myoblasts, those including negative regulators of the cell cycle such as the CKIs $p27^{KIP1}$ and $p57^{KIP2}$, RB, the negative regulator of fibroblast growth factor (FGF) signalling sprouty 1 (Spry1), and many others (Fukada et al., 2007; Liu et al., 2013). Upon their activation from quiescence, Pax7-expressing satellite cells induce the expression of Myf5 and MyoD (Mckinnell et al., 2008), thus allowing successive rounds of cell proliferation (Olguin and Olwin, 2004), while Pax7 downregulation prior to Myogenin activation favors cell cycle withdrawal and differentiation entry (Olguin et al., 2007; Olguin, 2011; Bustos et al., 2015).

On the other hand, MRFs' expression is also subjected to translational regulation, especially in quiescence. Several mechanisms are reported to repress Myf5 and MyoD mRNAs

translation, including sequestration by microRNAs in ribonucleoprotein granules (Crist et al., 2012), or by tristetraprolin (TTP)-mediated degradation (Hausburg et al., 2015), respectively. In addition, a global mechanism of translational repression, through eukaryotic initiation factor eIF2α phosphorylation, preserves the exit from quiescence and induction of the myogenic program (Zismanov et al., 2016).

At the onset of differentiation, MyoD needs to associate with E proteins to bind E boxes of muscle gene promoters and activate their transcription. In proliferating myoblasts, the formation of functional MyoD/E protein heterodimers is actively inhibited by different mechanisms. Moreover, inhibitor of differentiation (Id) proteins are highly expressed during proliferation, and interact with either MyoD or E proteins (**Figure i11)**. Since they lack a basic DNA-binding domain, Id proteins impede heterodimer formation and association at myogenic loci, thereby inhibiting myogenic progression. Following cell cycle exit, Id expression is downregulated, allowing the formation of functional heterodimers and promoting muscle-specific transcription (Puri and Sartorelli, 2000). Other proteins have been reported to act as MRF repressors either by its direct association or by sequestering their functional partners, such as Snail and ZEB1 (Buas et al., 2010; Siles et al., 2013; Soleimani et al., 2012b).

Remarkably, recent genome-wide analyses of MyoD binding have revealed that MyoD is found associated to an extensive number of gene promoters that are not regulated during myogenesis, in both myoblasts and myotubes (Cao et al., 2010; Soleimani et al., 2012b), and its binding correlated with local

histone hyperacetylation, suggesting a role for MyoD in the epigenetic landscape reprogramming through the recruitment of histone acetyltransferases (HATs) to regions across the genome.

4.2.2. Epigenetic regulation of myogenesis.

As we mentioned above, muscle gene expression is also modulated by a number of epigenetic mechanisms, including DNA methylation, chromatin remodeling, post-translational modifications of histones and regulation by a network of noncoding RNAs. These epigenetic modifications are generally dynamic and reversible and can be associated with gene activation or repression (Bergman and Cedar, 2013).

DNA methylation has been largely established as a permanent repressive epigenetic mark. Nevertheless, more recent studies through genome-wide analysis of DNA methylation in different genomic contexts have questioned this notion, since active DNA methylation and demethylation occur during cellular differentiation and tissue specification, suggesting a more dynamic regulation than previously anticipated (Carrió et al., 2015; Carrió et al., 2016; Carrió and Suelves, 2015; Suelves et al., 2016). In a recent work, DNA methylation dynamics of the major genes orchestrating myogenic determination and differentiation was addressed for the first time, by comparing pluripotent embryonic stem cells (ESCs), myogenic precursors from Pax7-inducible ESCs, proliferating muscle stem cells, and their respective myotube derivatives, and showed a common muscle-specific DNA

demethylation signature required to acquire and maintain the muscle-cell identity (Carrió et al., 2016).

Moreover, studies of human and mouse ESCs have outlined the role of the histone methyltransferase (HMT) families Polycomb group (PcG) and Trithorax group (TrxG) in modulating the pluripotency and lineage commitment in different cell types (Mohn et al., 2008). The TrxG family of proteins has been largely associated with transcriptional gene activation; trimethylation of histone 3 at lysine 4 ($H3^{K4me3}$) surrounding the transcription start sites (TSS), and trimethylation of histone 3 at lysine 36 ($H3^{K36me3}$) in the gene body, are generally associated with active gene transcription. Alternatively, the histone 3 trimethylation at lysine 27 ($H3^{K27me3}$) mediated by the PcG complex is associated with transcriptional repression (Kouzarides, 2007). Despite that the repressive $H3^{K27me3}$ mark is transmitted to daughter cells (Hansen et al., 2008) and is dominant over the permissive $H3^{K4me3}$ mark (Barski et al., 2007), transcriptional gene activation requires the demethylation of $H3^{K27me3}$, which is mediated by lysine-specific demethylase 6A (KDM6a) and KDM1 lysine-specific demethylase 6B (KDM6b) (Agger et al., De Santa et al., 2007; Lan et al., 2007). Thus, whereas polycomb repressive complex 2 (PRC2) establishes gene silencing at developmentally regulated loci, the TrxG and KDM6a/KDM6b families work together to antagonize the repressive activity of PRC2 and to promote gene expression in specific cell types.

Another epigenetic mechanism that has been largely involved in transcription by multiple mechanisms is histone acetylation of histone tails, associated with chromatin relaxation

and thus increasing the accessibility of transcription factors to their target genes (Shahbazian and Grunstein, 2007). Histone acetylation by histone acetyltransferases (HATs) can be recognized by transcriptional activators as binding regions. Conversely, histone deacetylation by deacetylases (HDACs) induces transcriptional repression by favoring the compactation of the chromatin structure (Ruthenburg et al., 2007). Both HATs and HDACs act on chromatin by associating with a variety of DNA-binding transactivator proteins. Lastly, small noncoding RNAs (miRNAs) have been largely associated to negative regulation of target mRNAs by different mechanisms (Nilsen, 2007), although miRNAs can also stimulate gene expression in response to specific cellular conditions or cofactors (Vasudevan, 2012).

In the context of satellite cell-dependent myogenesis, while the Pax7 gene must be expressed in quiescence, modulators of cell cycle progression and MRFs need to remain silent. Increasing evidence suggest that quiescent satellite cells are not in a dormant state but rather are primed for activation and differentiation in response to external stimuli (Cheung et al., 2012; Crist et al., 2012; Rodgers et al., 2014). At the chromatin level, this primed state is maintained by the general lack of the repressive mark $H3^{K27me3}$ throughout the genome and the concomitant presence of activation mark $H3^{K4me3}$ at the transcription start site (TSS) of many genes, including MyoD and Myf5 (Liu et al., 2013; Caretti et al., 2004). In addition, a large number of TSSs across the genome contain bivalent chromatin domains in quiescent satellite cells (Liu et al., 2013), which are characterized by the presence of both $H3^{K4me3}$ and $H3^{K27me3}$ marks, many of them corresponding to lineage-specific genes (Mikkelsen et al., 2007). Interestingly, Pax3 is the

only myogenic transcription factor having bivalent domains in quiescent satellite cells, whereas Pax7 is only marked by the $H3^{K4me3}$ activation mark. In clear contrast, Myogenin displays a significant enrichment of the $H3^{K4me3}$ mark at its TSS upon satellite cell activation (Liu et al., 2013). Together, these data suggest an interplay between the repressive Polycomb group and activating Trithorax group complexes. In addition, other methyl-transferases as PRDM2 (Cheedipudi et al., 2015), Carm1 (Kawabe et al., 2012) and Suv4-20H1 (Boonsanay et al., 2016) are implicated in the repression of cell cycle genes, allowing Pax7 expression, or altering chromatin structure leading to repression of MyoD expression, respectively. Moreover, miRNAs as miR-489 and miR-31 also contribute to maintain the quiescent state by different mechanisms (Cheung et al., 2012; Crist et al., 2012).

Upon satellite cell activation, epigenetic events are critical to maintain satellite cells in a proliferating state and prevent their premature differentiation. In response to different stimuli as muscle damage, satellite cells start to express cell cycle regulators, which are readily marked by permissive $H3^{K4me3}$ (Sebastian et al., 2009), and re-enter the cell cycle. After the asymmetric division, the committed myoblasts start to proliferate (Kuang and Rudnicki, 2008) and express Myf5 and MyoD genes, as well as genes that control cell cycle progression, all of which are characterized by the enrichment of the transcriptionally permissive $H3^{K4me3}$ mark at their TSSs (McKinnell et al., 2008). During myoblast proliferation, different classes of HDACs are also involved in the repression of transcription of muscle genes by opposing HATs activities. Class I and II HDACs contribute to the hypoacetylation of the MyoD gene and the inhibition of MEF2 transcription and activation,

respectively. MyoD also associates with HDAC1, and this interaction is responsible for silencing the MyoD-dependent transcription of $p21^{CIP1}$ and muscle-specific genes (Mal et al., 2001; Puri et al., 2001). Moreover, Class II HDACs are recruited by MEF2 and MyoD (Lu et al., 2000), which in turn recruits several histone methyltransferases (Suv39H1, G9a and MLL3/4) (Ling et al., 2012). A recent study of HDAC4 function and satellite cell proliferation reported that the HDAC4 levels positively correlate with the expression of Pax7 (Choi et al., 2014). Moreover, satellite cell proliferation is also promoted and maintained by miR-27a/b, miR-133a, miR-682, and miR-206 by different mechanisms (McFarlane et al., 2014; Crist et al., 2009; Chen et al., 2006; Chen et al., 2011; Liu et al., 2010).

During satellite cell differentiation, genes no longer needed for lineage progression are targeted for stable repression (Liu et al., 2013; Dilworth and Blais, 2011). Chromatin converts to a more repressed state by accumulating $H3^{K27me3}$ across the genome at both TSSs and intergenic regions, and this repressive mark is dramatically increased in differentiating muscle stem cells. More in detail, when satellite cells differentiate, PRC2 is released from muscle-specific genes as MyoD to translocate to loci that are typically repressed in differentiated myotubes. Indeed, Pax7 is progressively silenced during the transition from a transcriptionally permissive state ($H3^{K4me3}$) to a repressive state ($H3^{K27me3}$) via PRC2 throughout cell differentiation (Palacios et al., 2010), thus contributing to the switching off of satellite cell proliferation (Palacios et al., 2010). In a similar manner, a switch from permissive to repressive marks occurs on cell cycle-related genes, mediated by E2F transcription factors and by RB as the satellite

cell exits the cell cycle to terminally differentiate (Sebastian et al., 2009; Blais and Dynlacht, 2007; Blais et al., 2007). Furthermore, Pax7 associates with the Wdr5-Ash2L-MLL2 HMT complex, which mediates $H3^{K4me3}$ mark (McKinnell et al., 2008). The binding of the Pax7-HMT complex to Myf5 results in the establishment of $H3^{K4me3}$ on the surrounding chromatin. Thus, Pax7 also contributes in the induction of chromatin modifications that favor transcriptional activation of target genes to regulate the entry into the muscle differentiation program. At the same time, lysine-specific demethylase 4A (KDM4a), together with heterochromatin protein 1 (HP1) α, promotes the dimethylation of $H3^{K9me3}$ at muscle-specific promoters, enabling myoblast commitment (Sdek et al., 2013).

4.3. RB and cell cycle regulation in myogenesis.

In addition to orchestrating mitotic arrest and preventing cell cycle re-entry as a G1 checkpoint, RB controls cellular differentiation during embryogenesis and in adult tissues, regulates apoptotic cell death, maintenance of permanent cell cycle arrest and preservation of chromosomal stability (reviewed in Burkhart and Sage, 2008). RB is necessary for the completion of the muscle differentiation program and for myogenic-dependent transcription. In fact, in addition to induction and maintenance of permanent cell cycle withdrawal through negative regulation of E2F-responsive genes involved in proliferation (reviewed in De Falco et al., 2006), RB plays a positive role in the activation of muscle-specific genes (Gu et al., 1993; Schneider et al., 1994; Novitch et al., 1999; Lasorella et al., 2000; MacLellan et al., 2000; Puri et al., 2001; Benevolenskaya et al., 2005).

4.3.1. RB regulation in satellite cell homeostasis and myoblast differentiation.

RB null mice die before birth and lack differentiated muscles (Zacksenhaus et al., 1996; Takahashi et al., 2003), and RB-null muscle cells fail to exit the cell cycle and are susceptible to apoptotic cell death (Ferreira et al., 1998; Vandel et al., 2001; Nicolas et al., 2003; Frolov and Dyson, 2004). The acute suppression or permanent elimination of RB produced contradictory results regarding its effect in reversing differentiation and postmitotic arrest in skeletal muscle cells. In immortalized mammalian myoblast cell lines, such as C2C12 cells, RB inactivation by viral oncoproteins resulted in BrdU incorporation in nuclei of differentiated myotubes and S phase re-entry (Gu et al., 1993; Crescenzi et al., 1995), and this was also observed in other studies inactivating RB by the use of siRNAs (Blais et al., 2007). Conversely, in similar experiments with primary muscle cells isolated directly from mammalian muscle tissues, RB reduction or elimination by Cre-mediated excision failed to result in significant S phase re-entry (Sacco et al., 2003; Camarda et al., 2004; Huh et al., 2004).

In addition to its inhibitory role on cell proliferation, RB has also been shown to impact differentiation and muscle-specific gene expression. RB loss or suppression leads only to moderate impairment in differentiation in primary differentiated skeletal muscle cells (Camarda et al., 2004; Huh et al., 2004; Pajcini et al., 2010), as demonstrated by the reduced accumulation of Myogenin and MHC (Pajcini et al., 2010). These data suggest that RB loss in terminally differentiated muscle cells is not sufficient to induce

reversal of the postmitotic state in mammals, and other regulatory mechanisms may be involved in this regulation. Moreover, the simultaneous inactivation of RB and $p19^{ARF}$ generated an extensive impairment in differentiation in myocytes (Pajcini et al., 2010). The profound loss of architectural integrity and downregulation of Myogenin, MRF4, MHC, and MCK when RB and $p19^{ARF}$ were deleted together, suggest a role for these factors as potent stabilizers of the differentiated state (Pajcini et al., 2010).

Other studies reported that RB is required to inhibit apoptosis in myoblasts and autophagy in myotubes, but not to activate the differentiation program *in vitro* (Ciavarra and Zacksenhaus, 2010). Indeed, the study of pocket family members in double and triple knockout myoblasts showed that these cells can undergo robust myogenic differentiation in the absence of RB and one of its relatives, p107 or p130, under conditions where autophagy defects were rescued. Nevertheless, mutations in all RB family severely abrogated myogenic differentiation, indicating that myoblast fusion and myotube survival require at least one RB family member (Ciavarra et al., 2011).

Importantly, the loss of RB has also an impact in satellite cell homeostasis. Keller and collaborators explored the role of RB in muscle stem cell activation and proliferation *in vivo* using conditional mice where RB was only deleted in satellite cells (Hosoyama et al., 2011). RB loss generated a significant increase in satellite cell and myoblast numbers, while terminal differentiation was greatly impaired (Hosoyama et al., 2011). These mice also displayed muscle fiber hypotrophy as well as a delay of muscle regeneration, suggesting that cell cycle re-entry of quiescent

muscle stem cells is accelerated following RB loss, resulting in the expansion of both satellite cells and their derived myoblast in adult muscle and a defect in muscle fiber formation (Hosoyama et al., 2011). Moreover, the pharmacological inhibition of protein phosphatase activity, resulting in RB hypophosphorylation (inactivation), accelerated satellite cell activation and expansion in a transient manner (Hosoyama et al., 2011).

4.3.2. Role of RB in the transcriptional and epigenetic regulation during myogenesis.

During myogenesis, RB becomes hypophosphorylated, a state associated with its active role as E2F transcriptional repressor, and its mRNA and protein expression levels increase in a MyoD-and cyclic AMP responsive element binding protein (CREB)-dependent manner (Martelli et al., 1994; Corbeil et al., 1995; Magenta et al., 2003). When differentiation is induced, CREB transcription factor is upregulated and phosphorylated, and is able to recruit a multi-protein complex containing MyoD and the HATs p300 and PCAF on RB promoter to induce its expression (Magenta et al., 2003). Of note, despite the abundance of hypophosphorylated RB, p130-E2F repressor complexes are predominant in myotubes (Corbeil et al., 1995; Puri et al., 1997).

RB is thought to stimulate lineage-specific transcription factors, including MyoD, myogenin, and MEF2C (Gu et al., 1993; Schneider et al., 1994; Novitch et al., 1999), and sequester inhibitors of differentiation such as Id2, HDAC1, EID-1, and RBP2 (Lasorella et al., 2000; MacLellan et al., 2000; Puri et al., 2001;

Benevolenskaya et al., 2005). RB-deficient muscle cell lines are able to express normal levels of early differentiation effectors, as Myogenin and $p21^{CIP1}$, but display reduced levels of MHC and have a deficient differentiation (Frolov and Dyson, 2004), being fusion markedly impaired (Zacksenhaus et al., 1996). RB (and p130) are also able to bind the histone methyltransferase Suv39H1 that is able to methylate the lysine 9 residue of histone H3 (H3K9), which was suggested to be involved in the repression of E2F-dependent genes in quiescent cells (Vandel et al., 2001; Nicolas et al., 2003; Nielsen et al., 2001). Accordingly, E2F-dependent gene downregulation in myotubes correlated with an increase of H3K9 methylation by Suv39H1/2 (Ait-Si-Ali et al., 2004; Vandromme et al., 2008). Moreover, Trouche and collaborators investigated pocket protein binding to E2F-regulated promoters during muscle differentiation, demonstrating that RB bound equally well to E2F-promoters in quiescent cells compared with myotubes. This suggests that RB binding is not the exclusive determinant of permanent repression of E2F-regulated promoters during muscle differentiation (Vandromme et al., 2008).

4.4. p38 MAPK signaling in myogenesis.

4.4.1. Function of p38 MAPK at distinct stages of the myogenic process.

The p38 MAPK signaling pathway has an active role in each myogenic stage, as it has been demonstrated by the use of cellular models (myoblast cell lines, such as C2C12 cells, or satellite cell-derived primary myoblasts) able to recapitulate myogenesis *in vitro*.

Remarkably, p38 MAPK acts as a regulator of the proliferation-to-differentiation transition in myoblasts, being involved in the induction of cell cycle withdrawal and the expression of muscle-specific genes (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000; Li et al., 2000a, Keren et al., 2006; Lluis et al., 2005; Suelves et al., 2004; Serra et al., 2007; Perdiguero et al., 2007a; Ruiz-Bonilla et al., 2008; Simone et al., 2004; Forcales et al., 2012; Rampalli et al., 2007; Cuadrado et al., 2010; Segales et al., 2016a). Besides, p38 signaling functions as one of the main controllers of satellite cells' fate decisions (Jones et al., 2005; Palacios et al., 2010; Troy et al., 2012; Brien et al., 2013; Bernet et al., 2014; Hausburg et al., 2015; Charville et al., 2015) (**Figure i12**).

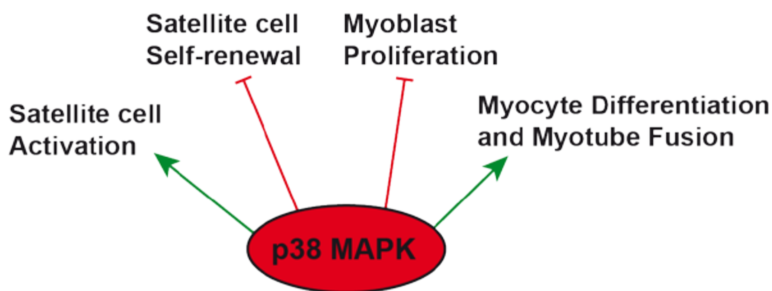

Figure 12: Role of p38α MAPK in satellite cell-dependent myogenesis. Diverse roles have been attributed to p38 signaling. Among them, p38α negatively regulates myoblast proliferation and favors myocyte differentiation and fusion into mature myotubes. p38 MAPK has also been also involved in the control of satellite cell activation and self-renewal.

Several stimuli, including inflammatory cytokines (as TNFα or amphotericin/HMGB1), growth factors (TGFβ) or cell-to-cell contact, can activate p38 MAPK in satellite cells (Krauss, 2010; Guasconi and Puri, 2009). Pioneer *in vitro* studies demonstrated the requirement of p38 MAPK pathway for skeletal muscle

differentiation through different mechanisms (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000). p38α signaling has been proven to be critical at the onset of muscle differentiation by a mechanism involving the inhibition of the proliferation-promoting JNK pathway by inducing the upregulation of the JNK phosphatase MKP-1, thus leading to cyclin D1 expression downregulation and induction of cell cycle withdrawal (Perdiguero et al., 2007b).

p38 MAPK also regulates skeletal muscle differentiation and fusion. Pharmacological inhibition of p38α/β signaling in myoblasts prevents the induction of muscle-specific genes and fusion into myotubes, while forced p38 MAPK activation by ectopic expression of a constitutively active MKK6 mutant in proliferating myoblasts was sufficient to induce both the expression of myogenic markers and the appearance of multinucleated myotubes (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000; Li et al., 2000). The study of the relative contribution of the four p38 isoforms in muscle stem cells indicated that they are not completely redundant during muscledifferentiation, and revealed a predominant role for p38α isoform in myogenic differentiation and fusion by regulating the whole myogenic transcriptional program at multiple stages (Ruiz-Bonilla et al., 2008; Wang et al., 2008; Liu et al., 2012), and by promoting myoblast fusion through tetraspanin CD53 upregulation (Liu et al., 2012). Otherwise, p38γ signaling contributes to myoblast proliferation by preventing premature differentiation through the induction of a repressive MyoD transcriptional complex (Gillespie et al., 2009), while the other isoforms (p38β and p38δ) appear to be dispensable for these processes (Perdiguero et al., 2007a; Ruiz-Bonilla et al., 2008).

Other studies have established a role for p38 MAPKs in the maintenance of satellite cells quiescence and activation. p38α/β signaling is required for satellite cell activation and MyoD induction (Jones et al., 2005), and once activated, p38α leads to tristetraprolin (TTP) inactivation and MyoD mRNA stabilization (Hausburg et al., 2015). Following activation, muscle stem cells enter the cell cycle and a subset undergoes asymmetric division to replenish the muscle stem cell pool. Remarkably, p38α/β MAPKs are asymmetrically activated in only one daughter cell, in which MyoD is induced, allowing cell cycle entry and commitment to generate a proliferating myoblast. Conversely, MyoD induction is prevented in the other daughter cell by the absence of p38α/β signaling, renewing the quiescent satellite cell pool (Troy et al., 2012).

The use of mice with conditional deletion of p38α in satellite cells disclosed a role of p38α in skeletal muscle growth and regeneration *in vivo*. Work from the Pell laboratory confirmed previous *in vitro* studies (Perdiguero et al., 2007a) and demonstrated that p38α restrains postnatal proliferation and promotes timely myoblast differentiation (Brien et al., 2013). The loss of p38α in the Pax7-lineage generated a postnatal growth defect together with an augmented number of muscle stem cells, resulting from increased myoblast proliferation postnatally. Furthermore, muscle regeneration after injury was delayed in the absence of p38α, with further increase of the satellite cell population (Brien et al., 2013). Interestingly, p38α ablation was accompanied by increased p38γ phosphorylation, and p38γ inhibition *ex vivo* significantly diminished the myogenic defect. As muscle regeneration can occur in the absence of p38γ quite

effectively (Ruiz-Bonilla et al., 2008), but is defective when p38α is absent (Brien et al., 2013), p38α arises as the master kinase for transcriptional reprogramming during satellite cell proliferation-to-differentiation transition, both *in vitro* and *in vivo*.

Similar to mice, p38 MAPK has a critical role in the regulation of human muscle stem cell functions (Charville et al., 2015). p38 is upregulated in activated human satellite cells compared with quiescent ones. Moreover, reversible p38α/β inhibition in cultured human satellite cells promoted its expansion and prevented differentiation. These expanded satellite cells displayed an enhanced self-renewal and engraftment capacity in transplantation experiments compared to freshly isolated satellite cells or cells cultured in the absence of p38α/β inhibitors (Charville et al., 2015).

4.4.2. Transcriptional and epigenetic regulation of myogenesis by p38 MAPK.

p38 MAPK pathway has an active role at the onset of myogenesis by modulating the expression and/or activity of several players implicated in the transcriptional and epigenetic regulation of myogenesis. p38α/β signaling induce MEF2 transcriptional activity and MyoD/E47 heterodimer formation by direct phosphorylation on MEF2 and E47 (Zetser et al., 1999; Lluis et al., 2005), thus enhancing RNA Pol II recruitment to myogenic loci, critical step in the transcriptional initiation of the differentiation program. Furthermore, p38α/β kinases phosphorylate the chromatin-associated protein BAF60c, contributing to the myogenic

transcriptosome assembly on the chromatin of muscle loci by promoting the recruitment of SWI/SNF chromatin remodeling complex (Simone et al., 2004; Serra et al., 2007; Forcales et al., 2012) and ASH2L-containing mixed-lineage leukemia (MLL) methyltransferase complex (Rampalli et al., 2007). p38α MAPK also phosphorylates and recruits SNF2-related CBP activator protein (SRCAP) subunit p18Hamlet to muscle loci, required for H2A.Z accumulation and activation of gene expression (Cuadrado et al., 2010). Moreover, in late stages of myogenesis, p38 phosphorylates MRF4, resulting in reduced MRF4-mediated transcriptional activity, a mechanism that led to downregulation of specific muscle genes (Suelves et al., 2004).

p38α is also involved in Pax7 transcriptional repression in differentiating satellite cells by a mechanism involving the phosphorylation of Ezh2, the catalytic subunit of the PRC2 complex. Ezh2 phosphorylation promotes the association between YY1 transcription factor and PRC2, leading to repressive chromatin on the Pax7 promoter and therefore modulating satellite cells' decision to proliferate or differentiate (Palacios et al., 2010; Mozzetta et al., 2011). Conversely, p38γ activation in muscle cells leads to MyoD phosphorylation, resulting in an enhanced MyoD occupancy on the Myogenin promoter together with markedly decreased transcriptional activity, which was associated with extensive methylation of histone H3K9 together with recruitment of the KMT1A methyltransferase, thus also affecting this myogenic decision (Gillespie et al., 2009). Furthermore, two independent studies proved that the activation of MSK1 (a p38α/β downstream kinase) leads to H3 phosphorylation on serine 28, favoring an interchange between Ezh2- and Ezh1-containing PRC2 complexes

on Myogenin promoter in differentiating myotubes (Stojic et al., 2011; Mousavi et al., 2012). Of interest, Ezh1-containing complexes were associated to RNA Pol II recruitment and transcriptional activation, questioning the paradigm of PRC2 complexes as chromatin repressors (Mousavi et al., 2012). Thus, depending on the engagement of specific p38 isoforms, the p38 MAPK pathway can either induce or repress transcription in muscle stem cells.

In response to stress, p38 MAPK (and Hog1, its homolog in yeast) acts directly at chromatin and, through its interaction with stress-responsive promoters, and activate transcription (de Nadal et al., 2004; Pokholok et al., 2006; Ferreiro et al., 2010b). Similarly, during myogenic differentiation, p38α associates to several muscle-specific genes, such as Myogenin, MCK, and MHC (Simone et al., 2004; Palacios et al., 2010). Remarkably, p38α also exerts its promyogenic function, at least in part, by binding and acting at chromatin (Segales et al., 2016a). Genome-wide localization analysis linked to gene expression profiling revealed that p38α associates to a large number of active promoters during the myoblast transition from proliferation to differentiation, demonstrating the relevance of kinase signaling pathways in the direct regulation of transcription (Segales et al., 2016a), being these results consistent with previous studies for other kinases (Bungard et al., 2010; Tiwari et al., 2012; Di Vona et al., 2015). Of note, p38α also associated to transcriptionally inactive or repressed promoters at the onset of myogenesis (Segales et al., 2016a). Thus, p38α is recruited to an extensive set of myogenic promoters to facilitate their activation or repression, suggesting a higher degree of complexity in their regulation. Lastly, p38α recruitment to

muscle loci probably involves the interaction with transcription and/or chromatin-regulatory factors, as it was reported for several stress-induced genes (Ferreiro et al., 2010b), given the observation that p38α-bound promoters are enriched with binding motifs for certain transcription factors, some of them known to be p38 MAPK substrates (Segales et al., 2016a).

4.5. Impact of aging on satellite cell functions.

The aging process affects all tissues of an organism, and can be explained by accumulation of damage at a molecular, cellular and tissular level. Common hallmarks of aging across different species have been proposed in the last years, including genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Lopez-Otin2013).

Skeletal muscle has a remarkable capacity to regenerate that declines with aging, although whether this is due to extrinsic environmental changes and/or to satellite cell-intrinsic mechanisms associated to aging has been a controversial question for a long time (Brack and Muñoz-Cánoves, 2016). Particularly in skeletal muscle, aging has been associated with a loss of muscle mass and function (sarcopenia), together with a decline in muscle regenerative potential of muscle stem cells (Garcia-Prat et al., 2013), resulting in an impaired capacity to maintain homeostasis and repair (Hikida, 2011; Zwetsloot et al., 2013). This has been at least partially ascribed to a numerical loss of the satellite cell pool

by approximately 50% with age (Sousa-Victor et al., 2014a,b; Hawke and Garry, 2001; Shefer et al., 2006, 2010; Zammit et al., 2002). Several studies have proposed that age-associated changes in the local microenvironment and systemic cues, as well as cell-intrinsic alterations, which alters the balance between quiescence and proliferation and have an impact in cell survival, as the main drivers of satellite cell numerical and functional decline during aging (Sousa-Victor et al., 2015; Segales et al., 2016b).

4.5.1. Intrinsic alterations in aged satellite cells.

Recent findings have suggested that the decline in satellite cell function has a strong cell-intrinsic component. These cell-intrinsic alterations include deficient mechanisms to maintain genome integrity (and lead to genomic instability), oxidative and DNA damage accumulation, and alterations in mitochondrial function and autophagy (Hasty et al., 2003; Ames, 2004; Golden et al., 2002; Pietrangelo et al., 2009; Zhang et al., 2016; Garcia-Prat et al., 2016).

Despite that satellite cells seem to be more resistant to DNA damage and more efficient repairing DNA lesions than their committed progeny (Vahidi Ferdousi et al., 2014), a long-time exposure to genotoxic stresses and a decline in antioxidant capacity with aging (Fulle et al., 2005) have a deleterious impact on their genomic integrity. When isolated from aged muscles, satellite cells display an increased number of foci containing phosphorylated histone H2AX, a marker of DNA damage (Sinha et al., 2014; Sousa-Victor et al., 2014a). Moreover, in mice deficient

for Ku80, a subunit in the non-homologous end-joining pathway (NHEJ) that repairs double-strand DNA breaks, skeletal muscle display an accelerated aging phenotype (Didier et al., 2012). Still, the role of DNA damage in satellite cell aging remains unknown, but it has been proposed to be responsible for age-associated muscle stem cell loss of function and regenerative capacity.

High-throughput gene expression profiling studies in young and aged human satellite cells disclosed remarkable differences in the transcriptional program that would explain the altered function with aging, including dysregulated expression of myogenic-specific genes, antioxidant enzyme-coding genes or genes related to protein folding (Bortoli et al., 2003; Pietrangelo et al., 2009; Charville et al., 2015). In agreement with these data, aged satellite cells have a reduced myogenic ability, including the capacity to fuse into myotubes (Collins et al., 2007; Chargé et al., 2002), and a lower ability to form myogenic colonies and to activate and proliferate (Conboy et al., 2003; Shefer et al., 2006; Shadrach and Wagers, 2011; Day et al., 2010; Baj et al., 2005). Moreover, aged satellite cells tend to adopt fibroblastic and adipogenic fates *in vitro* (Brack et al., 2007) and *in vivo*, particularly in pathological aging muscle (Pessina et al., 2015; Biressi et al., 2014), thus explaining the high levels of fat deposition and fibrotic tissue in aged mice muscle. These differences in age-associated gene expression could be partly attributed to epigenetic alterations (Liu et al., 2013).

In recent years, evidence linking metabolism, mitochondrial dynamics, and protein homeostasis (proteostasis) as essential regulators of stem cell functions has emerged, having a deep impact in satellite cell function during aging (reviewed in García-

Prat et al., 2017; García-Prat et al., 2016; Zhang et al., 2016). Entry of satellite cells into the activation state in response to damage-induced signals requires rapid changes in protein composition, eliminating proteins involved in quiescence maintenance and supplying new proteins involved in cell-cycle regulation and differentiation (García-Prat et al., 2017). Mitochondrial dysfunction, induced by calorie-dense diets or aging, can result from depletion of the oxidized form of nicotinamide adenine dinucleotide (NAD^+), whereas NAD^+ repletion with precursors such as nicotinamide riboside (NR) can reverse this process (Cantó et al., 2012; Pirinen et al., 2014; Mouchiroud et al., 2013; Yoshino et al., 2011; Gomes et al., 2013). Mitochondrial function has been linked to satellite cell maintenance and activation (Cerletti et al., 2012; Stein and Imai, 2014; Katajisto et al., 2015; Ryall et al., 2015). In a recent study, treatment with the NAD^+ precursor NR rejuvenated satellite cell in aged mice by protecting them from senescence and safeguarding muscle function (Zhang et al., 2016). In another recent work from our laboratory, we demonstrated that basal autophagy is essential to maintain the quiescent state in satellite cells. We reported that a failure in autophagy in aged resting stem cells leads to accumulation of damaged proteins and dysfunctional organelles (especially mitochondria), enhanced reactive oxygen species (ROS) and DNA damage accumulation, and ultimately leading to stem-cell exhaustion (García-Prat et al., 2016).

4.5.2. Extrinsic factors linked with satellite cells aging.

A proper stem cell function is supported and regulated by different extrinsic factors from the surrounding microenvironment,

usually referred to as the 'niche', and external cues generated by local inflammation or systemic sources (Jasper and Kennedy, 2012; Jones and Rando, 2011). The muscle fiber acts as a niche, and participates in the maintenance of the reversible quiescent state (Chakkalakal et al., 2012; Rezza et al., 2014; Chang and Rudnicki, 2014; Jung et al., 2014). Findings by Brack and coworkers strongly supported this notion, demonstrating that an increased expression of myofiber-derived fibroblast growth factor 2 (FGF2) with aging disrupted quiescence in a subset of muscle stem cells, leading to spontaneous mitogenic activity and reduction of the stem cell pool with aging (Chakkalakal et al., 2012). Remarkably, FGF2 activity neutralization or interference with their receptors (FGFRs) was sufficient to restore quiescence in old satellite cells (Chakkalakal et al., 2012). High FGF2 expression in quiescent satellite cells generated a downregulation of Sprouty1 (a negative regulator of FGF-induced signaling) (Chakkalakal et al., 2012), previously shown to be required for satellite cell self-renewal during regeneration (Abou-Khalil and Brack, 2010; Shea et al., 2010). Thus, FGF2/Sprouty1 axis dysregulation in old satellite cells would be an obstacle to maintain quiescence.

Other studies have documented the existence of additional niche-derived factors regulating satellite cell function. Increased levels of TGFβ have been reported in aged mice, which correlated with enhanced Smad transcription factor activation, thus dysregulating the endogenous Notch/Smad3 balance (Carlson et al., 2008).This lack of regulation resulted in inhibited satellite cell proliferation and limited aged muscle regenerative potential (Carlson et al., 2008). Other circulating factors as Wnt ligands are abundant in serum of aged individuals, and Wnt signaling was

found increased in old muscle, contributing to the promotion of satellite cell differentiation and reducing its potential to self-renew (Brack et al., 2007). Interactions between Notch and Wnt signaling have also been demonstrated by the fact that young satellite cells recapitulated the effects on Wnt signaling following Notch signaling inhibition, whereas delivery of active Notch could partially rescue muscle regeneration in aged mice (Conboy et al., 2003; Conboy et al., 2005; Conboy and Rando, 2005).

The contribution of the environment to the process of satellite cell aging was further reinforced by pioneer heterochronic tissue transplant experiments (Harrison, 1983; Carlson and Faulkner, 1983) and, more recently, by heterochronic parabiosis studies (Brack and Rando, 2007; Villeda et al., 2011; Conboy et al., 2005), both of which strongly suggested the existence of environmentally-derived factors that are altered with age and affect the muscle stem cells function. In these studies, the regenerative capacity of aged satellite cells could be rejuvenated by exposure to a young myogenic environment (Gutmann and Carlson, 1976; Carlson and Faulkner, 1989; Roberts et al., 1997) or to young systemic factors, achieved by connecting the circulatory system of young and old animals (Brack and Rando, 2007; Villeda et al., 2011; Conboy et al., 2005). Interestingly, the regenerative capacity of young satellite cells from the heterochronic pair was also impaired, supporting the notion of circulating age-related factors that contribute to muscle stem cell functional decline. Indeed, reestablishment of Notch signaling proved to be part of the mechanisms through which young serum could rescue aged satellite cell function in heterochronic parabiosis experiments, with

similar results observed *in vitro* (Wagers and Conboy, 2005; Conboy et al., 2005; Carlson and Conboy, 2000).

The reduced regenerative potential of aged satellite cells has also been associated with increased JAK/STAT signaling, which impairs muscle stem cell function by stimulating asymmetric division (Price et al., 2014). Furthermore, interleukin (IL)-6-activated STAT3 regulates the expression of the myogenic factor MyoD, promoting satellite cell differentiation in detriment to their expansion (Tierney et al., 2014). In a recent study, Hoxa9 developmental gene was described to act as a central hub required for the parallel induction of downstream targets of the Wnt, TGFβ and JAK/STAT pathways in aged satellite cells (Schworer et al., 2016). The aberrant global and site-specific induction of active chromatin marks in activated satellite cells from aged mice, resulted in the specific induction of Hoxa9 (Schworer et al., 2016). Conversely, inhibition of aberrant chromatin activation, or deletion of Hoxa9, improved satellite cell function and muscle regeneration in aged mice, whereas overexpression of Hoxa9 mimicked aging-associated defects (Schworer et al., 2016).

4.5.3. p38 MAPK signaling regulates satellite cell functions during aging.

Different signaling pathways have been found dysregulated in muscle stem cells from aged mice, including fibroblast growth factor receptor-1 (FGFR1), p38 MAPK, and Janus kinase–Signal transducer and activator of transcription (JAK/STAT), thus contributing to defective regulation of quiescence and

compromised self-renewal potential (Bernet et al., 2014; Cosgrove et al., 2014; Price et al., 2014; Tierney et al., 2014).

It is well established that the FGFR1 signaling pathway is altered during aging. It has been suggested that increased FGF2 signaling in aged muscle can generate the disruption of satellite cell quiescence (Chakkalakal et al., 2012). In a more recent study from the Olwin group it was proposed that the augmented FGF2 in the aged satellite cell niche constitutes a compensatory mechanism due to the loss of FGFR1 signaling (Bernet et al., 2014). Moreover, an altered FGF2 signaling in combination with elevated TNFα levels observed in old muscles, have been associated to constitutive and aberrant activation of the p38 MAPK signaling pathway, which ultimately leads to impaired self-renewal of aged satellite cells (Bernet et al., 2014). More in detail, proper and asymmetric p38α/β MAPK activation is required to promote self-renewal in satellite cells from young mice, generating a quiescent daughter and a lineage-committed daughter cell. The defect observed in self-renewal is due to elevated phosphorylated p38 in aged satellite cells, thus preventing asymmetric p38α/β MAPK signal transduction, and generating two lineage-committed daughter cells, and consequently, an exhaustion of quiescent satellite cell pool (Bernet et al., 2014).

Another work from Blau and colleagues confirmed that an age-dependent increase in p38α/β MAPK signaling could limit expansion of muscle stem cells during proliferation while favoring their permanent cell cycle exit, leading to diminished regenerative and self-renewal capacity in satellite cells from aged individuals (Cosgrove et al., 2014). Remarkably, the inactivation of p38α/β

signaling with pharmacological inhibitors partially restored the proliferative capacity and self-renewal of aged satellite cells evaluated in muscle transplantation experiments (Bernet et al., 2014; Cosgrove et al., 2014). It is noteworthy that the beneficial effects of p38α/β signaling inhibition were strongly enhanced in satellite cells cultured with p38α/β chemical inhibitors in the presence of a hydrogel matrix, mimicking the biomechanical properties of young muscles (Cosgrove et al., 2014).

4.5.4. Senescence in aging satellite cells.

Cellular senescence has been typically associated to tumor suppression and aging, both characterized by the accumulation of severe cellular damage. Damage-induced senescence can be attributed to different stimuli as telomere attrition (replicative senescence), DNA lesions (DNA-damage-induced senescence), environmental stress or ROS (stress-induced senescence) and oncogenes (oncogene-induced senescence) (Campisi, 2013). These diverse stimuli can activate different signaling pathways that frequently converge in the activation of p53, which in turn activate the CDK inhibitors $p16^{INK4a}$, $p15^{INK4b}$, $p21^{CIP1}$ and $p27^{KIP1}$. CDK/cyclin complexes inhibition results in an arrest in proliferation via hypophosphorylated RB. This proliferative arrest can be reversible at the beginning, although due to persistent signaling and activation of senescence mediators, it turns into an irreversible event (Muñoz-Espin and Serrano, 2014).

Other features of senescence include the absence of proliferative markers, high senescence-associated β-galactosidase

(SA-β-GAL) activity, and often also expression of DNA damage markers, nuclear foci of constitutive heterochromatin (Muñoz-Espin and Serrano, 2014). Senescent cells are thought to promote aging through their senescence-associated secretory phenotype (SASP), which involves the increased expression and secretion of a suite of inflammatory cytokines, chemokines, growth factors, and proteases (Coppé et al., 2010; van Deursen, 2014).

Satellite cells of aged mice (at geriatric age, 28 months or older) lose their reversible quiescent state in basal conditions due to induction of $p16^{INK4a}$, and adopt a pre-senescent state (Sousa-Victor et al., 2014a). In response to an injury, pre-senescent satellite cells from geriatric mice enter a full senescent state (geroconversion) and lose their regenerative potential, including diminished activation, proliferation and self-renewal capacities. Geroconversion could be partially reverted through the downregulation of $p16^{INK4a}$ expression, which also restored the self-renewal capacity in very old muscle stem cells. Mechanistically, the epigenetic repression exerted at the $p16^{INK4a}$ locus in young quiescent satellite cells, via PRC1-mediated monoubiquitination of histone H2A ($H2A^{K119Ub}$), was deregulated in geriatric cells. Moreover, $p16^{INK4a}$ upregulation correlated with diminished levels of phosphorylated RB and reduced E2F-regulated transcription, suggesting this pathway as a possible driver of the satellite cell conversion to senescence. Lastly, $p16^{INK4a}$ was also found upregulated in human geriatric satellite cells, highlighting these findings as particularly relevant for muscle stem-cell rejuvenation in sarcopenic muscles (Sousa-Victor et al., 2014a). In agreement with these results, Blau and coworkers reported a diminished regenerative and self-renewal potential of muscle stem cells from

aged mice, which was preceded by a cell-autonomous increase in the p38 MAPK pathway activity, a limited proliferation that favored an irreversible cell cycle arrest, and the expression of CDK inhibitors as $p16^{INK4a}$ and $p21^{CIP1}$, which associated with a senescent phenotype (Cosgrove et al., 2014).

The *in vivo* analysis of freshly isolated activated satellite cells from aged mice revealed a specific upregulation of Hoxa9, both at the mRNA and protein level (Schworer et al., 2016). Moreover, the impaired myogenic capacity of satellite cells in response to Hoxa9 over expression was associated with increased rates of apoptosis and decreased cell proliferation. This was associated with the suppression of several cell cycle regulators and induction of cell cycle inhibitors (such as $p16^{INK4a}$, $p15I^{NK4b}$, and $p21^{CIP1}$) and other senescence-inducing genes, as well as with increased staining for SA-β-GAL activity (Schworer et al., 2016).

A more recent work demonstrated that basal autophagy is essential to maintain the stem-cell quiescent state in mice (García-Prat et al., 2016). Failure in autophagy in aged resting stem cells leads to accumulation of damaged proteins and dysfunctional organelles, especially mitochondria, which generates enhanced ROS levels that generate DNA damage, senescence entry and stem-cell exhaustion. Moreover, ROS was disclosed as a key epigenetic regulator of the INK4a locus in aged satellite cells by impeding its silencing (associated with $H2A^{K119Ub}$ repressive mark), and being this situation reversed by treatment with antioxidants. Remarkably, the re-establishment of autophagy in mouse and human satellite cells was sufficient to reverse senescence and restored the regenerative functions in geriatric satellite cells

(García-Prat et al., 2016). In agreement with this, Auxwerx and coworkers demonstrated the importance of the amount of NAD^+ and its effect on mitochondrial activity as a pivotal switch to modulate satellite cell senescence during aging (Zhang et al., 2016). Treatment with the NAD^+ precursor NR rejuvenated satellite cells in aged mice by inducing the mitochondrial unfolded protein response and synthesis of prohibitin proteins, and prevented satellite cell senescence in a mouse model of muscular dystrophy (Zhang et al., 2016).

4.5.5. p38 MAPK and senescence.

A vast body of work has established a crucial role for p38 MAPK in the induction of the senescence program. Pioneer studies in mouse fibroblasts demonstrated that the genetic activation of p38 by $MKK6^{EE}$ was sufficient to induce a permanent and irreversible G1 cell cycle arrest, showing these cells biochemical features of senescence in a p38-dependent manner, including enhanced expression of $p21^{CIP1}$ (Haq et al., 2002). Ishikawa and collaborators defined p38 MAPK as a senescence-executing molecule in which heterogeneous types of cellular senescence converge, namely, Ras-induced, replicative, oxidative stress-induced and culture shock-induced senescence, being activated by both telomere-dependent and telomere-independent senescence-inducing stimuli (Iwasa et al., 2003). Human lung fibroblasts expressing a retroviral $MKK6^{EE}$ displayed retarded growth rates, stopped growing before reaching confluence, and did not progress into S phase. In addition, these cells showed a flat and large cytoplasm which was positive for SA-β-gal activity, and expressed

higher amounts of p16^{INK4a}, p21^{CIP1} and hypophosphorylated RB, but not that of p53 (Iwasa et al., 2003). Of note, the addition of SB203580 to these cell cultures abolished the reduced growth and the SA-β-gal activity. In this context, p38 is not directly activated by the initial stimuli, but indirectly as a cellular response to those initial events (Iwasa et al., 2003). This was confirmed in another work demonstrating that p38 gets activated in primary human fibroblasts during the onset of Ras-induced senescence, and the expression of an oncogenic Ras was sufficient to provoke premature senescence by sequentially activating the MEK-ERK and MKK3/6-p38 pathways, being this phenomenon abrogated upon p38 inhibition (Wang et al., 2002).

Upon its activation, p38 MAPK has been demonstrated to mediate senescence through its action on downstream effectors. Moreover, in primary mouse skin fibroblasts, p53 phosphorylation by p38-regulated/activated protein kinase (PRAK) following p38 activation was suggested to play an important role in Ras-induced senescence and tumor suppression (Sun et al., 2007). Interestingly, PRAK deficiency in mice was sufficient to enhance skin carcinogenesis, coinciding with compromised induction of senescence (Sun et al., 2007). In another study in the context of wound healing, the matricellular protein CCN1 induced DNA damage response and p53 activation in mouse primary fibroblasts, and activated the superoxide-generating RAC1-NOX1 complex to induce ROS production. In turn, ROS-activated ERK and p38 pathways led to the induction of the p16^{INK4a}/RB pathway and cellular senescence (Jun and Lau, 2010).

Other findings have assigned a novel role for p38 MAPKs in the senescence-associated secretory phenotype (SASP) regulation. Diverse senescence-inducing stimuli activate p38 MAPK in normal human fibroblasts, inducing the secretion of most SASP factors in a mechanism involving an increased NF-kB transcriptional activity (Freund et al., 2011).

p38 MAPK activation in response to increasing levels of ROS limited the lifespan of hematopoietic stem cells (HSCs) *in vivo* (Ito et al., 2006). The defect in the maintenance of HSC quiescence upon ROS-activated p38 signaling was rescued by treatment with p38 inhibitors. Moreover, prolonged treatment with an antioxidant or p38 inhibitors extended the lifespan of HSCs from wild-type mice in serial transplantation experiments (Ito et al., 2006). In a more recent study, p38 MAPK was reported to be relevant in the premature senescence entry of human endometrium-derived mesenchymal stem cells (hMESCs) under sublethal doses of oxidative stress (Borodkina et al., 2014). Exogenous H_2O_2 induced the activation of DNA damage response (DDR), which in turn activated the p53/ $p21^{CIP1}$/RB and the p38 MAPK/MAPKAPK-2 pathways, both suggested to establish the irreversible cell cycle arrest preceding senescence. The further stabilization of senescence required prolonged DDR signaling activation, provided by permanent ROS production, which in turn was regulated by both p38 MAPK and the increased functional mitochondria. Remarkably, cell treatment with SB203580 was sufficient to recover partially the senescence phenotype, block the ROS elevation, decrease the mitochondrial function, and finally rescue proliferation (Borodkina et al., 2014).

As mentioned above, the age-related activation of the p38 MAPK signaling pathway in muscle stem cells ultimately leads to impaired self-renewal (Bernet et al., 2014; Cosgrove et al., 2014), and a diminished regenerative capacity (Cosgrove et al., 2014). In the later work, this deficiency correlated with a higher incidence of cells that express senescence markers ($p16^{INK4a}$ and $p21^{CIP1}$) and a persistently elevated p38α/β MAPK activity. This situation was partially reverted by transient p38α/β signaling inhibition in conjunction with culture on soft hydrogel substrates, which rejuvenated aged satellite cell potential for regeneration and serial transplantation (Cosgrove et al., 2014). In agreement with these results, one study with a dominant negative haploinsufficient p38α mouse (DN-$p38\alpha^{AF/+}$) demonstrated that *in vivo* attenuation of p38α activity delayed the age-associated decline in the gastrocnemius of the aged mutant. These mice showed attenuated expression of $p16^{INK4a}$ and $p19^{ARF}$ and a decline of the senescent progenitor cell pool level, among others signs of rejuvenation (Papaconstantinou et al., 2015). All these studies support the notion that attenuation of p38 signaling functions as a major mechanism that delays senescence and age-associated diseases.

II. OBJECTIVES

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p38 MAPK mediates stress adaptive responses, as well as other physiological processes such as skeletal muscle differentiation, or pathological situations like cancer. This work is the result of a collaboration between two laboratories with common interests: getting insights into the role p38 in these diverse processes. In this PhD Thesis we explored in further detail the function of p38 signaling in cellular stress and skeletal muscle differentiation. For practical reasons, I divided this study in three different parts.

Part I: Role of RB phosphorylation by p38 in stress adaptation, cell proliferation and cancer.

Cell cycle progression is delayed upon stress. In this study we aimed to understand the mechanisms involving the modulation exerted by p38 MAPK in cell cycle. The objectives for this part were:

1. To explore the role of p38 MAPK in cell cycle modulation upon stress by phosphorylation of the essential regulator RB.
2. To decipher the functional relevance of this phosphorylation in RB transcriptional activity, cell cycle progression and survival.
3. To evaluate the impact of this phosphorylation in cell proliferation and cancer cell growth.
4. To elucidate the mechanism by which p38 phosphorylation on RB modulates its activity.

Part II: Role of RB phosphorylation by p38 MAPK in muscle cell differentiation.

p38 and RB are both essential in the transition from proliferation to differentiation that leads to myofiber formation. We aimed to assess the importance of RB phosphorylation by p38 MAPK in several muscle cell differentiation models. Our aims were:

1. To investigate the biological relevance of RB phosphorylation by p38 in a physiological process different from stress adaptation, such as skeletal myogenesis.
2. To explore the contribution of a p38-phosphorylated RB in a muscle-related cancer model as rhabdomyosarcoma.

Part III: Role of p38α in muscle stem cell aging.

The number and the functionality of muscle stem cells (satellite cells) decline dramatically during aging. How satellite cells avoid acquisition of the senescence program until advanced age is largely unknown. The objectives for this study were:

1. To determine the contribution of p38α MAPK to satellite cell function during aging.
2. To assses the physiological relevance of p38-deficiency in satellite cells from aged mice.
3. To study the mechanisms underlying the phenotypic effects observed in aged satellite cells upon p38 loss.

III. EXPERIMENTAL PROCEDURES

III. EXPERIMENTAL PROCEDURES

Part I: The N-Terminal Phosphorylation of RB by p38 Bypasses Its Inactivation by CDKs and Prevents Proliferation in Cancer Cells.

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Details regarding oligonucleotides, cell lines, plasmids, viruses, and antibodies, as well as detailed information regarding the methods and other experimental procedures including bacterial expression and purification of recombinant proteins, *in vitro* kinase assay, western blotting, immunoprecipitation, chromatin immunoprecipitation (ChIP) and immunocytochemistry, are included in the 'Supplemental Experimental Procedures' section in the attached paper.

Cell culture, Transfection, and Infection.

HEK293T, wild type mouse embryo fibroblasts (MEFs), *p38α*$^{-/-}$ MEFs, *RB*$^{-/-}$ MEFs, MCF7, PK9, 235J and MDA-MB-231 cells were all maintained in Dulbecco's modified Eagle's medium (Biological Industries) containing 10% fetal calf serum (Sigma) and supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml Penicillin and 100 µg/ml Streptomycin (GibCO) and were cultured in a 5% CO2 humidified incubator at 37 ºC. When

indicated, cells were treated with 100-250 mM NaCl, 400-1200 μM H_2O_2 or 5-50 ng/ml Anisomycin. When indicated, cells were incubated with 1 μM SB203580 (Calbiochem) for 30 minutes or 0.5 μM BIRB796 (Merck-Millipore) for 2 hours prior to the treatments.

Cells were transiently transfected with the indicated plasmids using the FuGENE 6 transfection reagent (Roche Applied Science), Lipofectamin LTX or Lipofectamin 3000 (Invitrogen) according to the manufacturer's directions.

Regarding infection, supernatants containing lentiviruses were produced in transfected HEK293T cells. Briefly, HEK293T cells were cotransfected with the lentiviral vector pLVX along with the lentiviral packaging and envelope vectors pMDG2 and psPAX2 and were left for 48 hours before harvesting the media. After a brief centrifugation to remove cell debris, cleared supernatants were added directly to cell culture dishes in which MDA-MB-231 cells were growing.

Plasmids and constructs.

The following plasmids were used. pGEX2TK-RB (human) was provided by Dr. Mayol (IMIM, Barcelona). RB fragments were obtained by digestion of the full length pGEX2T-RB. The myc-tagged RBs were obtained by PCR amplification using the full-length RB of pGEX2TK-RB as a template, and cloned into the pCDNA3-myc N-terminal tagged. The RB mutants were generated using the Quickchange XL site directed mutagenesis kit from Stratagene following the manufacturer's instructions. pEFmlink-$MKK6^{DD}$ (with the two phosphorylation sites Ser 207 and Thr 211 in

the activation loop of MKK6 changed to Glu) has been described (Alonso et al., 2000). pGEX2T1-MKK6^{DD} was obtained by PCR amplifying MKK6^{DD} from pEFmlink-MKK6^{DD} and cloning it the pGEX2T1 plasmid. pMT3-HAp38α was obtained from Addgene (12658). pGEX5x3-p38α was obtained by PCR amplifying p38α from pMT3-HA-p38α and cloning it into the pGEX5x3 plasmid. Mutagenesis primer pairs and probes for cloning are detailed in the '**Supplemental Experimental Procedures**' section.

pRC-HA-CDK4, pRC-HA-CycD, pRC-HA-CDK2, pRC-HA-CycA2, and pRC-HA-CycE1 were a gift from Dr. Watson (Institute of Clinical Sciences, University of Birmingham) and can be found in Addgene (1876, 8958, 1884, 8959 and 8963, respectively). pGL2-E2F-luc, pRC-HA-E2F1 and pcDNA3-HA-DP1 were a gift from Dr. Tauler (UB, Barcelona). The lentiviral vectors pMD2G and psPAX2 were obtained from Dr. Trono (EPFL, Lausanne) and pLVX-TetOne-Puro was bought from Clontech.

Antibodies.

Anti-E2F1 (sc-251), anti-RB (sc-50), anti-cycA (sc-596), anti-GAPDH (sc-32233) and anti-p38 (sc-535) were obtained from SantaCruz. Anti-RB (9309), anti-p38α (9228), anti-phospho-S795-RB (9301), anti-phospho-S780-RB (9307), anti-phospho-p38 (9215), anti-phospho-S608-RB (2181) and anti-phospho-S807/811-RB (9308) were purchased from Cell Signaling. Anti-RB (BDPharmingen, 554136), anti-tubulin (Sigma, S9026), anti-GST (GE Healthcare), anti-phospho-T373-RB (Abcam, ab52975) and anti-RNA Pol II (8WG16) (Abcam) were also obtained for the study.

Mouse monoclonal anti-HA and mouse monoclonal anti-myc were from the 12CA5 and 9E10 hybridomas, respectively.

Rabbit polyclonal antibodies specifically targeting RB phosphorylation at S249/T252 were generated by Genscript Corporation. Horse Radish Peroxidase-conjugated anti-rabbit and anti-mouse antibodies and the Enhanced Chemiluminiscence kit were purchased from GE Healthcare.

Bacterial expression and purification of recombinant proteins.

GST-fused proteins were purified as described in the '**Supplemental Experimental Procedures**'. GST-p38α was activated *in vitro* by mixing with GST-MKK6^{DD}, and it was used to phosphorylate either GST-fused RB purified from *E. coli* or immunoprecipitated RB from mammalian cells.

Luciferase reporter assays.

Cells were transfected with E2F-luc, HA-E2F1, and HA-DP1 in the presence or absence of myc-RB, myc-RB$^{249A/T252A}$, myc-RB$^{249E/252E}$, and/or HA-tagged CDK2, CDK4, CycE, and CycA, as indicated in the figures. Treated cells were washed twice with cold PBS and were lysed with cell culture lysis reagent (Promega). Cell lysates were cleared by microcentrifugation. Luciferase reporter activity in cell supernatants was measured using a Luciferase Reporter Assay kit (Promega) and a Microlumat LB 960 luminometer (Berthold Technologies). The total amount of protein in the cell extracts was measured using the Bradford reagent (Bio-Rad). Protein-corrected luciferase reporter activities are shown.

RNA purification and Real Time (RT)-PCR mRNA analysis

Total RNA was purified from MEF cells using the RNeasy kit (Qiagen) following the manufacturer's instructions. Total RNA (100 ng) was then converted to cDNA using the reverse transcriptase Superscript kit (Invitrogen) according to the manufacturer's protocol. cDNA was analyzed by real-time PCR using a DNA Biosystems 7700 sequence detector and the SYBR Green kit (Applied Biosystems). Real-time PCRs were performed in triplicate and were referenced to the GAPDH mRNA levels. The following oligonucleotides were used:

	Forward primer	Reverse primer
MAD2	AGGATGAAATTCGCTCAG	CATTGACAGGGGTTTTGT
E2F1	GGATCTGGAGACTGACCA	CTCCAGGACATTGGTGAT
BRCA1	ACTGAAAGGCATCCAGAA	GAACCTGCCTGTCGTTAC
CycA2	GTCCTTCATGGAAAGCAG	ACGTTCACTGGCTTGTCT
CycE1	CCTCCAAAGTTGCACCAGTT	GGACGCACAGGTCTAGAAGC
p107	TCTTGTATGCGGAATCCT	ATCTCCATTCCATGAAGC
MCM7	ACCTATGTCCACCAGCAC	CCAGAGCAGTGGAAAGTC
PCNA	GGCAATGGGAACATTAAG	GTCCCATGTCAGCAATTT
RRN2	AGGATGAGCCGTTACTGA	TAAATCGCTCCACCAAGT
GAPDH	AATTCAACGGCACAGTCAAGG	GGATGCAGGGATGATGTTCTG

Chromatin-immunoprecipitation (ChIP) assays.

Cells were cross-linked with formaldehyde. Immunoprecipitation of DNA fragments was analyzed by real-time PCR. RT-PCR results were referenced to the inputs, and they are represented as fold induction over the mock-transfected cells. For

ChIP assays of tagged proteins, a mock ChIP where the corresponding empty vector plasmid had been transfected was carried out; DNA amplified from the mock IP is regarded as background. A detailed ChIP protocol is described in '**Supplemental Experimental Procedures**'. Immunoprecipitated DNA fragments were analyzed using PCR or RT-PCR as described above. PCR primers for the individual genes assayed are the following:

	Forward primer	Reverse primer
hCycA2	CCCCAGCCAGTTTGTTTC	TCAAGTTCATAG
hE2F1	GCGTTAAAGCCAATAGGA	CTTTTACGCGCCAAATC
mCycA2	CTCCCGCCCTGTAAG ATTC	AGTTCAAGTATCCCGCGACT

h, human and m, mouse.

RT-PCR results were referenced to the inputs, and are represented as fold induction over the mock transfected cells. For ChIP assays of tagged proteins, a mock ChIP where the corresponding empty vector plasmid had been transfected was carried out; DNA amplified from the mock IP is regarded as background.

Cell-cycle analysis and cell viability.

Exponentially growing wild type, RB-/- MEFs and MCF7 cells were stressed with NaCl, H_2O_2 or anisomycin as indicated in the figures, and treated with nocodazole (Sigma) after 1 hour (final concentration 100 ng/ml) to trap the cells in the G2/M phase. DNA was labeled *in vivo* with Hoechst 33342 (Sigma, 8 μM) for 1 hour before trypzinization of the cells and collection for fluorescence-

activated cell sorting (FACS) analysis of cell-cycle. Cell viability in response to stress was assessed by labeling living cells with propidium iodide (PI, 1 µg/ml for 10 minutes), followed by FACS analysis. The stained cells were acquired on an LSR flow cytometer (Becton Dickinson) using CellQuest software. Cell cycle profiles and viability were then analyzed using WinMDI software.

Colony formation assays.

For colony formation assays, cells were transfected with the indicated constructs. Cells were tryspinized, counted and then seeded out in appropriate dilutions to form colonies in 1-2 weeks. Colonies were fixed with paraformaldehyde (3.7%) for 10 minutes and then stained with crystal violet (0.05%) for 10 minutes, washed twice with water and left inverted until dry. Colony area occupancy was analyzed using ImageJ software.

***In vivo* xenografts.**

GFP-positive sorted, lentivirally transduced MDA-MB-231 cells were suspended in PBS containing 75% Matrigel (BD Bioscience). The cells (106) were injected subcutaneously into 8-10 week-old Hsd:Athymic Nude-Foxn1nu female mice (Envigo, Barcelona, Spain) (n=5-7/group) and the mice were housed under specific pathogen-free conditions at CNIO (Spanish National Cancer Research Center). Once the mean tumor volume reached 0.1 cm^3, mice were randomized to receive, or not, a doxycycline-containing diet. Tumor diameters were measured with a digital caliper and the tumor volume was calculated using the formula: volume = (width)2 x length/2. Animal procedures were approved by

the Ethics Committee for Research and Animal Welfare of Instituto de Salud Carlos III and the General Guidance of the Environment of Madrid Community, and were performed following the guidelines for Ethical Conduct in the Care and Use of Animals as stated in "The International Guiding Principles for Biomedical Research involving Animals", developed by the Council for International Organizations of Medical Sciences.

In silico modeling.

For *in silico* modeling, the structure of the N-terminal domain region 244-269 of RB was modeled by different approaches (among them, Swiss-Model server and iTASSER) due to the lack of templates with sufficient similarity and percentage of identity to be used as templates. Details about *in silico* modeling are extensively described in '**Supplemental Experimental Procedures**'

Statistical analysis.

Statistical analysis was performed using Prism version 3.0 (GraphPad). Data were analyzed using Student's t test and one-way ANOVA, followed by post hoc Bonferroni tests when appropriated. Data are presented as means ± SE and $p < 0.05$ was considered significant. Experiments were performed in triplicate and repeated at least three times independently.

Part II: Role of RB phosphorylation by p38 MAPK in muscle cell differentiation.

Cell culture, Transfection, and Infection.

C2C12 cells were cultured in DMEM containing 10% FCS, 100U/ml penicillin, 100µg/ml streptomycin, 0.001% Fungizone, (GM growth medium). The medium was changed daily and cultures were passaged 1:3 as they reached 60-70% confluence. To induce differentiation, GM was replaced by differentiation medium DM (DMEM supplemented with 2% horse serum, 2 mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 0.001% Fungizone) at myoblast subconfluence. Rh30 cells were cultured in RPMI 1640 medium containing 20% FCS, 100U/ml penicillin, 100µg/ml streptomycin, and 0.001% Fungizone. To induce differentiation, GM was replaced by DM (same as with C2C12 cells) at subconfluence.

C2C12 and Rh30 cells were transiently transfected with the indicated plasmids using the Lipofectamin 3000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were infected as it was detailed in the first part (Gubern et al., 2016). Lentiviral particles were obtained cotransfecting pLVX-GFP-RBs (wild-type and mutants) or pLVX-myc-MKK6^{DD} with the lentiviral packaging vectors (pMD2G and psPAX2) in HEK293T cells.

Plasmids.

The HA-tagged RB mutants were obtained by digestion and cloning using the full-length myc-tagged RBs as a template

(Gubern et al., 2016) into a pEFmlink-HA-RB vector backbone. The pLVX-myc-MKK6^{DD} vector was obtained by digestion and cloning using the pEFmlink-MKK6^{DD} as a template and the pLVX-TetOne-Puro as a backbone (Clontech).

Antibodies.

Anti-phospho-p38 (Cell Signaling, 9215), anti-p38 (Cell Signaling, L53F8), anti-RB (BDPharmingen, 554136), anti-tubulin (Sigma, S9026) and rabbit polyclonal antibody specifically targeting RB phosphorylation at S249/T252 (Genscript) were used as in the previous part (Gubern et al., 2016). Mouse monoclonal anti-Myogenin (F5D) and anti- eMHC (F1.652) were obtained from the Developmental Studies Hybridoma Bank.

RNA purification and Real Time (RT)-PCR mRNA analysis.

Total RNA was purified from C2C12 cells using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Total RNA (300 ng) was then converted to cDNA using the RT Superscript III kit (Invitrogen) according to the manufacturer's protocol. cDNA was analyzed by RT-PCR using a DNA Biosystems 7700 sequence detector and the SYBR Green kit (Applied Biosystems). RT-PCRs were performed in triplicate and were referenced to the GAPDH mRNA levels. The following oligonucleotides were used:

	Forward primer	**Reverse primer**
MyoG	GGTGTG TAAGAGGAAGTCTGTG	TAGGCGCTCAATGTACTGGAT
Cav3	GGATCTGGAAGCTCGGATCAT	TCCGCAATCACGTCTTCAAAAT

Part III: Role of p38α in muscle stem cell aging.

Generation of conditional p38α$^{\Delta Pax7}$ mice.

Mice carrying the floxed p38α, allele were generated by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, USA). Floxed p38α mice were crossed to Pax7Cre line (kindly provided by M. Capecchi) to obtain p38α$^{\Delta Pax7}$ knockout mice. All animal experiments and isolation of satellite cells from wild type and knockout mice were approved by the ethics committee of the Barcelona Biomedical Research Park (PRBB) and by the Catalan Government and used sex-,age- and weight-matched littermate animals.

Induction of muscle regeneration.

Young adults (2-3 months) and aged (18-24 months) mice were anaesthetized with ketamine/xylazine (80/10 mg kg^{-1}, intraperitoneally). Regeneration of skeletal muscle was induced by intramuscular injection of cardiotoxin (CTX, Latoxan; 10−5 M) in the tibialis anterior (TA) muscle of the mice as described (Suelves et al., 2007; Sousa-Victor et al., 2014; García-Prat et al., 2016). At the indicated time after injury (7 days), mice were killed and TA muscles were dissected, frozen in isopentane cooled with liquid nitrogen, and stored at −80 °C until analysis. Histology and immunohistochemistry in muscle cryosections was performed as previously described (Sousa-Victor et al., 2014). Regeneration was assessed in young (n=2) and aged (n=3) wild-type and p38α$^{\Delta Pax7}$ mice.

Satelite cell isolation by FACS and culture.

FACS isolation of satellite cells and subsequent culture were performed as previously described (Sousa-Victor et al., 2014). Briefly, muscles were 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 then incubated in lysing buffer (BD Pharm Lyse) for 10 min on ice, resuspended in PBS with 2.5% goat serum and counted. PE-Cy7-conjugated anti-CD45 (Biolegend 103113/14), anti-CD31 (Biolegend 102418), anti-CD11b (Biolegend 101215/16) and anti-Sca-1 (Biolegend 108113/14) antibodies were used to exclude the Lin (−) negative population and Alexa647-conjugated anti-CD34 (BD Pharmigen 560230) and PE-conjugated anti-α7-integrin (Ablab AB10STMW215) were used for double-positive staining of quiescent satellite cells. Cells were sorted using a FACS Aria II (BD).

Primary myoblast cultures were maintained on a routinary basis on collagen-coated dishes in Ham's F10 medium supplemented with 20% FCS, 100U/ml penicillin, 100μg/ml streptomycin, 0.001% Fungizone and 5 ng/ml bFGF (GM). Experiments were performed by plating cells on Matrigel™ (BD Biosciences) Basement Membrane Matrix coated dishes.

Proliferation assay.

Proliferation assays were performed as it was previously described in other works from our laboratory (Perdiguero et al., 2007a; Sousa-Victor et al., 2014a; García-Prat et al., 2016). Briefly,

activated satellite cells were plated in 12-well plates and, after 24h proliferating, pulsed with BrdU (1.5 μg ml−1; Sigma) 1h prior to fixation in 3.7% formaldehyde for 10 minutes. BrdU-labelled 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 by counting the cells under a microscope and results were expressed as a percentage of the total number of cells analyzed. For this assay, the number of mice (isolated satellite cells) per group analyzed is the following: young $p38\alpha^{WT}$ n=3, young $p38\alpha^{\Delta Pax7}$ n=2, aged $p38\alpha^{WT}$ n=10, aged $p38\alpha^{\Delta Pax7}$ n=3.

Senescence-associated β-galactosidase activity.

Senescence-associated β-galactosidase (SA-β-gal) activity was detected in freshly isolated satellite cells using the Senescence β-Galactosidase Staining kit (Cell signaling), according to the manufacturer's instructions. For this assay, the number of mice (isolated satellite cells) per group analyzed is the following: young $p38\alpha^{WT}$ n=5, young $p38\alpha^{\Delta Pax7}$ n=2, aged $p38\alpha^{WT}$ n=10, aged $p38\alpha^{\Delta Pax7}$ n=4.

Antibodies.

Anti-p38 (Cell Signaling, L53F8), anti-tubulin (Sigma, S9026) and $p16^{INK4a}$ (Santa Cruz, sc-1207), and anti-eMHC (Developmental Studies Hybridoma Bank, F1.652) antibodies were used.

RNA purification and Real Time (RT)-PCR mRNA analysis.

Total RNA was purified from freshly isolated or cultured satellite cells using the RNeasy Micro kit (Qiagen) following the manufacturer's instructions. Total RNA (300 ng) was then converted to cDNA using the reverse transcriptase Superscript III kit (Invitrogen) according to the manufacturer's protocol. cDNA was analyzed by real-time PCR using a DNA Biosystems 7700 sequence detector and the SYBR Green kit (Applied Biosystems). Real-time PCRs were performed in triplicate and were referenced to the GAPDH mRNA levels. The following oligonucleotides were used:

	Forward primer	**Reverse primer**
$p16^{INK4a}$	CAT CTG GAG CAG CAT GGA GTC	GGG TAC GAC CGA AAG AGT TCG

For this assay, the number of mice (isolated satellite cells) per group analyzed is the following: young $p38\alpha^{WT}$ n=6, young $p38\alpha^{\Delta Pax7}$ n=2, aged $p38\alpha^{WT}$ n=5, aged $p38\alpha^{\Delta Pax7}$ n=4.

Chromatin immunoprecipitation (ChIP) assays of $H2A^{K119Ub}$ repressive mark.

$H2A^{K119Ub}$ ChIPs in different regions of the INK4a locus were performed as previously described (Sousa-Victor et al., 2014; García-Prat et al., 2016). Briefly, FACS-isolated satellite cells from resting muscle of $p38\alpha^{\Delta Pax7}$ and wild-type mice from different ages were cross-linked with 1% formaldehyde for 15 min at room temperature. For each ChIP, 20,000 cells were lysed and

chromatin was sonicated in a Bioruptor (Diagenode) for 12 cycles (30″on/30″ off). Sonicated chromatin was then diluted and subjected to immunoprecipitation using ChIP Low Cell kit (Diagenode) following the manufacturer's recommendations, with specific anti-$H2A^{K119ub}$ (noted as H2Aub) antibody (ABE569 Millipore) plus rabbit anti-mouse IgM antibody (Millipore 12-488). Bound fraction and input were analysed by qPCR using specific primer sets for the INK4a locus:

	Forward primer	**Reverse primer**
$RD^{INK4a/ARF}$	GGTCTCCCCTAGCAGGATTC	GCCTGTCATTAAACAGGGTGA
INK4a Exon1a	CCGGAGCCACCCATTAAACTA	CAAGACTTCTCAAAAATAAGA CACTGAAA
INK4a Exon2	CCCAACACCCACTTGAGGAA	CAGAGGTCACAGGCATCGAA

Whole muscle graft.

Whole muscle grafts experiments, also called heterografts, were performed as follows: the Extensor digitorum longus (EDL) muscle of aged mice was grafted onto the tibialis anterior (TA) muscle of 3 months-old wild-type mice (n=3 mice were analyzed). 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). When indicated, p38AF-containing adeno-associated virus (AAV2/1) were

used to inhibit p38 signaling by localized slow injection of a 15 µl PBS solution into the EDL muscle just before grafting, using a Hamilton syringe. Viral dose was 0,2-0,4 x 10^{10} PFU/muscle. AAVs were generated by cloning a dominant-negative mutant of p38 (p38AF), kindly donated by David Engelberg, into an AAV2/1 backbone provided by the Centre for Animal Biotechnology and Gene Therapy (CBATEG, UAB Barcelona), where the viral particles were produced.

IV. RESULTS

IV. RESULTS

Part I: The N-Terminal phosphorylation of RB by p38 bypasses its inactivation by CDKs and prevents proliferation in cancer cells.

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The p38 MAPK controls cell proliferation through the activation of different cell cycle checkpoints, and accordingly, it has been considered to act as a tumor suppressor (Ambrosino and Nebreda, 2001; Bulavin and Fornace, 2004). Upon different types of stress, p38 has a critical role in cell cycle arrest at G1/S and G2/M phase transitions, dependent on different mechanisms (Reinhardt et al., 2007; Cuadrado et al., 2009; Lafarga et al., 2009; Joaquin et al., 2012a,b).

The Retinoblastoma (RB) tumor suppressor protein is also critical for cell cycle progression from the G1 to S phase in mammalian cells. RB negatively regulates E2F transcription factors (Henley and Dick, 2012) through its association with other binding partners, as chromatin-remodeling complexes (Brehm and Kouzarides, 1999), In G1, RB acts as a transcriptional repressor, and becomes inactivated following CDK phosphorylation at the G1/S transition, releasing the repression machinery from E2F-

dependent promoters and allowing cells to progress into S phase (Henley and Dick, 2012).

In this work we showed that p38 MAPK maximizes cell survival upon stress by downregulating the E2F transcriptional program through the direct targeting of RB. RB phosphorylation by p38 in its N terminus results in an RB insensitive to CDK inactivation. Moreover, this phosphorylation on RB increases its affinity towards E2F, represses E2F-driven transcription, and delays cell-cycle progression. Remarkably, the expression of a phosphomimetic RB mutant in cancer cell lines reduces colony formation and decreases their proliferative and tumorigenic potential in a mouse xenograft model.

1. p38 and RB mediate the downregulation of cyclin expression upon stress.

p38 is critical for cell cycle arrest at G1 phase upon stress (Reinhardt et al., 2007; Cuadrado et al., 2009; Lafarga et al., 2009; Joaquin et al., 2012a,b). We assessed whether G1 arrest upon p38 (α isoform) activation occurs through regulation of gene expression. Upon p38 activation by different stresses (NaCl, H_2O_2 or Anisomycin) (**Figures 1A and S1A**) or genetically by a constitutively active form of its upstream kinase ($MKK6^{DD}$) (**Figure S1D**), we observed a clear downregulation of E2F-dependent gene expression (not observed in p38α knockout cells ($p38\alpha^{-/-}$)). As in $p38\alpha^{-/-}$ cells, RB knockout cells ($RB^{-/-}$) were unable to downregulate E2F-dependent gene transcription upon stress (**Figures 1A, S1A, S1C and S1D**). Therefore, we hypothesized that downregulation of

cell-cycle gene expression could be mediated by p38 through RB regulation.

2. The p38 MAPK interacts with and phosphorylates Ser249 and Thr252 at the N-terminal region of RB.

Previous studies reported that p38 directly associates with RB (Li and Wicks, 2001; Cho et al., 2010). We confirmed this interaction *in vivo* by co-immunoprecipitation assays (**Figure S1E**), and endogenously with specific antibodies (**Figure S1F**). Moreover, p38 directly targeted RB in an *in vitro* kinase assay using purified proteins. As p38 efficiently phosphorylated full length RB (**Figure S2A**), we determined in which of the 16 putative S/TP MAPK consensus residues takes place this phosphorylation. Truncated versions of RB were subjected to *in vitro* phosphorylation, being the N-terminal fragment the only one phosphorylated by p38 (**Figure S2B**). By single and double S/TP site mutants analysis, we elucidated the specific residues phosphorylated in the N terminus (Ser249 and Thr252) (**Figure S2C**). RB phosphorylation by p38 was completely abolished *in vitro* following the combined mutation of these sites into alanine in a full-length RB ($RB^{S249A/T252A}$) (**Figure 1B**).

To further investigate RB phosphorylation by p38 we generated a specific polyclonal antibody against the doubly phosphorylated RB (**Figure S2D**). We detected p38α-mediated phosphorylation *in vitro* in a purified full-length RB (**Figure S2E**), and *in vivo* in cells challenged with different stresses or genetic activation by $MKK6^{DD}$, while it was abolished in $RB^{-/-}$ and $p38\alpha^{-/-}$

cells or upon p38αβ inhibition (SB203580) (**Figures 1C, 1D and S2F**). A wild-type RB expressed in cells was specifically phosphorylated upon p38 activation, differently from the not-phosphorylatable $RB^{S249A/T252A}$ mutant in the same conditions (**Figures 1E and S2G**). *In vivo* RB phosphorylation by p38 was also observed upon different stresses by immunofluorescence (**Figure 1F**). All these data confirmed that p38 phosphorylates the N-terminal domain of RB in Ser249 and Thr252 sites, both *in vitro* and *in vivo*.

3. Phosphorylation of RB by p38 increases RB activity as a transcriptional repressor.

To evaluate the functional relevance of RB phosphorylation by p38, we took advantage of an E2F reporter system (Krek et al., 1993) to monitor RB activity. The decrease in E2F-driven luciferase activity upon stress was fully suppressed upon p38 inhibition, reinforcing its dependence on p38 activity (**Figures 2A and S3A**). In stress conditions, differently from wild-type RB, the $RB^{S249AT252A}$ mutant could not repress E2F-mediated transcription (**Figure 2B**), while it was as effective as the wild-type in the absence of stress (**Figures 2B, S3B and S3D**). Then, we hypothesized that the expression of a phosphomimetic mutant ($RB^{S249ET252E}$) would be able to repress transcription even in the absence of stress or p38 activation. Indeed, the $RB^{S249ET252E}$ mutant repressed E2F transcription in non-stressed cells, in a similar manner than the stressed wild-type (**Figure 2B**).

To further validate these results, we monitored endogenous E2F-regulated gene expression. Upon stress, cells expressing a wild-type RB downregulated the expression of CycA2 and E2F1 transcripts, but this downregulation did not take place inhibiting p38 activity or when transcription was regulated by the $RB^{S249AT252A}$ mutant (**Figures 2C, 2D and S3E**). Moreover, even without stress, the $RB^{S249E/T252E}$ mutant repressed endogenous CycA2 and E2F1 expression (**Figures 2D and S3E)**.

Since RB is able to inhibit transcription by direct association with E2F-dependent promoters (Dick and Rubin, 2012), we tested whether the observed E2F-mediated transcriptional repression resulted from an increased RB association to the E2F promoters. We first observed that RNA pol II binding to the CycA2 promoter decreased in wild-type cells upon different stresses, but this was not the case in $RB^{-/-}$ cells (**Figure S3F**). Then, we assessed RB association to CycA2 and E2F1 promoters, which was clearly increased in wild-type RB expressing cells following p38 activation, but this did not occur upon p38 inhibition or in cells expressing the $RB^{S249AT252A}$ mutant (**Figures 2E, 2F and S3G)**. Moreover, the phosphomimetic mutant associated with these promoters in the absence of stress, similarly as the stressed wild-type RB (**Figure 2F and S3G**). Thus, an increased binding of RB with E2F-dependent promoter would be the cause of the decreased gene expression observed upon RB phosphorylation. Altogether, these results highlighted the relevance of the N-terminal phosphorylation by p38 in transcriptional repression upon stress.

4. RB phosphorylation by p38 leads to cell cycle delay and increased cell survival upon stress.

At this point, we assessed the biological relevance of RB activity regulation upon stress. To do that, we evaluated the effect of RB phosphorylation by p38 on cell cycle progression in wild-type and *RB*$^{-/-}$ cells. We monitored cell cycle progression from G1 to G2/M phase (in nocodazole-treated cells), where stress-mediated p38 activation promoted a G1 arrest that was partially suppressed in *RB*$^{-/-}$ cells, highlighting the requirement of a functional RB to block the cell cycle in this context (**Figure 3A**). Furthermore, cell cycle arrest upon stress was rescued when we reintroduced a wild-type RB in *RB*$^{-/-}$ cells, but this was not observed when we expressed the RB^{S249AT252A} mutant, which behaved similarly to RB knockout cells (**Figure 3A**). Thus, N-terminal phosphorylation of RB by p38 is crucial for the cell-cycle delay caused by stress.

Cell cycle delay in the G1 phase is critical for cell survival in response to stress in yeast and mammals (Escoté et al., 2004; Joaquin et al., 2012a,b). We assessed cell viability upon different stresses, turning to be *RB*$^{-/-}$ cells more sensitive than RB wild-type cells (**Figures 3B and S4B**). *RB*$^{-/-}$ cells were able to restore cell survival upon stress when we reconstituted the expression of a wild-type RB, but this did not occur when we expressed the RB^{S249AT252A} mutant, where survival was as low as in the *RB*$^{-/-}$ cells (**Figure 3B**). Given all these data, we concluded that the delay in cell-cycle progression mediated by p38-phosphorylated RB maximizes cell survival upon stress.

5. p38-phosphorylated RB is resistant to inactivation by CDKs.

During cell cycle, RB is phosphorylated by CDKs mostly in the C terminus and its pocket domain, inactivating RB activity by promoting its dissociation from E2F (Rubin, 2013; Munro et al., 2012). Conversely, RB phosphorylation by p38 takes place in the N-terminal domain with an opposite effect. We wanted to assess whether the activating phosphorylation by p38 could override the inactivating RB phosphorylation by CDKs. To demonstrate this, we expressed wild-type or the mutant RBs in combination with CDK4/CycD and CDK2/CycA. Of note, the mutant versions of RB were phosphorylated by CDKs to the same extent as wild-type RB (**Figures 4A and 4B**). We monitored RB activity upon CDK phosphorylation, observing a clear increase in E2F-mediated transcription when we overexpressed CDK activity in wild-type RB cells, but this effect was suppressed in the presence of stress (**Figure 4C**). Even upon stress, cells expressing the $RB^{S249AT252A}$ mutant were unable to repress E2F-driven transcription mediated by CDKs, while cells expressing the $RB^{S249ET252E}$ mutant did not show any increase in transcription and behaved as the stressed wild-type regardless of CDK expression, (**Figure 4C**).

Given these observations, we analyzed RB association with an endogenous E2F-regulated promoter (CycA2) in this context. In the absence of stress, RB dissociated from the CycA2 promoter upon CDK activation, but it remained bounded upon stress (**Figure 4D**). On the other hand, the dissociation of the $RB^{S249AT252A}$ mutant from this promoter occurred even in stress conditions. Moreover, the phosphomimetic $RB^{S249ET252E}$ mutant strongly associated with

the CycA2 promoter in the absence of stress, and this association was not affected by high CDK activity (**Figure 4D**). We obtained similar results when we assessed RB association to E2F1 promoter (**Figure S4C**). We concluded that the phosphorylation of RB by p38 prevents RB inactivation by CDKs. Consistent with these data, the association between E2F with RB decreased upon CDK2 phosphorylation, whereas it was more efficient upon p38 phosphorylation (**Figure 4E**). Remarkably, the association of p38-phosphorylated RB with E2F was not altered by CDK2 phosphorylation, supporting the notion that p38 phosphorylation is predominant over the effect of CDK phosphorylation.

A large body of evidence supports that CDK activation results in RB inactivation, which promotes cell proliferation as a consequence of a suppressed RB function (Hassler et al., 2007; Burke et al., 2010, 2012). We hypothesized that the expression of an RB insensitive to CDK inactivation should inhibit cell proliferation, even in a background of high CDK activity. We observed that, conversely to wild-type, expression of the $RB^{S249ET252E}$ mutant was sufficient to prevent proliferation in the presence of high CDK activity (**Figures 4F and S4D**). Therefore, the p38-mediated phosphorylation of RB, or the mutation that mimics it, results in a RB insensitive to CDK regulation that is able to repress E2F-mediated transcription and inhibit cell proliferation.

6. The mutant that mimics RB phosphorylation, $RB^{S249ET252E}$, prevents cancer cell proliferation.

A common feature in the vast majority of cancers is a dysregulated proliferation, generally as a consequence of mutations that lead to an increased CDK activity (Burkhart and Sage, 2008; Stone et al., 2012; Manning and Dyson, 2012; Hanahan and Weinberg, 2011; Chinnam and Goodrich, 2011). We investigated if the p38-mediated phosphorylation of RB could inhibit the proliferation of cancer cells with a high CDK background, as it was proved to be dominant over CDK phosphorylation. We assessed cell growth in a breast cancer cell line (MCF7) where, differently from cells expressing wild-type RB, those expressing the phosphomimetic mutant exhibited a clear defect in proliferation after 72 hours (**Figures 5A and S5A**), remained in G1 phase for a longer time (**Figure 5B)** and were more efficient repressing E2F-mediated transcription than cells carrying an empty vector or expressing a wild-type RB (**Figure 5D**). At a long-term, the colony-forming ability of MCF7 cells was strongly reduced by the expression of the $RB^{S249ET252E}$ mutant (**Figures 5C and S5B**). We obtained similar results in PK9 (pancreatic cancer) and 235J (bladder cancer) cells regarding the effects of the $RB^{S249ET252E}$ mutation on cell proliferation and E2F-mediated transcription (**Figures 5C, 5D and S5B**). Furthermore, cells expressing the $RB^{S249AT252A}$ allele did not display any effect on cell proliferation (**Figure S5C**). We concluded that the expression of a mutant mimicking the p38-mediated N-terminal phosphorylation of RB is able to inhibit the proliferation of cancer cells.

Given all these observations, we decided to test the ability of the $RB^{S249ET252E}$ mutant to inhibit cell proliferation *in vivo* in a mouse xenograft model. MDA-MB-231 breast cancer cells were transduced with a Tet-On-inducible lentiviral plasmid expressing wild-type RB or the $RB^{S249ET252E}$ mutant fused to GFP. We recapitulated the effect in E2F-mediated transcription in cells expressing the GFP-fused RB forms with the E2F reporter system (**Figure S5D**). We sorted GFP-positive cells that were injected into immunodeficient mice, which received doxycycline (DOX) once the tumor was formed (**Figures 5E-5G**). As a result, we confirmed *in vivo* that the expression of a phosphomimetic RB mutant leads to reduced tumor formation and inhibition of cancer cell proliferation.

7. RB N-terminal phosphorylation increases its affinity toward E2F.

At this point, we investigated whether p38 phosphorylation in the N-terminal region of RB could generate a conformational change that would promote its association with E2F, and as a result, an effect in RB activity. Unluckily, none of the published crystal structures of RB contained the loop region comprehending the p38 specific residues (e.g., Hassler et al., 2007 and Burke et al., 2010, 2012). We used different approaches to model this region, which predicted a similar structure to the C-terminus where RB binds to E2F1 and DP1 coactivator (Bonet et al., 2014) (**Figure 6A**). We hypothesized that, upon p38 phosphorylation, RB would suffer a conformational change, disposing the N-terminal region to interact with E2F1 and DP1 in a similar manner than it does with the C-terminal loop of RB (**Figure 6A**). Moreover, the

phosphomimetic mutations in the N-terminal fragment were predicted to improve RB binding affinity compared to wild-type (**Figure 6B**) and conserved the structural basic features of the C-motif with a better similitude than the wild-type RB or the unphosphorylatable mutant (**Figures S6A and S6B**).

Based on these modeling predictions, the p38-mediated phosphorylation of RB, or the introduction of phosphomimetic mutations in the N-terminal region of RB would favor the interaction to E2F (**Figure 6B**). GST-purified N-terminal RB was able to bind E2F (**Figure S6C**), and the affinity was higher when RB was phosphorylated *in vitro* by p38 (**Figure 6C**). Consistent with this, the interaction of E2F with the N-terminal RB, that contained the EE mutations, was stronger than that with the wild-type RB or the $RB^{S249AT252A}$ mutant (**Figure 6D**). Full-length purified GST-RB also co-precipitated more efficiently with E2F1 when it was phosphorylated by p38 (**Figure 6E**). Similar results were obtained when p38 was activated *in vivo* by stress. Remarkably, the interaction of $RB^{S249ET252E}$ with E2F under non-stressed conditions was similar to that of phosphorylated RB upon stress and was stronger than that of $RB^{S249AT252A}$ (**Figure 6F**). These results confirmed that the phosphorylated RB and the phosphomimetic mutant display a stronger affinity toward E2F. The combined data indicated that p38-mediated RB phosphorylation leads to an increase in affinity of the N-terminal toward E2F, creating an alternative E2F-binding surface insensitive to CDK phosphorylation.

Personal contribution to this work.

I have been involved in the development of this project by designing and executing experiments related to:

1. *In vitro* and *in vivo* phosphorylation of RB by p38.
2. Evaluation of the dominance of p38-mediated phosphorylation of RB over those by CDKs.
3. Assessment of cell proliferation in HEK293T cells and several cancer cell lines expressing the wild-type and the phosphomimetic RB mutant, performing Colony formation assays (CFA).
4. Development and setup of molecular biology tools as cloning the RB mutants into adequate plasmids and lentiviral particle production, infection and maintenance of several cell lines included in the paper.

Gubern, A., Joaquin, M., Marquès, M., **Maseres, P**., Garcia-Garcia, J., Amat, R., González-Nuñez, D., Oliva, B., Real, F.X., de Nadal E.*, and Posas, F.*

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The N-Terminal Phosphorylation of RB by p38 Bypasses Its Inactivation by CDKs and Prevents Proliferation in Cancer Cells.

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Part II: Role of RB phosphorylation by p38 MAPK in muscle cell differentiation.

8. Role of Ser249/Thr252 RB phosphorylation in a cellular model of differentiation.

Previous experiments in our laboratory (Gubern et al., 2016) showed that p38 is able to phosphorylate RB protein *in vitro* and *in vivo*. We identified the specific residues phosphorylated by p38 (Ser 249 and Thr 252) by site-directed mutagenesis. Also, using an E2F-luciferase reporter system, we observed that p38 is able to modulate RB activity (addressed by the pharmacological inhibition of p38 and an RB unphosphorylatable mutant). Moreover, the phosphomimetic mutant of these specific phosphorylation sites downregulated the transcription of cell cycle related genes and inhibited cell proliferation in several cancer cell lines. Remarkably, RB phosphorylation by p38 was dominant over those by CDKs, which could be critical in processes where an irreversible cell cycle arrest is needed, as in the case of muscle differentiation (Gubern et al., 2016).

Taking into account the role of RB in the transition from myoblast proliferation to differentiation, we hypothesized that p38, besides controlling the expression of muscle-specific genes (reviewed in Segalés et al, 2016b), and regulating MKP-1/JNK/c-Jun axis in cell cycle withdrawal (Perdiguero et al., 2007a,b), could also regulate cell cycle exit via RB phosphorylation (**Figure 8**). To address this possibility, we used C2C12 mouse myoblasts, a cell

model extensively used to recapitulate muscle differentiation *in vitro*. When shifted to differentiation medium (DM, with low levels of serum), C2C12 myoblasts stopped proliferating and entered into a terminal differentiation program, with fusion into myotubes after 48h in DM (**Figure 9A**).

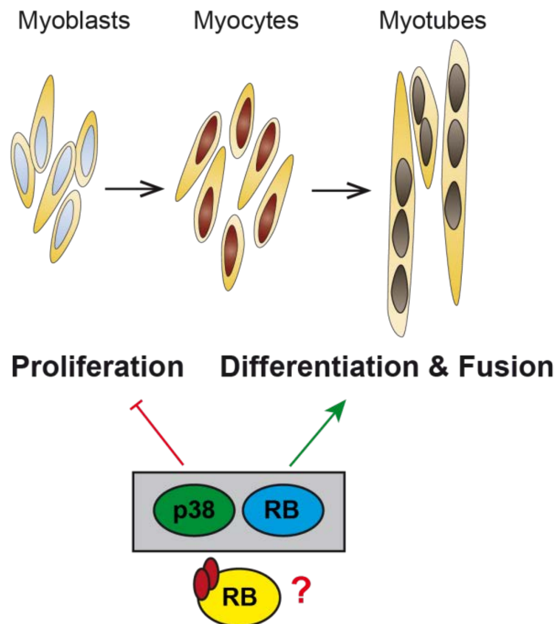

Figure 8: Hypothetic model of the role of RB phosphorylated by p38 in muscle cell differentiation. Besides controlling myogenic gene expression, p38 could regulate the irreversible cell-cycle arrest at the onset of differentiation through RB phosphorylation at N-terminal specific sites.

In order to assess the biological relevance of p38-mediated RB phosphorylation during myogenic differentiation, we did a time-course of C2C12 cell differentiation and analyzed several differentiation markers and the phosphorylation of RB at the p38-specific sites with a phospho-specific antibody (Gubern et al., 2016). As myoblasts differentiated, we observed the induced

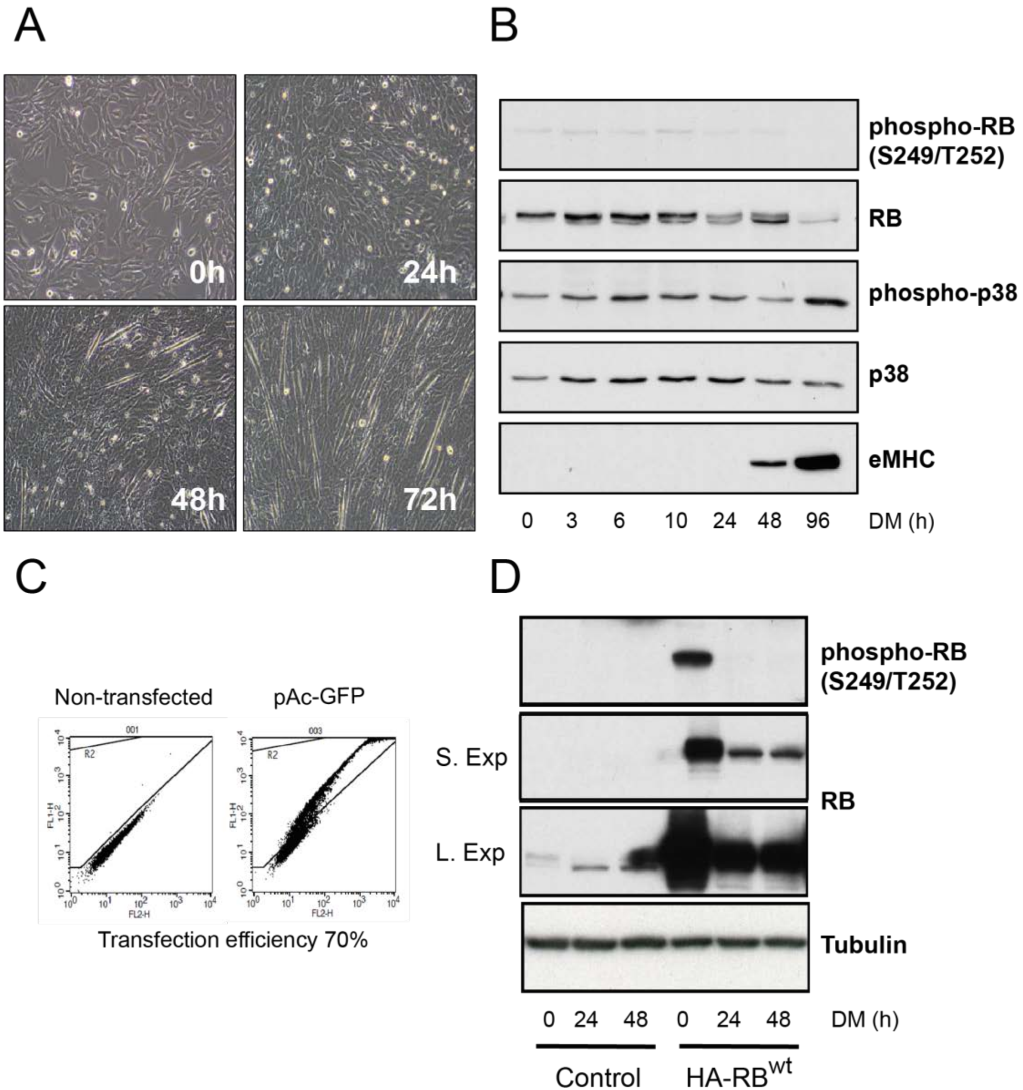

Figure 9: Role of Ser249/Thr252 RB phosphorylation in myogenic differentiation. A, C2C12 myoblasts cultured in growth medium were shifted to differentiatiation conditions for 72h. Bright field images showing myotube formation over 72h. **B,** RB and p38 phosphorylation in differentiating conditions (96h) were assessed with the indicated antibodies **C,** Transfection efficiency of C2C12 cells (24h post-transfection). **d,** Time course of differentiation in myoblasts overexpressing an HA-tagged wild-type RB (HA-RB^{wt}).

expression of myogenic differentiation markers such as embryonic myosin heavy chain (eMHC). We also monitored the progressive inactivation of RB, from a hyperphosphorylated slow-migrating form (inactive RB) to a hypophosphorylated fast-migrating RB (active form) over differentiation. Moreover, we observed the progressive activation of p38 with specific antibodies. Nevertheless, even though p38 was activated during this process, we could not detect significant levels of endogenous RB phosphorylation by p38 (**Figure 9B**).

As an experimental alternative, we transfected a plasmid with wild-type RB (HA-tagged wild-type RB), for its overexpression before shifting the myoblasts to differentiation conditions in DM. We achieved a transfection efficiency of about 70% (using a GFP plasmid as transfection reporter) (**Figure 9C**). In C2C12 cells transfected with HA-RB, we were able to detect p38-phosphorylated RB at the onset of differentiation (DM0h), but it was lost over the process. HA-RB expression was also lost progressively over myogenesis, probably due to a transient effect of cell transfection (**Figure 9D**).

9. RB mutants in the p38-phosphorylated specific sites do not interfere with myoblast differentiation.

Next, we evaluated if expression of a wild-type RB or its phosphorylation mutants affected the differentiation of myoblasts. Accordingly, we transfected C2C12 cells with plasmids carrying an HA-tagged wild-type RB and mutant versions, the non-phosphorylatable $RB^{S249AT252A}$ mutant and the phosphomimetic

RB^{S249ET252E} mutant. Cells were shifted to DM for different time points, and we assessed mRNA expression of different myogenic markers such as Myogenin and Caveolin 3, relative to GAPDH (**Figure 10A**). We did not find significant differences in the expression of these markers between cells transfected with wild-type RB or the mutants.

We also checked the effect of wild-type RB or mutants on the myogenic differentiation markers Myogenin and MHC at the protein level (representative time course is shown in **Figure 10B**). We did not detect significant differences in the expression levels of these proteins in cells expressing wild-type or mutant RB, although there was high variability between experiments (likely due to small differences in cell confluence or in the efficiency of transfection). Of note, the expression of RB mutants was also reduced over differentiation, as previously observed in the case of the wild-type RB (**Figure 9D**).

As an alternative approach, we infected C2C12 cells with lentiviral particles (non-inducible and inducible vectors) to generate C2C12 cells with stable (or inducible) expression of the wild-type RB and mutants. This system is suited to express transgenes in a stable and uniform way. Unfortunately, while the system was working properly in other cell lines, we did not succeed in stably expressing wild-type and mutant RB forms in C2C12 cells despite the high number of attempts, due to technical reasons. Hence, we were not able to reach any conclusion regarding the effect of RB phosphorylation by p38 in myoblast differentiation, and therefore additional tools or approaches are needed to further test our hypothesis.

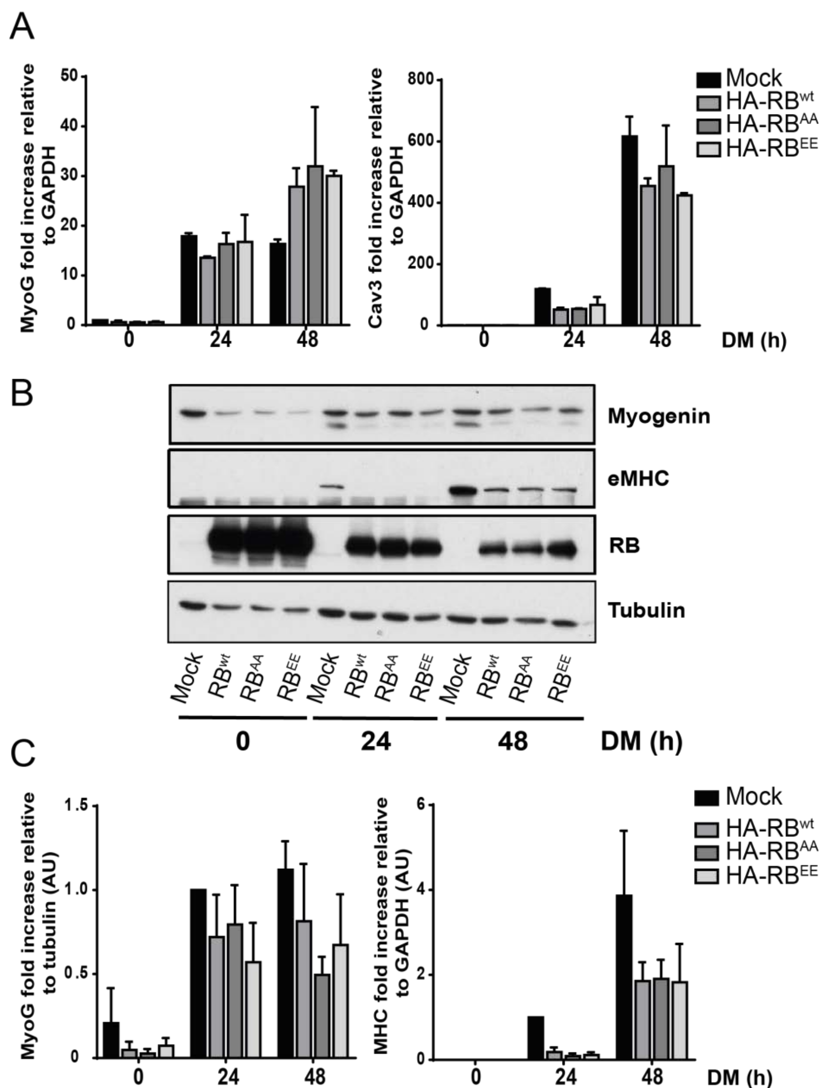

Figure 10: RB mutants in the p38-phosphorylated specific sites do not interfere with myoblast differentiation. A, Time course of myogenic differentiation in C2C12 cells transfected with plasmids carrying HA-tagged versions of wild-type RB (RB^{wt}), the non-phosphorylatable $RB^{S249AT252A}$ mutant (RB^{AA}) or the phosphomimetic $RB^{S249ET252E}$ mutant (RB^{EE}). mRNA levels of the differentiation markers Myogenin and Caveolin 3 were quantified and represented (2 biological replicates). **B,** Protein expression analysis of differentiation was assessed after 24 and 48h in differentiation conditions. **C,** Quantification of Myogenin and MHC (relative to tubulin) for 4 independent experiments is represented. In **A,** and **C,** means are represented with their SEM. Statistical analysis showed non-significant differences.

10. The expression of a phosphomimetic RB mutant is not sufficient to recover the differentiation defect in human rhabdomyosarcoma cells.

We also wanted to explore the relevance of the p38-RB pathways in the context of a muscle-related cancer model. We described previously that the expression of a phosphomimetic RB mutant was sufficient to inhibit the proliferation of cancer cells (Gubern et al., 2016). We analyzed the role of this phosphorylation in a model of rhabdomyosarcoma (RMS), one of the most common tumors of childhood, in which muscle precursor cells fail to complete the differentiation program, despite the expression of the muscle regulatory proteins MyoD and Myogenin (Dias et al. 1992; Tapscott et al. 1993).

Puri and collaborators described that RMS cell lines express p38 MAPK but its activity was not induced under differentiation conditions (scheme in **Figure 11**). After the stable expression of a constitutively active form of MKK6 ($MKK6^{EE}$), RMS cells stopped proliferating, and upon transfer into DM, they formed multinucleated myotubes. These studies further showed that activation of p38 MAPK by $MKK6^{EE}$ could restore MyoD activity and override the differentiation block in RMS cells (Puri et al., 2000). In this context, we hypothesized that the phosphorylation of RB by p38 could be important to stop RMS cell proliferation and induce differentiation. To test this, we started to work with a well-known human alveolar rhabdomyosarcoma cell line (Rh30 cells) that has been extensively used in the study of this type of cancer, including the mentioned work (Puri et al., 2000).

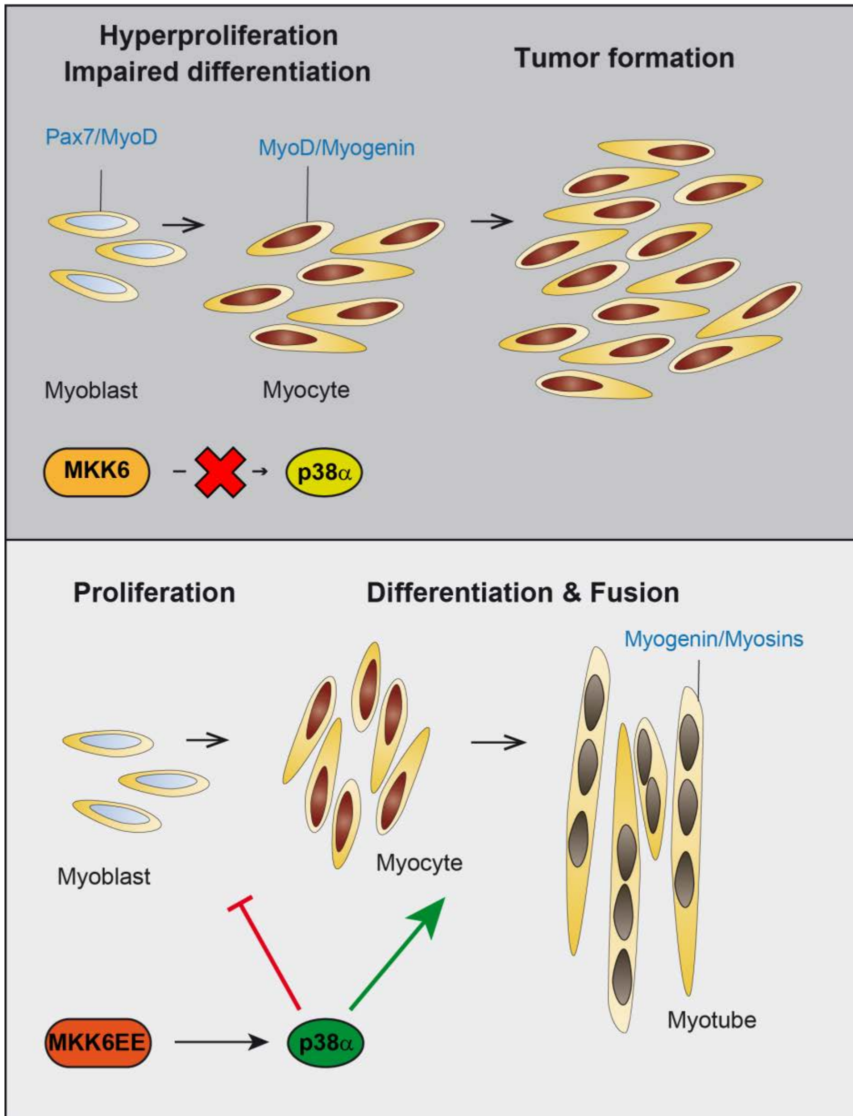

Figure 11: Role of p38α in terminal differentiation of human rhabdomyosarcoma cells. p38 MAPK is expressed in RMS cells; however, these cells cannot induce its activity in differentiating conditions, and are not able to achieve terminal differentiation, thus promoting tumor formation. The activation of p38 MAPK by MKK6^{EE} restores MyoD activity and RMS cells override the differentiation block, and are able to form myotubes.

We infected Rh30 cells with the lentiviral particles of the wild-type and the phosphomimetic RB mutant (with a doxycycline inducible promoter) in order to generate cell lines stably expressing our proteins of interest. These lentiviral constructs allowed selection of the infected population with puromycin resistance. Of note, it is known that viral transduction place both in dividing and non- diving cells.

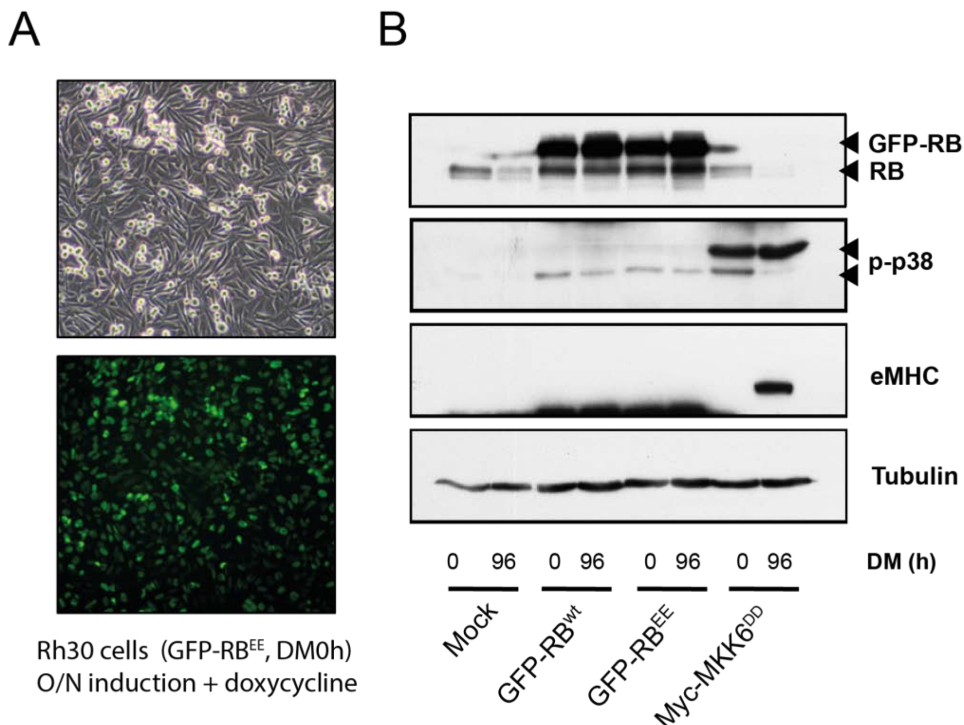

Figure 12: The expression of a phosphomimetic RB mutant form is not sufficient to recover the differentiation defect of human rhabdomyosarcoma cells. **A,** Transduction efficiency in Rh30 cells infected with an inducible lentivirus carrying a GFP-fused RB^{S249ET252E} mutant, 24 hours after doxycycline administration. **B,** Cells transduced with wild-type (GFP-RB^{wt}) or phosphomimetic RB (GFP-RB^{EE}) were challenged to differentiate for 92h. Cells expressing a myc-MKK6^{DD} and an empty vector were used as controls. Doxycycline (1 mg/ml) was refreshed every 24 hours.

Transduction efficiency was evaluated in puromycin-resistant Rh30 cells infected with an inducible lentiviral plasmid carrying a GFP-fused wild-type or the phosphomimetic $RB^{S249ET252E}$ mutant, 24h after doxycycline administration (**Figure 12A**). In contrast with C2C12 cells, the vast majority of Rh30 cells were able to express both RB forms, as shown by GFP expression. Cells transduced with wild-type or phosphomimetic RB were challenged to differentiate under the same conditions as those previously used for C2C12 cells. Cells transduced with a Myc-$MKK6^{DD}$ or with an empty vector were used as controls. Of note, the decay in RB expression previously observed in transfected C2C12 cells (**Figures 9D and 10B**) was avoided by doxycycline administration (1 mg/ml) every 24 hours. Remarkably, $MKK6^{DD}$ expressing cells clearly restored its potential to differentiate, showing eMHC expression after 96 hours (**Figure 12B**). That was not the case in cells expressing the phosphomimetic RB mutant (GFP-RB^{EE}). We concluded that the restoration in differentiation of RMS cells by activation of the p38 signaling pathway do not occur through the phosphorylation of RB in p38 specific residues, or this is not sufficient to rescue its differentiation defect.

Personal contribution to this work.

I have been fully involved in the design, execution and discussion of the experiments and results described in this part. In these studies we assessed the biological relevance of RB phosphorylation by p38 in physiological processes different from stress adaptation, such as skeletal myogenesis, as well as its contribution to the terminal differentiation of human rhabdomyosarcoma cells.

Part III: Role of p38α in muscle stem cell aging.

The physiological function for p38 MAPK signaling *in vivo* has been determined by the use of specific chemical inhibitors and by the generation of genetically modified mice. p38α knockout mice die during embryonic development (Adams et al., 2000; Mudget et al., 2000; Tamura et al., 2000). For this reason, the generation of tissue-specific conditional knockout mice is necessary to study the role of p38α pathway in a tissue-specific manner in the adult.

Pell and collaborators showed a role for p38α in skeletal muscle growth and regeneration in mice with conditional deletion of p38α in satellite cells (p38α$^{\Delta Pax7}$) (Brien et al., 2013). In these mice, p38α ablation is driven by Cre-recombinase expression under the control of the Pax7 promoter, thus affecting exclusively the Pax7 lineage (satellite cells). This study confirmed previous *in vitro* findings showing a key role for p38α in the switch of myoblast proliferation to differentiation (Perdiguero et al., 2007a), as they demonstrated that p38α restricts excessive myoblast proliferation during the muscle postnatal hyperproliferative phase and promotes myoblasts towards a differentiated state (**Figure 13**) (Brien et al., 2013). Accordingly, the loss of p38α caused a postnatal growth defect together with an augmented number of satellite cells, as a consequence of an increased myoblast proliferation *in vivo*. Muscle regeneration in young mice after a cardiotoxin-induced injury was delayed in the absence of p38α, with further increase of the satellite cell population (Brien et al., 2013). Moreover, myoblasts lacking p38α had increased expression of proliferation-enhancing

genes, mostly related with cell cycle and DNA replication (Brien et al., 2013). Since p38α also controls human satellite cell (huSCs) regenerative potential (Charville et al., 2015), p38α MAPK emerges as a key regulator of the transition from proliferation to differentiation of satellite cells both in mice and humans.

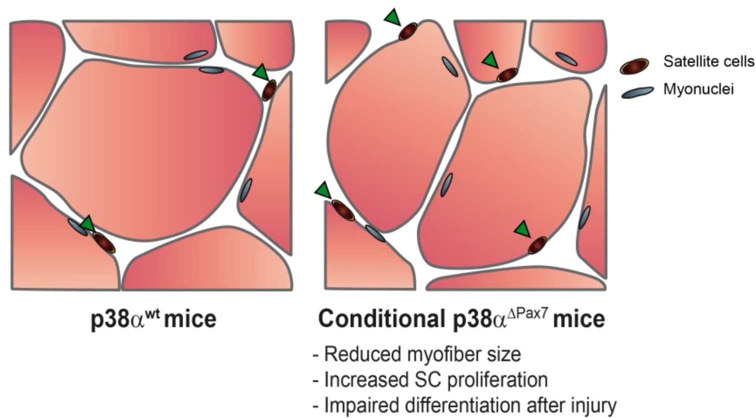

Figure 13: Young mice with conditional deletion of p38α in satellite cells show alterations in skeletal muscle growth. p38α$^{\Delta Pax7}$ mice showed a postnatal defect in skeletal muscle growth, with reduced myofiber size and unchanged total fiber number. These mice also exhibited a defective regeneration by extended satellite cell proliferation and an impaired differentiation. Brien et al., 2013.

11. p38α deletion has a different impact in the regenerative capacity of satellite cells during aging.

Aging is characterized by a loss of muscle mass and function (sarcopenia), especially at geriatric age (28 months-old mice or older). Sarcopenia is accompanied by a dramatic drop in the regenerative potential of aged skeletal muscle (Sousa-Victor et al., 2014a). Although the muscle regenerative decline in old mice

can be partially reversed by a youthful environment (Conboy et al., 2005), this decline is known to occur at the expense of intrinsic changes in the satellite cell population (Brien et al., 2013; Cosgrove et al., 2014; Sousa-Victor et al., 2014a). Importantly, old satellite cells have a cell-autonomous increase in the activity of the p38 MAPK pathway (Cosgrove et al., 2014; Bernet et al., 2014), and the use of pharmacological inhibitors together with cell culture in soft hydrogel (mimicking the properties of young muscle) had beneficial effects in old satellite cell engraftment and muscle regeneration (Cosgrove et al., 2014). Based on these findings using pharmacological strategies, we were interested in exploring the impact of the genetic loss of p38α in satellite cells on muscle regeneration during aging.

We generated p38α$^{\Delta Pax7}$ mice (after intercrossing p38α$^{f/f}$ mice with Pax7-CRE mice) and brought them to old age, and analyzed comparatively the changes in the satellite cell population in p38α$^{\Delta Pax7}$ mice and their age-matched wild-type littermates with aging. In agreement with previous data (Zammit et al., 2002; Hawke and Garry, 2001; Shefer et al., 2006, 2010; Sousa-Victor et al., 2014a), we observed a decline in the number of Pax7^{+} cells (per mg of tissue) during aging (**Figure 14A**). Differently of the phenotype observed in postnatal juvenile mice (Brien et al., 2013), p38α-deficiency did not induce a significant difference in the number of satellite cells in young/adult and aged p38α$^{\Delta Pax7}$ mice compared to age-matched wild-type mice.

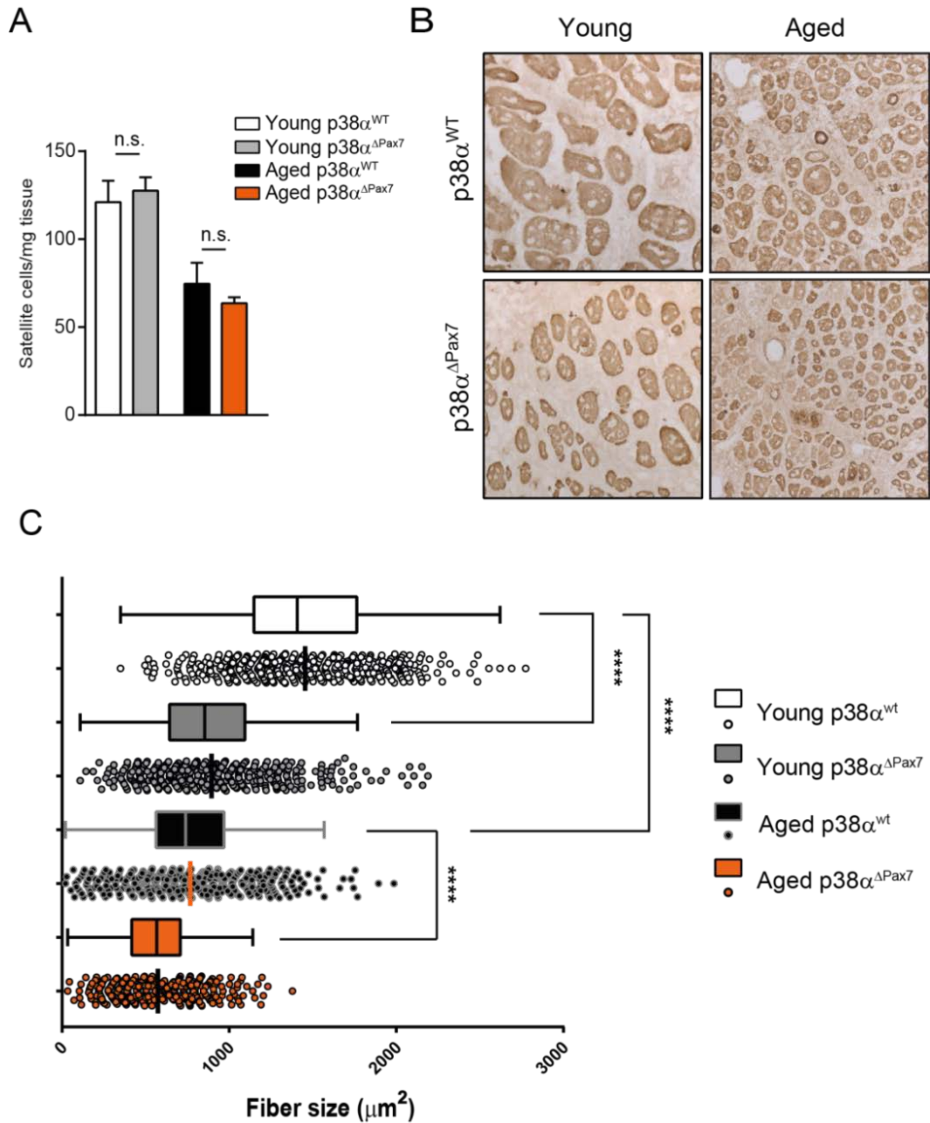

Figure 14: p38α ablation impacts differently on the regenerative capacity of young and aged satellite cells. **A,** Satellite cell numbers (per mg of tissue) in young (2-3 months) and aged (18-24 months) p38α-deficient mice and their wild-type littermates. **B,** Representative pictures of newly formed fibers (eMHC staining) in wild-type and p38α-deficient mice after cardiotoxin (CTX) injection (7 days). **C,** Distribution of the regenerated fibers in CTX-injured mice. Box and whiskers (median) and scatter dot blot (mean) are represented. Two-tailed parametric unpaired t-test analysis of the means (Young p38α^{WT}, 1454μm^2; Young p38α$^{\Delta Pax7}$, 895μm^2; Aged p38α^{WT}, 767,8μm^2; Aged p38α$^{\Delta Pax7}$, 574,3μm^2; ****, $p<0,0001$).

To check if the regenerative potential of p38α-deficient satellite cells was affected, we performed muscle injury by intramuscular injection of cardiotoxin (CTX), a well-known model of injury-induced muscle regeneration (Perdiguero et al., 2007a; Ruiz-Bonilla et al., 2008; Sousa-Victor et al., 2014a; García-Prat et al., 2016), in p38α$^{\Delta Pax7}$ mice at different ages and in age-matched wild-type controls. We determined the formation and size of new myofibers after CTX injection, by measuring the cross-sectional area (CSA) of the regenerating fibers stained with embryonic myosin heavy chain (eMHC) (representative images in **Figure 14B**). We observed that, in the absence of p38α, young mice had a markedly reduced capacity of regeneration, as shown by the 40% decrease in mean regenerating fiber size compared to wild-type mice, similar to previous studies by the Pell lab (Brien et al., 2013) (**Figure 14C**). In contrast, in aged mice, the drop in regenerative capacity was attenuated in p38α$^{\Delta Pax7}$ mice compared to wild-type mice, and loss of p38α only caused a 25% decrease in regenerating fiber size, indicating that the loss of p38α was less detrimental for the regenerative potential of aged satellite cells compared to young counterparts (**Figure 14C**). These age-dependent differences could be explained by intrinsic changes in the satellite cell population, in their microenvironment and/or by additional functions of p38α (other than myogenic ones) associated to aging. Thus, p38α deletion has a different impact in the regenerative potential of satellite cells during aging.

12. Loss of p38α in young and aged satellite cells results in increased proliferation.

To evaluate the potential altered functions of satellite cells upon p38α ablation and the influence of age, we isolated satellite cells from wild-type and p38α$^{\Delta Pax7}$ young and aged mice by FACS sorting (α7-integrin^{+}/CD34^{+} cells). We assessed satellite cell proliferation capacity by BrdU incorporation *in vitro*, and BrdU-labelled cells were detected by immunostaining using a specific antibody (representative images shown in **Figure 15**). As expected, young satellite cells from p38α$^{\Delta Pax7}$ mice showed an increase in proliferation with respect to wild-type cells (**Figure 15**). We observed that that the decline in proliferation in aged satellite cells (BrdU^{+} cells) was reversed by the lack of p38α, reaching values of young wild-type cells. This indicates that aged satellite cells deficient for p38α maintain high proliferative potential over time.

13. Lack of p38α during aging results in reduced senescence.

One of the most prevalent pathologies associated to aging are sarcopenia and reduced muscle regenerative capacity. Recent studies have associated satellite cell senescence as an underlying cause of age-associated loss of regenerative potential. Aged satellite cells displayed positive senescence-associated β-galactosidase activity (SA-β-gal) staining, a classical senescence marker, and accumulated senescence-associated proteins like p16^{INK4a} (Sousa-Victor et al., 2014a; Cosgrove et al., 2014; Du et

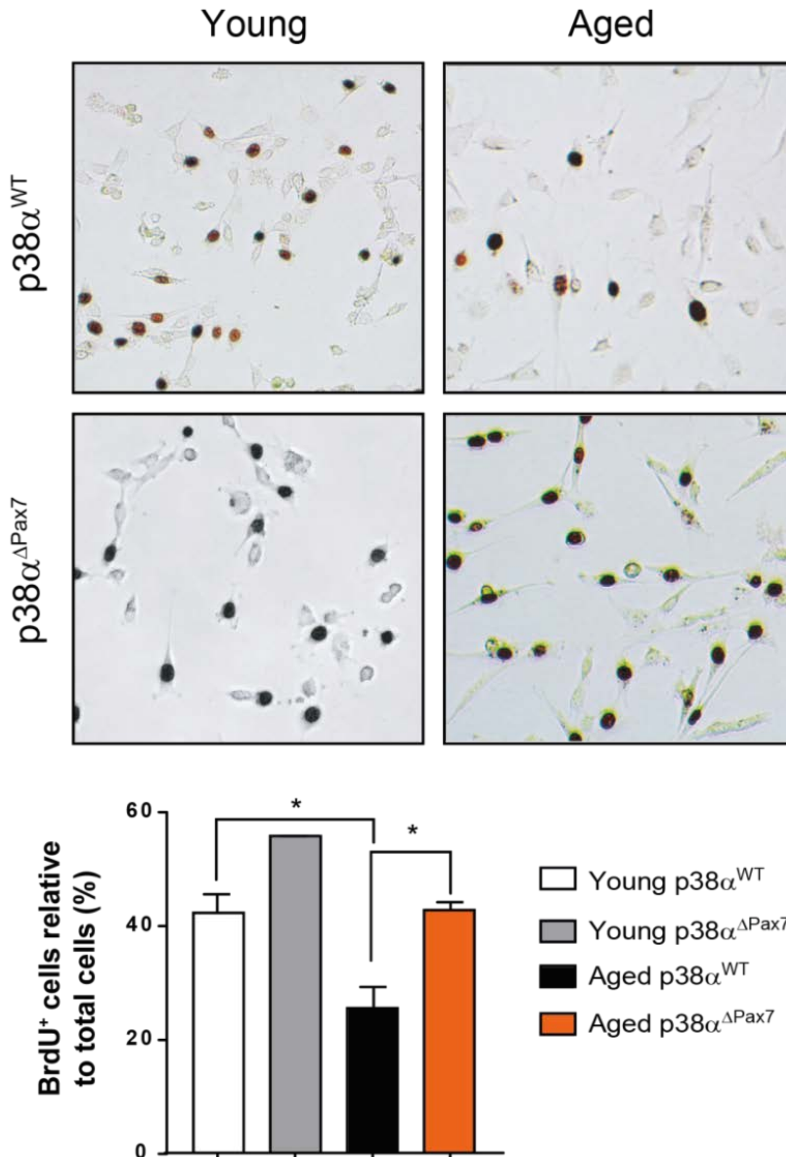

Figure 15: p38α deletion in aging satellite cells results in increased proliferation. Proliferation analysis of satellite cells isolated from p38α$^{\Delta Pax7}$ and wild-type mice from different ages and cultured in growth medum *in vitro*. Proliferation was assessed after 1h of BrdU incorporation by positive stained cells (BrdU^{+} cells) relative to total cells (%). Upper panel, representative images from proliferating satellite cells stained for BrdU. Lower panel, mean proliferation rates of satellite cells from mice of different ages represented with their SEM Two-tailed parametric unpaired t-test analysis of the means (*, Young p38α^{WT} vs. Aged p38α^{WT} p=0,0334; Aged p38α^{WT} vs. Aged p38α$^{\Delta Pax7}$ p=0,0256).

al., 2014) and Igfbp5, together with an increased γH2AX DNA damage/repair signal (Sousa-Victor et al., 2014a).

To analyze the potential impact of p38α on senescence of aged satellite cells, we assessed SA-β-gal activity in satellite cells isolated from wild-type and p38α$^{\Delta Pax7}$ mice of different ages. Cells were cultured in GM (proliferating medium) for 4 days and the relative percentage of positive cells (SA-β-gal^{+} cells) was evaluated (representative images are shown in **Figure 16**). We observed that, under proliferative conditions, around 20% of the aged satellite cells underwent a fast entry into senescence (geroconversion) when compared with young satellite cells, similarly to previous results (Sousa-Victor et al., 2014a). Importantly, aged cells isolated from p38α$^{\Delta Pax7}$ mice did not enter into senescence, as shown by the negligible numbers of SA-β-gal^{+} cells, showing therefore a similar behavior as satellite cells from young wild-type mice (**Figure 16**). Thus, p38α deficiency is beneficial for aged cells, by preventing geroconversion under proliferative conditions.

14. p38α loss reverses the epigenetic derepression of the p16^{INK4a} locus during aging.

The genetic inactivation of p16^{INK4a} (Sousa-Victor et al., 2014a) or chemical inhibition of p38α/β (Cosgrove et al.,2014; Bernet et al., 2014) had a similar output in the performance of aged satellite cells, since both treatments were sufficient to revert the regenerative impairment associated with aging. This suggested a potential link between p38α and p16^{INK4a} expression in satellite cells.

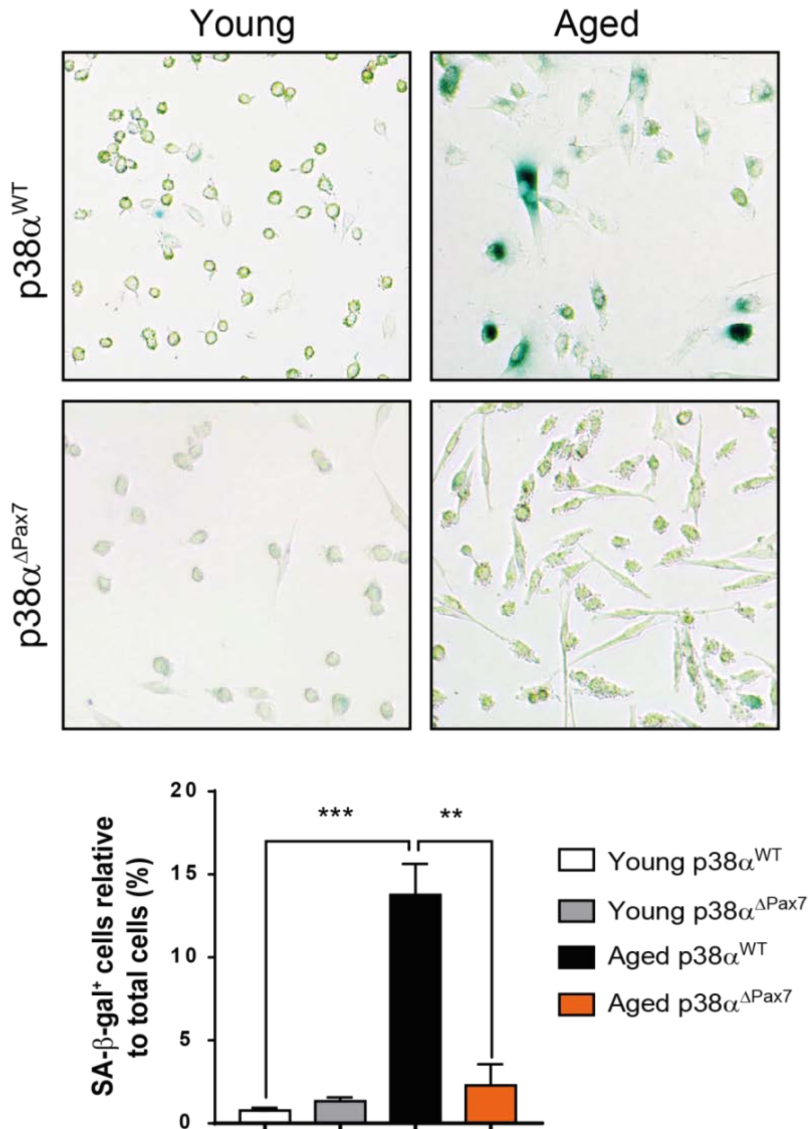

Figure 16: Lack of p38α results in reduced senescence entry of aged satellite cells. Senescence in wild-type and p38α-deficient satellite cells from different ages was assessed by senescence-associated β-galactosidase activity (SA-β-gal) staining of cells cultured for 4 days in growth medium (GM). Upper panel, representative images of positively stained aged satellite cells (SA-β-gal^{+} cells). Lower panel, mean of the relative percentages of positively stained cells (SA-β-gal+ cells) from wild-type and p38α$^{\Delta Pax7}$ mice at different ages are represented with their SEM. Two-tailed parametric unpaired t-test analysis of the means (Young p38α^{WT} vs. Aged p38α^{WT} p=0,0003; Aged p38α^{WT} vs. Aged p38α$^{\Delta Pax7}$ p=0,0031).

We analyzed $p16^{INK4a}$ expression in satellite cells isolated from wild-type and $p38\alpha^{\Delta Pax7}$ mice. Both protein (**Figure 17A**) and mRNA expression (**Figure 17B**) were evaluated in proliferating cells, and we observed that, while $p16^{INK4a}$ expression increased in aged satellite cells correlating with senescence status, it was abrogated in aged p38α-deficient cells both at protein and mRNA levels (**Figures 17A and 17B**). Thus, aged p38α knockout cells prevented $p16^{INK4a}$ upregulation and this could explain, at least in part, how these cells were able to bypass entry into senescence under high proliferative conditions.

We wanted to further elucidate the regulation of the INK4a locus at a mechanistic level in aging satellite cells. The epigenetic repression of the INK4/ARF locus, which codes for $p16^{INK4a}$ and other CDK inhibitors such as $p15^{INK4b}$ and $p19^{ARF}$, was described to be lost in aged satellite cells, leading to $p16^{INK4a}$ upregulation (Sousa-Victor et al., 2014a). The increased expression of $p16^{INK4a}$ had been previously implicated in promoting the irreversible cell cycle arrest that precedes senescence (Dai and Enders, 2000; Beausejour et al., 2003; Takahashi et al., 2006; Collado and Serrano, 2006). This regulation is mediated by chromatin associated protein complexes, and mainly by Polycomb repressive complex 1 and 2 (PRC1 and PRC2), which are implicated in cellular senescence (Agherbi et al., 2009; Lanigan et al., 2011). Interestingly, p38α has been shown to phosphorylate Ezh2 (the catalytic subunit of PRC2) in the regulation of the Pax7 locus in muscle regeneration (Palacios et al., 2010).

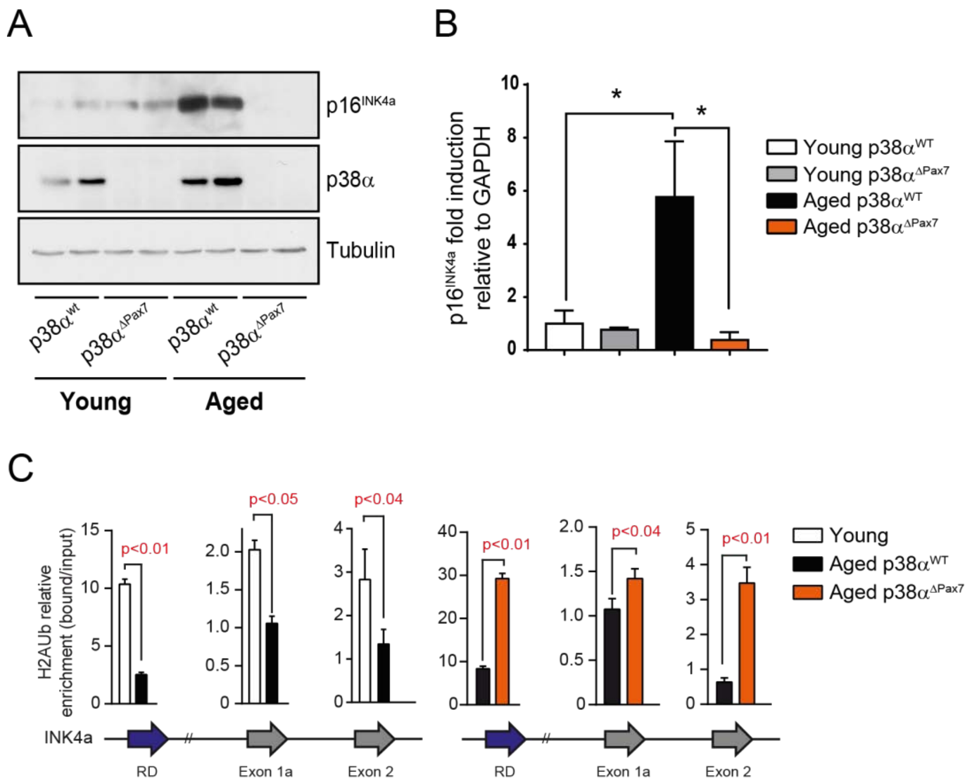

Figure 17: p38α loss alters the epigenetic regulation of the INK4a locus during aging. A, p16^{INK4a} protein and **B,** mRNA (relative to GAPDH) expression in proliferating satellite cells *in vitro*. p16^{INK4a} mRNA levels relative to young p38α^{WT} satellite cells. One-tailed parametric unpaired t-test analysis of the means (*, Young p38α^{WT} vs. Aged p38α^{WT} p=0,0289; Aged p38α^{WT} vs. Aged p38α$^{\Delta Pax7}$ p=0,0296). **C,** ChIP analysis for H2AK119ub (H2Aub) repressive mark at different key sites of the INK4a locus in sorted satellite cells from resting muscle.

In previous reports from our laboratory, we observed that the association of the H2A monoubiquitination at lysine 119 (H2A^{K119Ub}) repressive mark catalyzed by PRC1 to key regions of the INK4a locus, the INK4a regulatory domain (RD) and exons 1a and 2 (Agherbi et al., 2009), was reduced in aged satellite cells (Sousa-Victor et al., 2014). Preliminary results using ChIP analysis of satellite cells from p38α^{WT} and p38α$^{\Delta Pax7}$ mice demonstrated that

the H2A^{K119Ub} repressive mark was still present in aged cells lacking p38α (**Figure 17C**), which would explain the observed effect at the level of p16^{INK4a} gene expression. These findings support the notion that the lack of p38α in aged satellite cells confers them resistant to geroconvertion by preventing epigenetic derepression at the INK4a locus.

15. The inhibition of p38 activity results in a regenerative advantage of aged satellite cells *in vivo*.

Our initial results show that lack of p38α in satellite cells had milder regenerative consequences in aged than in young mice after muscle injury, correlating with reduced senescence in aged satellite cells. We hypothesized that specific interventions inhibiting p38 activity *in vivo,* could rescue the impaired regeneration observed in aged mice.

To address this hypothesis, we used a whole muscle graft approach, consisting on isolating digitorum longus (EDL) muscles from aged mice and grafting them onto the tibialis anterior (TA) muscle of a recipient host, thus preserving a young host environment and allowing to test the intrinsic defects of aged satellite cells, with or without p38 inhibition. In this model, myofibers of the grafted EDL muscle degenerate and formation of new regenerating myofibers relies on the EDL resident satellite cells. We infected the whole EDL muscle from aged donors with recombinant adeno-associated virus (AAV) particles carrying a dominant negative p38 mutant (AAV-GFP-p38AF) (Diskin et al., 2007; Tzarum et al., 2007), and then grafted it onto TA muscles of

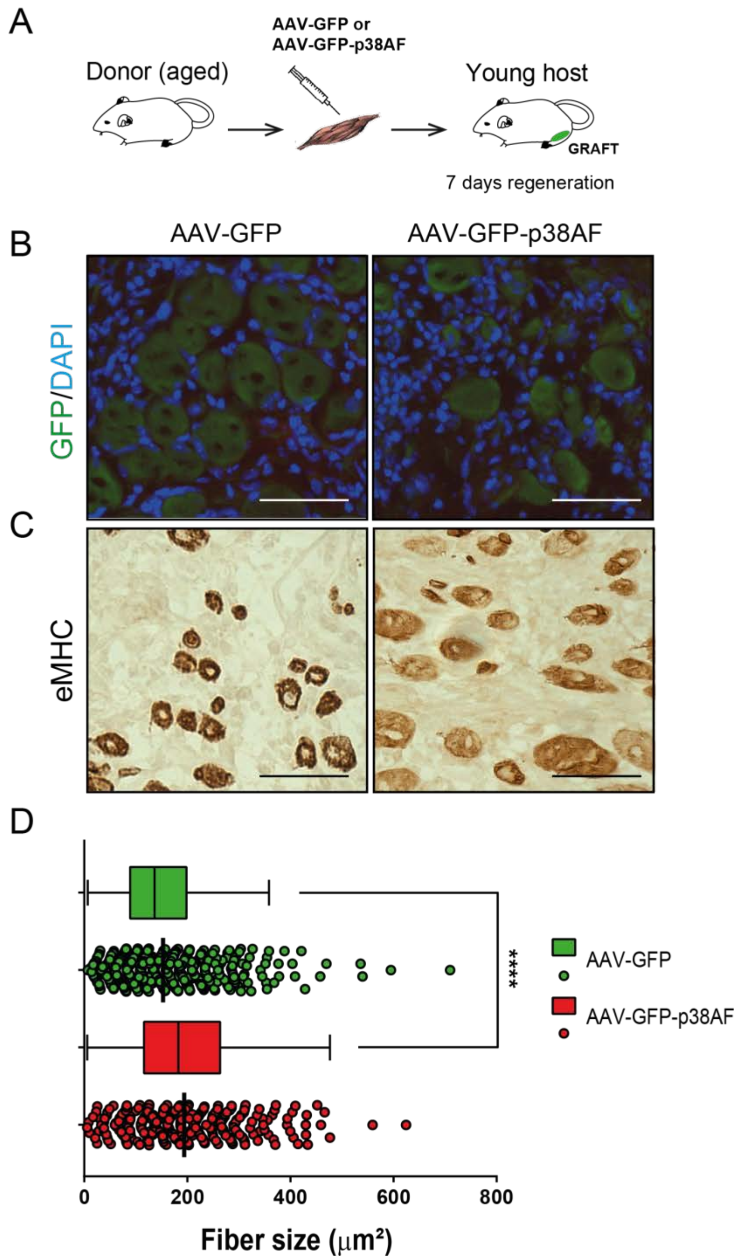

Figure 18: The regenerative potential of aged satellite cells increases upon inhibition of p38 activation. A, EDL muscle from aged donors was infected with an adeno-associated virus (AAV) containing a dominant negative p38 (p38AF, activation sites mutated) and its counterpart with a AAV-GFP alone. Then, the donor EDL was grafted on top of the TA muscle of a young recipient host, and the regeneration was assessed after 7 days. **B,** Formation of GFP^{+} fibers to assess satellite cell infection

by AAV particles. **C,** Generation of new regenerating fibers after 7 days of muscle grafting following p38 activity inhibition, was evaluated by eMHC staining. **D,** Regenerative fiber distribution by size is shown. Box and whiskers (median) and scatter dot blot (mean) are represented. Two-tailed parametric unpaired t-test analysis of the means (Mean AAV-GFP 153,03µm^2; AAV-GFP-p38AF 194,10µm^2; ****, p<0,0001). **B,** and **C,** Scale bar 50 µm.

young mice. This vector also encodes an IRES GFP, allowing the labeling of new fibers derived from infected satellite cells. Whole muscle grafts infected with AAV-GFP where used as controls (see scheme in **Figure 18A**). Effectiveness of the infection was assessed by immunostaining with an antibody against GFP (GFP$^+$ fibers) (**Figure 18B**).

We evaluated the regenerative potential of aged satellite cells upon p38 inhibition, measured as CSA of the new generated fibers stained for eMHC (representative images shown in **Figure 18C**). The regenerative potential significantly increased in muscles infected with AAV-GFP-p38AF particles, where we found a significant increase in regenerative fiber size (21%) after 7 days of grafting (**Figure 18D**). Thus, we confirmed that excessive p38 activity is deletereous in the context of muscle regeneration during aging, where constitutive p38 signaling in satellite cells would lead to impaired regeneration by preventing cell proliferation in combination with rapid entry into senescence. In this context, the inhibition of p38 signaling pathway would entail beneficial effects in regeneration.

Personal contribution to this work.

I have been fully involved in the design, execution and discussion of the experiments and results described in this part. For the analysis of muscle regeneration in young and aged mice, I used genetically modified mice and some muscle biopsies that were already available in our laboratory that I integrated in this project.

V. DISCUSSION

V. DISCUSSION

Part I: The N-Terminal Phosphorylation of RB by p38 Bypasses Its Inactivation by CDKs and Prevents Proliferation in Cancer Cells.

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Many studies in the past decades have brought into focus the essential role of RB regulation in G1/S transition and cell proliferation. One of the most common genetic events leading to cancer is the inactivation of the RB pathway, generally through a series of mechanisms that converge in a deregulation in the upstream control by CDK phosphorylation. Here, we describe a novel and essential mechanism, where the N-terminal region of RB is phosphorylated in two specific residues upon stress situations, leading to cell cycle arrest. Remarkably, this phosphorylation renders RB insensitive to CDK regulation, converting it into a strong suppressor of cell proliferation.

A delay in cell cycle progression is required for cell adaptation upon diverse environmental stresses, in order to orchestrate proper responses and maximize cell survival (Duch et al., 2012). Several studies in the yeast p38-ortholog Hog1 have demonstrated its relevance in the progression from G1 to S phase,

regulating cell cycle arrest by the combined action over the CDK inhibitor Sic1 and cyclin expression (Escoté et al., 2004; Adrover et al., 2011; González-Novo et al., 2015). Furthermore, this regulation has also been demonstrated in mammalian cells, where p38 activation lead to a delay in cell cycle progression by directly targeting the CDK inhibitors (CKIs) $p57^{KIP2}$, $p21^{CIP1}$ and $p27^{KIP1}$ (Cuadrado and Nebreda, 2010; Joaquin et al., 2012b; Cuadrado et al., 2009; Lafarga et al., 2009; Bulavin and Fornace, 2004). In this work, we reported a new mechanism involved in cell cycle regulation through a direct effect on RB transcriptional repressor, leading to a downregulation in E2F-mediated transcription. Remarkably, the mechanisms regulating the G1/S-phase transition are highly conserved across eukaryotes, and its inactivation through mutations (as in the case of $p57^{KIP2}$ and RB) leads to a strong reduction in cell survival upon stress.

The regulation of RB has been classically associated to its inactivation following CDK phosphorylation, which results in RB dissociation from E2F, leading to transcription activation of genes involved in cell cycle progression (DeCaprio et al., 1992; Lees et al., 1991). The inactivating phosphorylations by CDKs generally occur at the pocket and C-terminal domains of RB, although the N-terminal phosphorylation on T373 has also been described to be relevant for its inactivation (Dick and Rubin, 2013; Rubin, 2013). In this study we show that, in stress conditions, p38α phosphorylates RB at Ser249 and Thr252 residues in the N terminus, both *in vivo* and *in vitro*. These specific sites have been reported to be phosphorylated *in vitro* by p34 (CDK1), known to participate in the regulation of G2 and M phase checkpoints (Lees et al., 1991). In another work, RB inactivation by JNK and p38 MAPKs was

proposed to occur upon Fas stimulation (Wang et al., 1999); nevertheless, the direct targeting of RB and the underlying mechanism by these kinases was never proved.

Differently to the inactivating phosphorylation by CDKs, we demonstrated that the p38 phosphorylation at Ser249 and Thr252 sites had a direct effect inducing RB activity, by increasing the affinity of the N-terminal region towards E2F, promoting a stronger RB association to the E2F-responsive promoters, and repressing E2F-mediated gene expression. The region surrounding these phosphorylation sites at the N terminus have not been included in the known crystal structures of RB. Nevertheless, molecular modeling predictions suggested that this region displays a similar structure to that of the E2F-interacting RB C-terminal region, generating a potential additional surface in RB for E2F interaction. Moreover, its affinity toward E2F was predicted to increase notably only when phosphorylated at these sites. Our data confirmed these predictions, since we observed an increased affinity of the p38-phosphorylated RB N-terminal region (or its phosphomimetic mutant) toward E2F. Considering that this interaction surface between E2F and RB is different than that of the C-terminal RB targeted by CDKs, this opened the possibility that, once E2F and RB had interacted through the N-terminal surface, it might become insensitive to the effects of CDK phosphorylation. This mechanism could provide a new framework for RB N-terminal phosphorylation to bypass its inactivation in backgrounds of high CDK activity, as in the case of many cancers.

In this work we show that the p38-mediated phosphorylation of RB leads to transcriptional repression of E2F target genes, even

in the presence of CDK-mediated RB phosphorylations that are known to promote its dissociation from E2F. Accordingly, an $RB^{S249T252E}$ mutant mimicking the phosphorylation by p38 repressed gene expression and associated with E2F promoters, even in the presence of high CDK activity, a typical condition under which wild-type RB is unable to repress transcription. Consistent with these findings, we demonstrated that the expression of the $RB^{S249T252E}$ mutant, differently from wild-type RB, restricted cellular growth in colony-forming assays and in mouse xenograft models.

CDK hyperactivation is one of the most common mechanisms leading to the loss of function of RB in cancers, mediated by a variety of mechanisms including amplification of genes coding for cyclins, CDK mutation, and loss or mutation of CKIs such as $p16^{INK4a}$ and $p21^{CIP1}$ (Sage and Cleary, 2012). A lot of effort has been made trying to reconstitute cell cycle control through inhibition of CDK activity, which has emerged as a promising alternative in the development of targeted cancer therapy. In the past few years, several oral drugs that selectively target CDK4/6 have been tested such as palbociclib, abemaciclib, and LEE011. Preclinical studies demonstrated optimal activity in hormone receptor positive breast cancers that display, in some cases, biologic features that suggest a particular dependence on the CDK4/cyclinD1/RB interaction (reviewed in Asghar et al., 2015).

A possible therapeutic strategy would involve the activation of the p38 signaling pathway, favoring the N-terminal phosphorylation of RB, which may lead to potential beneficial effects inhibiting cancer cell proliferation through a CDK

independent mechanism. The difficulty to implement this knowledge into therapeutics would be that direct targeting on p38 signaling could be detrimental, given that the activation of this pathway is also implicated in many more functions besides cell proliferation, as inflammation, migration and metastasis (Wagner and Nebreda, 2009). For this reason, the advantage that would entail the deregulation of the p38 pathway for this purpose would be obscured by its potential negative effects. Theoretically, a strategy targeting with small molecules the N-terminal region to mimic the phosphorylation of RB, rendering RB insensitive to CDK phosphorylation, would be a very precious therapeutic tool, especially in cancers with a background of high CDK activity. For this reason, a drug that could stimulate RB activity, as the phosphorylation does, and bypass the regulation by CDKs, effectively inhibiting proliferation, would be a promising tool for cancer therapeutics.

Part II: Role of RB phosphorylation by p38 MAPK in muscle cell differentiation.

The p38 MAPK signaling pathway has been shown to control all myogenic stages, acting as a regulator of the activation of satellite cells from quiescence into activation, and of proliferation-to-differentiation transition in myoblasts, by controlling the induction of cell cycle withdrawal and the modulation of the muscle-specific gene expression program (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000; Li et al., 2000a, Keren et al., 2006; Lluis et al., 2005; Suelves et al., 2004; Serra et al., 2007; Perdiguero et al., 2007a,b; Ruiz-Bonilla et al., 2008; Simone et al., 2004; Forcales et al., 2012; Rampalli et al., 2007; Cuadrado et al., 2010; Segales et al., 2016a; Jones et al., 2005; Palacios et al., 2010; Troy et al., 2012; Brien et al., 2013; Bernet et al., 2014; Hausburg et al., 2015; Charville et al., 2015).

RB is also a regulator of skeletal muscle differentiation during embryogenesis and in adult tissues (Zacksenhaus et al., 1996; Takahashi et al., 2003; Ferreira et al., 1998; Vandel et al., 2001; Nicolas et al., 2003; Blais et al., 2007; Sacco et al., 2003; Camarda et al., 2004; Huh et al., 2004; Pajcini et al., 2010). In addition to its inhibitory role on cell proliferation, RB has also been shown to regulate the muscle-specific differentiation gene expression (Gu et al., 1993; Schneider et al., 1994; Novitch et al., 1999; Lasorella et al., 2000; MacLellan et al., 2000; Puri et al., 2001; Benevolenskaya et al., 2005). Moreover, the loss of RB has also an impact on satellite cell homeostasis, by impairing cell activation and proliferation *in vivo* (Hosoyama et al., 2011).

Based on these reports, it was tempting to hypothesize that the novel mechanism of cell cycle regulation by p38 via RB phosphorylation (Gubern et al., 2016) could be also involved in the irreversible cell cycle arrest preceding myogenic differentiation. Importantly, in stress conditions, this novel mechanism is dominant over CDK control, and functions as a potent repressor of proliferation in several cell lines. We tested this hypothesis in the C2C12 mouse myoblast cell line. In this model, we could not detect the endogenous RB phosphorylation by p38 with the phospho-specific antibody (Gubern et al., 2016), being this phosphorylation only detectable when we overexpressed a wild-type RB at the onset of differentiation. As an alternative, we overexpressed the phosphorylation mutants in myoblasts to determine the effect during differentiation. Myoblasts expressing these mutants did not display significant differences in myogenic differentiation, as revealed by the similar expression of myogenic markers. Given these results, we could not reach conclusion regarding the effect of a p38-phosphorylated RB in myoblast differentiation. Other approaches are therefore needed to throw light in this hypothetical issue.

We next explored the potential role of this phosphorylation in a muscle-related cancer model (rhabdomyosarcoma (RMS)). RMS cells cannot modulate p38 MAPK activity under differentiation conditions; nevertheless, the forced p38 MAPK activation by a constitutively active MKK6 ($MKK6^{EE}$) restores MyoD activity and overrides the differentiation block in these cells (Puri et al., 2000). Thus, we hypothesized that the phosphorylation of RB by p38 could be important to stop proliferating and start differentiating in this model. We observed that, while forced p38 activation through

MKK6^{DD} restored terminal differentiation in Rh30 cells, a well-known cell line of alveolar RMS, as previously described (Puri et al., 2000), the expression of a phosphomimetic (RB^{S249ET252E}) mutant was not sufficient to rescue its differentiation defect. These results suggest that other p38 targets different from RB could be involved in the regulation of this process.

Altogether, we have not observed a significant impact of the p38-phosphorylated RB (or the phosphomimetic mutant) in physiological muscle differentiation or in pathological conditions. This could be explained based on several studies suggesting that the effect of RB in skeletal muscle differentiation would be indirect. For example, studies from the Zacksenhaus lab reported that RB is not required to activate the muscle differentiation program *in vitro,* and its effects are more related to the inhibition of apoptosis and autophagy in myoblasts and myotubes, respectively (Ciavarra and Zacksenhaus, 2010). In fact, under conditions rescuing the autophagy defects, robust myogenic differentiation was observed in myoblast lacking RB and one of its relatives, p107 or p130, implying that RB loss can be partially compensated by other pocket proteins. However, mutations in all RB family severely abrogated myogenic differentiation, indicating that myoblast fusion and myotube survival require at least one RB family member (Ciavarra et al., 2011). Moreover, despite the abundance of hypophosphorylated RB, p130-E2F repressor complexes are predominant in myotubes (Corbeil et al., 1995; Puri et al., 1997), questioning the importance and requirement of RB in the activation and the maintenance of the myogenic program. On the other hand, the selective and sustained p38 MAPK activation that is required for muscle differentiation (Puri et al., 2000; Perdiguero et al, 2007a)

is distinct from the acute activation following the stress-activated response (Joaquin et al, 2012; Ferreiro et al., 2010b; Gubern et al., 2016), which may explain differences involving the dynamics in RB phosphorylation and its requirement for cell cycle withdrawal at the onset of differentiation.

Part III: Role of p38α in muscle stem cell aging.

Skeletal muscle aging is associated with a progressive decline in satellite cell number and function resulting in defective tissue regeneration (Brack et al., 2005; Chakkalakal et al., 2012; Sousa-Victor et al., 2014a; Verdijk et al., 2012, 2014; Zwetsloot et al., 2013; Conboy et al., 2003, 2005; Garcia-Prat et al., 2016; Collins et al., 2007; Cosgrove et al., 2014). During aging, satellite cell function has been proposed to be influenced by several extrinsic factors, often increased in aged skeletal myofibers (the cellular niche of satellite cells) and/or systemically in the circulation, but also involves cell-intrinsic (cell-autonomous) mechanisms that are likely consequences of irreversible damage to old cells (reviewed in Sousa-Victor et al., 2015). Indeed, many of the defined primary hallmarks of aging (Lopez-Otin 2013), as well as deregulation of key transcriptional circuits necessary for stem cell identity and activity, have been found to be deregulated in aged satellite cells (Cornelison and Perdiguero, 2017).

In vivo, p38α signaling has been shown to restrict excessive myoblast proliferation and allow cell cycle exit and entrance into myogenic differentiation during the muscle growth phase in postnatal mice and during muscle regeneration after injury in young/adult mice (Brien et al., 2013). This p38α-mediated proliferative restriction is exerted, at least in part, through the control of the expression of proliferation-enhancing genes, mostly related with cell cycle and DNA replication (Brien et al., 2013). Remarkably, aged satellite cells have a cell-autonomous increase

in the activity of the p38 MAPK pathway (Cosgrove et al., 2014; Bernet et al., 2014), and the use of pharmacological inhibitors for p38α/β together with culture in soft hydrogel (mimicking the stiffness properties of young muscles) had beneficial effects in aged satellite cell engraftment and formation of new regenerating myofibers (Cosgrove et al., 2014). Here, we explored the impact of the genetic loss of p38α, the central p38 isoform in myogenesis (Perdiguero et al., 2007a; Ruiz-Bonilla et al., 2008), in satellite cells during aging by using p38α$^{\Delta Pax7}$ mice.

We first observed that aged mice suffered a decrease in satellite cell number, as previously reported (Zammit et al., 2002; Hawke and Garry, 2001; Shefer et al., 2006; Shefer et al., 2010; Sousa-Victor et al., 2014). Nevertheless, p38α-deficiency did not induce a significant difference in the number of quiescent satellite cells in young/adult or aged mice, and this is in contrast with the increased number of satellite cells observed at early postnatal stages prior to quiescence entry at postnatal day 21 (Brien et al., 2012). Secondly, we analyzed the endogenous muscle regenerative potential of p38α$^{\Delta Pax7}$ mice at different ages. While the young mice suffered a remarkable drop in its muscle regenerative potential upon p38α loss in satellite cells (around 40%), this decrement was more discrete in aged mice (25%). This result suggested that p38α deficiency in satellite cells is less detrimental at old than at young age, despite that other age-related niche factors and/or satellite cell-intrinsic defects could also be contributing to the muscle regeneration phenotype. Based on these findings, and considering that the p38 MAPK pathway may also crosstalk with other signaling cascades (Bain et al., 2007), we hypothesized that the inhibition of p38 activity in aged satellite cells

could rescue their impaired regeneration *in vivo*. Indeed, in muscle grafting experiments, we observed a significant increase in the regenerative capacity (21%) of aged satellite cells impaired in p38 activation (after lentiviral delivery of a dominant negative p38 mutant form) compared to wild-type cells. This indicates that attenuation of p38 signaling in aged satellite cells has a beneficial effect for muscle regeneration. In agreement with these results, recent reports demonstrated that transient p38α/β MAPK inhibition (Cosgrove et al., 2014), or attenuation of p38 signaling in a dominant negative haploinsufficient p38α mouse (DN-p38α$^{AF/+}$) (Papaconstantinou et al., 2015), had a positive impact on muscle regeneration.

Global epigenetic alterations (Liu et al., 2013) are also a hallmark of aging. Key loci such as INK4a are typically derepressed (Sousa-Victor et al., 2014a; García-Prat et al., 2016; Cosgrove et al., 2014; Carlson et al., 2008; Elabd et al., 2014), showing modifications in repressive chromatin marks such as H3^{K4me3} and H3^{K27me3} in old satellite cells (Elabd et al., 2014) and H2A^{K119ub} in geriatric satellite cells (Sousa-Victor et al., 2014a; García-Prat et al., 2016). This loss of regulation leads to elevated p16^{INK4a} expression and satellite cells become pre-senescent, turning into full cellular senescence upon persistent proliferative signals (Sousa-Victor et al., 2014a).

The role of p38 signaling in the induction of p16^{INK4a} expression and promotion of cellular senescence has been studied in human fibroblasts (Iwasa et al., 2003), and the inhibition of p38 activity was sufficient to extend the proliferative lifespan of fibroblasts (Tivey et al., 2013), pancreatic islet cells (Wong et al.,

2009) or hematopoietic stem cells (Ito et al., 2012; Zou et al., 2012). In aged muscle stem cells, $p16^{INK4a}$ upregulation and the loss of regenerative potential has been linked to the activation of p38α/β MAPK, since its pharmacological inhibition increase proliferation, thus promoting an amelioration of muscle regeneration in satellite cell transplantation experiments (Cosgrove et al., 2014; Bernet et al., 2014). However, the p38 MAPK – $p16^{INK4a}$ link was not uncovered in those studies. The epigenetic regulation of the INK4/ARF locus, which codes for $p16^{INK4a}$ and other CDK inhibitors, was lost in quiescent satellite cells from aged mice. In proliferative conditions, these cells adopted a senescent-like state, showing well-known senescence-associated characteristics, such as an increased SA-β-gal activity and $p16^{INK4a}$ upregulation, and leading to a loss in quiescence and a drop in its regenerative potential (Sousa-Victor et al., 2014a). In the present study, we show that satellite cells from aged $p38\alpha^{\Delta Pax7}$ mice did not enter geroconversion *in vitro,* and had an increased proliferative capacity and reduced levels of senescence-associated markers, such as SA-β-gal activity and $p16^{INK4a}$ expression (both at mRNA and protein levels).

At a mechanistic level, the regulation of the INK4a locus is mainly exerted by PRC1 and PRC2 complexes, known mediators of cellular senescence (Agherbi et al., 2009; Lanigan et al., 2011). Sousa-Victor and coworkers reported that the association of the $H2A^{K119Ub}$ repressive mark, catalyzed by the PRC1 catalytic subunit Bmi1, to chromatin was reduced in aged satellite cells in key regions of the INK4a locus, the INK4a regulatory domain (RD) and exons 1a and 2, (Sousa-Victor et al., 2014). Our present study shows that the observed INK4a locus derepression in aged satellite

cells does not occur in the absence of p38α, being the $H2A^{K119Ub}$ repressive mark still associated at key regions of the INK4 locus.

In future studies, we aim at getting further insight into the mechanism by which p38α regulates the derepression of the INK4a locus. Several reports indicated that PRC proteins may be targets for MAPKAP kinase 2 (MK2) (Yannoni et al., 2004) and 3 (MK3) (Voncken et al., 2005), well-known downstream kinases of p38 MAPK. Following stress stimulation, MK2/3 phosphorylate components of the PRC1 complex, such as Bmi1, which generates the release of the entire PRC1 complex from the chromatin, and resulting in the derepression of the INK4a locus in fibroblasts (Voncken et al., 2005). Given the persistent increase of p38 activity in aged satellite cells, a p38-MK2/3-Bmi1 axis may explain the loss of regulation in the INK4a locus, and its repression upon p38α ablation. To prove this possibility, we plan to assess MK2/3 phosphorylation in satellite cells from aged $p38α^{ΔPax7}$ mice.

Interestingly, in neural stem cells, epidermal growth factor (EGF)-induced AKT phosphorylation renders Bmi-1 resistant to proteasomal degradation, leading to its stabilization and accumulation in the nucleus. However, inhibition of the AKT-dependent Bmi-1 stabilizing process by p38 MAPK signaling reduces the levels of Bmi-1 in these cells (Kim et al., 2011). This could be a complementary mechanism linking p38 and Bmi1, which could be also operative in aged satellite cells, which may explain the persistent repression of the INK4a locus upon p38α ablation during aging.

Other components of the PRC2 complex (initiation complex) have also been described to be substrates of p38 MAPK. In the context of muscle regeneration, PRC2 represses Pax7 expression following inflammation-activated signaling in satellite cells (Palacios et al., 2010). TNFα-activated p38α MAPK promotes the interaction between YY1 transcription factor and PRC2, via threonine 372 phosphorylation of Ezh2, the enzymatic subunit of the complex, leading to the formation of repressive chromatin on the Pax7 promoter (Palacios et al., 2010), and therefore modulating the decision of satellite cells to proliferate or differentiate. Since YY1 is known to be implicated both in gene activation and repression, it is tempting to hypothesize that p38 phosphorylation of Ezh2 may lead to INK4a locus activation during aging.

Alternatively, another possibility is that p38 MAPK specifically regulates the expression of the $p16^{INK4a}$ locus through direct phosphorylation of other downstream targets. HMG box-containing protein 1 (HBP1) transcriptional repressor has been reported as a putative substrate for p38 MAPK in cell-cycle arrest (Xiu et al., 2003) and in Ras- and p38 MAPK-induced premature senescence (Zhang et al., 2006). HBP1 regulates endogenous $p16^{INK4a}$ expression through direct sequence-specific binding at the INK4 promoter in lung fibroblasts (Li et al., 2010). Taking this into account, p38α ablation in satellite cells during aging may lead to a deficient HBP1 phosphorylation, thus contributing to inefficient binding to INK4a promoter and avoiding the expression of $p16^{INK4a}$. We plan to explore this putative mechanism in satellite cells by assessing HBP1 phosphorylation during aging, and also its association to the previously reported specific binding region at the INK4 promoter.

p38α has also been reported to associate to specific promoters during muscle differentiation (Simone et al., 2004; Palacios et al., 2010; Segalés et al., 2016) and in response to distinct types of stress (Ferreiro et al., 2010b). By its direct association, p38α is able to regulate the transcription of genes involved in cell cycle control and survival in these situations. Hence, it is tempting to hypothesize that p38α could be directly recruited to the INK4 locus, participating in its epigenetic regulation, by direct binding to its promoter (or other key regions), or by associating with elements that would lead to a loss of repression of this locus with aging. Our preliminary results point towards this scenario (data not shown).

Other cell-intrinsic defects remain to be explored in satellite cells of aging p38α$^{\Delta Pax7}$ mice. It has been reported that although quiescent satellite cells seem more resistant to DNA damage and more efficient in repairing DNA lesions than their committed progeny (Vahidi Ferdousi et al., 2014), lifetime exposure to genotoxic stresses and a decline in antioxidant capacity with aging (Fulle et al., 2005) have a deleterious impact on their genomic integrity. When isolated from aged muscles, satellite cells display an increased number of foci containing phosphorylated histone H2AX, which is a well know DNA double strand break and genomic instability marker (Sinha et al., 2014; Sousa-Victor et al., 2014a). Preliminary results from our group suggest that the loss of p38α in freshly isolated aged satellite cells ameliorated the accumulation of foci containing phosphorylated histone H2AX *in vitro* (data not shown). We are currently testing if this decreased DNA damage accumulation in aged p38α-deficient satellite cells also occurs *in*

vivo, by staining of sections of regenerating muscle from aged and young mice of both genotypes.

Another important mechanism that could link aging with senescence and $p16^{INK4a}$ accumulation is oxidative damage, a well-known activator of the p38 MAPK pathway (Iwasa et al., 2003; Wang et al., 2002). Autophagy failure in aged resting muscle stem cells leads to accumulation of damaged proteins and dysfunctional organelles, specially mitochondria, which generates enhanced ROS levels that may cause DNA damage and senescence entry (García-Prat et al., 2016). ROS was uncovered as a key epigenetic regulator of the senescence-promoting gene INK4a in aging stem cells, by impeding PRC1-mediated $H2A^{K119Ub}$ repressive mark, required for INK4a locus silencing. Consistent with this, treatment of geriatric mice with antioxidants not only restored PRC1-mediated INK4a locus repression and prevented senescence in aged satellite cells, but also restored their regenerative capacity (García-Prat et al., 2016). Taking together, we hypothesize that the milder intrinsic regenerative failure of satellite cells from aged $p38\alpha^{\Delta Pax7}$ mice could be a consequence of their ability to cope with oxidative damage. We are currently evaluating the levels of ROS in wild-type and p38α-deficient satellite cells at different ages trying to unravel this question. We have preliminary data showing that freshly isolated cells from geriatric $p38\alpha^{\Delta Pax7}$ mice accumulate less ROS levels in proliferating conditions (data not shown); this hypothesis must be confirmed *in vivo*, since ROS measurements can be misleading when cells are grown in high-oxygen cell culture conditions.

VI. CONCLUSIONS

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1. The p38 MAPK represses the E2F transcriptional program through direct control of RB.
2. p38 interacts with RB and phosphorylates the N-terminal Ser249 and Thr252 residues upon different stresses.
3. N-terminal RB phosphorylation by p38 increases RB activity as a transcriptional repressor of the E2F program.
4. RB phosphorylation by p38 leads to cell cycle delay and increases cell survival upon stress.
5. p38-phosphorylated RB is resistant to CDK inactivation.
6. The phosphomimetic mutant, $RB^{S249ET252E}$, inhibits cancer cell proliferation, both *in vitro* and *in vivo*.
7. The N-terminal phosphorylation of RB by p38 increases its affinity towards E2F.
8. RB phosphorylation by p38α does no show a clear contribution to muscle cell differentiation.
9. Genetic loss of p38α in muscle stem cells from young and aged mice results in increased proliferation, and provides a functional advantage for aged stem cells.
10. p38α deficiency in aged satellite cells results in reduced senescence entry.
11. p38α deficiency reverses the epigenetic derepression of the INK4a locus in aged satellite cells, thus preventing age-associated $p16^{INK4a}$ induction.
12. The inhibition of p38 activity results in a regenerative advantage for aged satellite cells.

VII. REFERENCES

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