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Doctoral thesis submitted by Carla Granados Colomina in candidacy for the degree of Ph.D. in Biochemistry, Molecular Biology and Biomedicine from the Universitat Autònoma de Barcelona.

Thesis performed at the Department de Bioquímica i Biologia Molecular, under the supervision of Dr. Víctor J. Yuste Mateos, Prof. Josep Vendrell Roca and Dr. Irantzu Pallarès Goitiz.

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A mis padres,

A mi Hermana,

A Ricardo

SUMMARY

Latexin is a recently discovered and poorly known retinoic acid (RA)-responsive gene whose cellular function is scarcely known. Latexin expression appears downregulated through promoter hypermethylation in several types of cancer, suggesting it can function as a tumor suppressor. With the aim to elucidate its potential role in cancer, we employed neuroblastoma-derived cells since they represent canonical and well-established models of apoptosis and differentiation. We first reveal that the lack of latexin expression appears as a common hallmark in neuroblastoma-derived cells although it can be modulated by RA treatment. The stable overexpression of latexin does not significantly alter cell proliferation or cell death responses in SH-SY5Y cells. However, the overexpression of latexin remarkably favors the emergence of S-type cells (Schwannian lineage) upon RA treatment. Consistently, latexin overexpression also facilitates the appearance of the S-type phenotype upon exposure to 5-bromo-2'-deoxyuridine (BrdU). Moreover, latexin-overexpressing cells display enhanced Akt activation upon RA or BrdU stimuli, or even in basal growth conditions. This activation allows latexin-overexpressing cells to survive for long periods under unfavorable extracellular conditions and to undergo cellular senescence. Indeed, the inhibition of the PI3K/Akt axis impedes the activation of cellular senescence. These evidences suggest that latexin could be a critical determinant of cell fate choices between apoptosis or senescence in neuroblastoma cells likely by modulating this cascade. These results encouraged us to unveil the landscape of gene expression promoted by latexin to identify key targets involved in the aforementioned latexin-mediated effects. Interestingly, latexin upregulated a large number of genes, most of them involved in cell adhesion, cell development and morphogenesis processes. Surprisingly, the vast majority of genes upregulated by latexin either in the presence or in the absence of RA belong to the extracellular matrix (ECM), therefore suggesting the potential involvement of the ECM in latexin-promoted effects in SH-SY5Y cells. When extending our results to other cellular models, we observe that latexin also promotes cellular senescence upon the adequate stimuli in the neuroblastoma cell line SK-N-LP and in the Glioblastoma Multiforme (GBM)-derived cell line LN-18. Intriguingly, latexin knock down in U87-MG cells results in astrocytic-like differentiation upon treatment with the genotoxic drug doxorubicin. Remarkably, U87-MG cells with decreased levels of latexin expression remarkably impair the activation of cellular senescence. Altogether, these findings disclose a novel functional role of latexin in regulating cell specification towards the acquisition of a senescent phenotype in different cellular models.

RESUMEN

Latexin es un gen de respuesta a ácido retinoico (RA) cuya función celular se conoce escasamente. Latexin se encuentra silenciada por un mecanismo de hipermetilación en numerosas líneas tumorales, sugiriendo que ésta podría estar ejerciendo una función como supresor tumoral. Con el objetivo de elucidar su potencial implicación en cáncer, empleamos líneas derivadas de neuroblastoma ya que representan modelos adecuados para estudiar tanto procesos de apoptosis como de diferenciación. En este estudio revelamos que la escasa expresión de latexin parece una característica común de las líneas de neuroblastoma y que dicha expresión puede ser modulada mediante el tratamiento con RA. La sobreexpresión estable de latexin no altera los procesos de proliferación celular ni de muerte celular en la línea SH-SY5Y. Sin embargo, su sobreexpresión sí que tiene un efecto significativo promoviendo la aparición de células del tipo S (del linaje Schwanniano) cuando son tratadas con RA. Además, su expresión también facilita la emergencia del fenotipo S cuando las células se exponen al agente 5-bromo-2'-deoxyuridine (BrdU). De hecho, la inhibición del axis PI3K/Akt impide la activación de senescencia. Estas evidencias sugieren que latexin podría ser un determinante en la decisión del destino celular entre apoptosis y senescencia en células de neuroblastoma, probablemente modulando dicha cascada de señalización. Estos resultados nos alentaron a tratar de desvelar el panorama de expresión génico que promueve latexin con el objetivo de identificar piezas claves en los efectos previamente mencionados promovidos por dicha proteina. Interesantemente, latexin promueve la expresión de un gran número de genes, principalmente involucrados en procesos de adhesión celular, desarrollo y morfogénesis. Curiosamente, la mayoría de genes promovidos por latexin, ya sea en presencia o ausencia de RA, pertenecen a la matriz extracelular (ECM), sugiriendo una potencial asociación entre la ECM y los efectos favorecidos por latexin en células SH-SY5Y. Cuando extendimos nuestros resultados a otros modelos celulares, observamos que latexin también fomenta el proceso de senescencia en respuesta al estímulo adecuado en la línea de neuroblastoma SK-N-LP y en la línea derivada de Glioblastoma Multiforme (GBM) LN-18. Sorprendentemente, una bajada en la expresión de latexin en las células U87-MG resulta en diferenciación tipo astrocitario cuando las células son tratadas con el agente genotóxico doxorubicina. Cabe remarcar que las células U87-MG con una expresión disminuida de latexin tienen remarcablemente perturbada la activación del proceso de senescencia. De una manera general, estos descubrimientos revelan una nueva función de latexin en la regulación de la especificación celular hacia la adquisición de un fenotipo senescente en diferentes modelos celulares.

INDEX

LIST OF ABREVIATIONS

5-aza-dC	5-Aza-2'-deoxycytidine
AKT	Protein Kinase B
ALK	Anaplastic Lymphoma Kinase
ATRA	All-*trans* Retinoic Acid
BrdU	5-Bromo-2'-deoxyuridine
CDK	Cyclin-Dependent Kinases
CP	Carboxypeptidase
CPi	Carboxypeptidase Inhibitor
CRABP	Cytosolic Retinoic Acid-Binding Protein
DAVID	Database for Annotation, Visualization Integration Discovery
DDR	DNA Damage Response
DEG	Differential Expressed Gene
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DSB	Double Strand Break
ECM	Extracellular matrix
EdU	5-Ethynyl-2′-deoxyuridine
EMT	Epithelial to Mesenchymal Transition
EV	Empty Vector
FABP5	Fatty Acid Binding Protein 5
FAK	Focal Adhesion Kinases
FBS	Fetal Bovine Serum
FC	Fold Change
FDR	False Discovery Rate
GBM	Glioblastoma Multiforme
GO	Gene Ontology

hCPA4	Human Carboxypeptidase A4
HSC	Hematopoietic Stem Cells
ID1	DNA-binding Protein Inhibitor
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDH	Lactate Dehydrogenase
LXN	LXN
MCP	Metalocarboxypeptidase
MMP	Matrix Metalloprotease
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NC	Neural Crest
NHEJ	Non-Homologous End Joining
NR	Non-Relevant
NSE	Neuron Specific Enolase
NT	Non-Treated
OIS	Oncogene Induced Senescence
PDK1	3-phosphoinositide dependent kinase 3
PI3K	phosphoinositide 3-kinase
PRAS40	Proline-Rich AKT Substrate 1
PTEN	Phosphatase and Tensin homolog
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Elements
RARRES	Retinoic Acid Receptor Responder
RARβ	Retinoic acid receptor beta
Rb	Retinoblastoma
RA	Retinoic Acid
RNAseq	RNA-Sequencing
RXR	Retinoic X Receptor
SAHF	Senescence-Associated Heterochromatin Foci
SASP	Senescence-Associated Secretory Phenotype

SA-β-gal	Senescence-Associated-β-Galactosidase
siRNA	Short Interference RNA
STP	Staurosporine
VAD	Vitamin A Deficiency

I. INTRODUCTION

1. CANCER BARRIERS

From a general point of view, cellular homeostasis requires a precise balance between cell survival and cell death. In this sense, cell attrition normally occurs through apoptosis whereas survival comprises cell proliferation, cell cycle arrest and cell differentiation, among other processes. Accordingly, cells respond to specific stimuli or stresses by promoting these different cell fates, depending on the specific requirements of the cellular context. Such stimuli include mitogenic signals, growth factors, trophic factors, and a broad repertoire of distinct stresses that activate these different cellular responses. In a healthy physiological context, these cellular processes are tightly orchestrated and temporally programmed to ensure the correct cell homeostasis (Evan & Vousden 2001).

Because cells of long-lived multicellular organisms are particular susceptible to mutations, they are endowed with mechanisms to remove or repair injured cells. Survival strategies are based on the repair of the induced damage or, alternatively, on the avoidance of the propagation of these lesions through processes like senescence or autophagy. On the other hand, unrepaired damage can also prompt the elimination of damaged cells by processes like apoptosis or necrosis, among other forms of cell death (Roos et al. 2016).

When the progressive accumulation of damaged cells leads to the deterioration of several functions, aging is manifested. Aging is accompanied by several detrimental effects known as age-related pathologies such as osteoporosis, macular degeneration, neurodegeneration, diabetes, cancer and sarcopenia, among others (Kirkwood & Austad 2000; López-Otín et al. 2013).

In addition, mutations can deregulate cellular processes like proliferation, differentiation or cell death, leading to the development of cancer. In fact, cancer and aging are highly connected, being the latter one of the most important triggering and influencing factors. Cancer arises because of the accumulation of inherited and acquired mutations resulting in a misbalance between cell proliferation and cell attrition. As a result of such variety of mutations, tumor cells, rather than homogeneous masses of proliferating cells, are a mixture of cell types with different degrees of cell differentiation, proliferation, vascularity or inflammation. In spite of such heterogeneity, there are some alterations commonly shared by many cancer cells. According to these common traits, Hanahan and Weinberg proposed the existence of different hallmarks to collectively define neoplastic diseases (Hanahan & Weinberg 2000). These hallmarks comprise: sustained proliferative signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), replicative immortality, sustained angiogenesis, tissue invasion and metastasis. However, based on more recent evidences, these hallmarks were revisited and extended. Concretely, two more hallmarks were considered to promote tumor progression: the ability to modify the metabolism to support proliferation and the evasion of immunological elimination by cancer cells. Aside from these hallmarks, these authors also documented the importance of the tumor

microenvironment actively influencing the biology and clinical outcome of tumor cells. They highlighted the importance of the extracellular matrix (ECM), playing a fundamental role in tumor initiation and progression (Hanahan & Weinberg 2011). The ECM is a complex network composed of secreted factors that provides cell support (Barros et al. 2011; Hynes & Naba 2012). Such structure is regulated by several enzymes, mainly proteases, that control the bioavailability and release of a broad repertoire of factors (Noël et al. 2012). Moreover, the ECM senses extracellular cues and activates intracellular signaling pathways by binding to cell surface receptors, mainly integrins (Brizzi et al. 2012). The interaction between the ECM and these cell adhesion receptors influences, in turn, most of the cellular responses that characterize the hallmarks of cancer (Pickup et al. 2014). Likewise, the ECM can support cell growth via regulating ERK or PI3K/Akt pathways (Pylayeva et al. 2009), or by counteracting the growth inhibitory function of p21 (Chen et al. 2008). In addition, changes in the ECM leading to enhanced rigidity also promote epithelial-mesenchymal transition (EMT), a key event during metastasis (Barkan et al. 2010; Leight et al. 2012). As well, the ECM might influence, both compromising or supporting, the tumor immune response (Sorokin 2010).

These hallmarks are acquired by tumor cells at different times conferring them the abilities to adapt and proliferate in different environments. In this sense, mutations leading to the activation or inactivation of oncogenes and tumor suppressors, respectively, result in the evasion of proliferative constraints and other barriers to the spread of cellular damage (Evan & Vousden 2001; Lowe et al. 2004). In this context, complex organisms have developed two main barriers to control the development of cancer: apoptosis and senescence. While apoptosis removes damaged cells, senescence induces cell cycle arrest in cells at risk of malignant transformation (Labi & Erlacher 2015). However, other barriers to halt cancer progression include cell differentiation or autophagy (Vicencio et al. 2008).

In the next sections, after a brief description of apoptosis, cellular senescence and cell differentiation are reviewed as important barriers against the development of cancer.

1.1. APOPTOSIS

Apoptosis is an essential physiological mechanism of programmed cell death that allows the removal of damaged cells and whose deregulation gives rise to a broad repertoire of pathologies including cancer or aging (Pollack et al. 2002). Apoptosis is a tightly controlled process involving biochemical and morphological events. Morphological features characterizing apoptotic cells are membrane shrinkage, chromatin condensation (pyknosis), nuclear fragmentation, plasma membrane *blebbing* and formation of apoptotic bodies (Galluzzi et al. 2007). There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic signaling pathway is activated upon ligand binding to death receptors, leading to the recruitment of adaptor proteins to form the death-inducing signaling complex (DISC) (Locksley et al. 2001). This complex associates with initiator pro-caspase-8 promoting its dimerization and activation (Kischkel et al. 1995). The extrinsic pathway is activated upon stress conditions or DNA damage

(Martinou & Green 2001). This pathway involves the permeabilization of the outer mitochondrial membrane (MOMP) resulting in the release of cytochrome c (Kroemer et al. 2007). Therefore, cytochrome c associates with apoptosis protease-activating factor-1 (APAF-1) which, in turn, activates caspase-9 (Cai et al. 1998; Slee et al. 1999). The key effectors of apoptosis are a set cysteine-aspartate proteases, the so-called caspases. Caspases are synthetized as inactive pro-enzymes that become activated after the specific cleavage by another protease or by auto-activation (Cohen 1997). Based on their activity, caspases can be broadly classified into 'initiator' (caspase-2, -8, -9 and -10) or 'effector' caspases (caspase-3, -6, -7) (Riedl & Shi 2004).

1.2. CELLULAR SENESCENCE

1.2.1. Overview

In 1961, Hayflick and Moorhead discovered that human diploid fibroblasts, after prolonged passages in culture, exhibited limited replicative potential. They reported that those cells, after achieving a certain number of doublings, entered into a cellular program they coined cellular senescence. Under their experimental settings, senescent cells, remained metabolically active despite arresting their cell cycle. They hypothesized that ''*the finite lifetime of diploid cell strains in vitro may be an expression of aging or senescence at the cellular level*''. Remarkably, they already proposed a correlation between cellular senescence and aging, although they could not corroborate it experimentally at the time (Hayflick & Moorhead 1961).

Early observations identified telomere shortening as the main event driving cellular senescence, although compelling data have more recently demonstrated the involvement of other cellular stimuli (Serrano et al. 1997; Itahana et al. 2003). Therefore, the concept of cellular senescence has evolved from being regarded as a cellular response against telomere shortening to be considered a stable cell cycle arrest in response to different stresses, in order to prevent the spread of cellular damage (Campisi & d'Adda di Fagagna 2007; Collado et al. 2007). Interestingly, such limited replicative potential contrasts sharply with the limitless growth capability associated to tumor cells. In fact, senescence is considered an important safeguard against cancer (Campisi 2005).

1.2.2. Features Associated to Cellular Senescence

Although several hallmarks have been proposed to define the senescent state, none is strictly specific of this cellular process. In addition, senescent cells do not normally exhibit all the senescent markers defined. However, there are some traits commonly employed to identify senescent cells (**Table 1**) (Campisi et al. 2011; Campisi 2013; Althubiti et al. 2014).

Stable cell cycle arrest - Cell cycle arrest irreversibility allows distinguishing senescence from other non-cycling cellular states including quiescence or terminal differentiation. Thus, the process of senescence theoretically implies a stable and imperturbable cell cycle arrest regardless any other cellular stimuli. However,

the irreversibility of cellular senescence has been challenged by studies demonstrating that genetic modifications, such as p53 inactivation, can reverse this cellular process (Beauséjour et al. 2003; Rodier & Campisi 2011). Nevertheless, whether this reversal can occur under physiological conditions, remains to be elucidated (Kuilman et al. 2010)

Morphology transformation - Senescence also entails striking morphological changes. Cells undergoing senescence tend to increase their size, being even able to double the size of their non-senescent counterparts (Hayflick 1965). Senescent cells usually adopt a spindle-shaped or a flattened morphology, being the latter the most commonly found. Interestingly, cell morphology often depends on the senescence inductor (Michaloglou et al. 2005; Denoyelle et al. 2006).

Senescence-associated β-galactosidase (SA-β-gal) - In 1995, Dimri and co-workers evidenced that senescent cells express a characteristic β-galactosidase activity detectable at pH 6, thus distinguishable from the canonical lysosomal β-galactosidase activity, with an acidic optimal pH (4.0-4.5). This senescence-specific activity is currently known as senescence-associated β-galactosidase (SA-β-gal) (Dimri et al. 1995; Debacq-Chainiaux et al. 2009; Itahana et al. 2013). However, it was later demonstrated that SA-β-gal activity results from a raise in lysosomal activity entailed by cellular senescence. Accordingly, SA-β-gal activity measures the residual activity of the lysosomal β-galactosidase, which can be detected at a suboptimal pH (Kurz et al. 2000). Consistently, SA-β-gal was reported to be encoded by the same lysosomal-β-galactosidase gene (GLB1) (Lee et al. 2006). These early studies defined SA-β-gal activity as a specific senescence biomarker that was only detected in senescent cells but not in quiescent or terminally differentiated cells (Dimri et al. 1995). However, more recent reports have revealed SA-β-gal activity in non-senescent cells, therefore questioning the specificity of this assay (Severino et al. 2000; Yang & Hu 2005). Nonetheless, SA-β-gal is still the most extensively used method to detect cellular senescence, although complementary senescence markers are required to confirm a senescent phenotype (Itahana et al. 2013).

Activation of tumor suppressor pathways - The INK4a/ARF locus encodes $p16^{INK4a}$ (activator of the retinoblastoma (Rb) pathway) and $p14^{ARF}$ (activator of the p53 pathway), two essential tumor suppressors associated to cell cycle arrest and senescence. $p16^{INK4a}$ represents an important, although non-specific, marker of cellular senescence since cells undergoing this process exhibit high levels of $p16^{INK4a}$ expression whereas these are generally reduced either in normal, quiescent or terminally differentiated cells (Serrano et al. 1997). In addition, $p16^{INK4a}$ expression also increases with age in several human tissues (Baker et al. 2011). The involvement of $p16^{INK4a}$ in senescence is rationalized by the role of this protein as an activator of the Rb pathway via inhibition of cyclin-dependent kinases (CDKs) that control cell cycle progression (Serrano et al. 1997; Burd et al. 2013). Rb is complexed with E2F transcription factor to regulate the expression of genes involved in the G1/S cell cycle transition. In addition, Rb alters heterochromatin structures, thus reinforcing the senescent state (Narita et al. 2003). On the other hand, p53 is a tumor suppressor and a key regulator of senescence induced by different stimuli such as telomere shortening, DNA damage or oncogenes (Itahana et

al. 2001). $p14^{ARF}$ activates p53 by sequestering the ubiquitin ligase Mouse Double Minute 2 (MDM2) which directs p53 to degradation. In turn, phosphorylated p53 protein activates the expression of p21, which mediates cell cycle arrest either through the inhibition of CDK-complexes or by silencing the transcription of genes involved in cell cycle progression (Vassilev et al. 2004; Abbas & Dutta 2009). p53 is an important sensor of telomere length and DNA damage; low DNA damage transiently activates its expression, while sustained activation occurs upon intense levels of DNA damage (Itahana et al. 2001; Sulli et al. 2012).

Senescence-associated heterochromatic foci (SAHF) - In senescent cells, chromosomes often appear compacted in structures named senescence-associated heterochromatin foci (SAHF), characterized by a punctuated nuclear pattern (d'Adda di Fagagna 2008). SAHFs are enriched in Lys-9-methylated histone H3, heterochromatin protein 1 (HP1), and macroH2A, (Funayama et al. 2006). These foci prevent the transcription of genes involved in cell cycle entry, thus promoting the senescent phenotype (Di Micco et al. 2011).

DNA-SCARS - DNA damage response (DDR) is a signaling network that senses DNA damage, avoids its propagation and induces its repair. This response is normally initiated at double strand break (DSB) sites, where many repair proteins and DDR signaling proteins such as H2AX and p53-binding protein 1 (53BP1) are recruited, and can be detected, allowing the identification of DNA damage foci. In the event of reversible damage, cell cycle is transiently arrested supplying the required time for the cells to repair the lesion. Then, damaged foci are resolved and cells can resume cell growth. In contrast, irreparable DNA damage results in persistent DDR, inducing cells to senesce. In this case, persistent nuclear foci are present, namely DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) (Rodier et al. 2011; Campisi 2013; Roos et al. 2016).

Metabolism - In spite of their stable cell cycle arrest, senescent cells remain metabolically active. Indeed, the senescence response is usually associated to particular metabolic changes including increase of mitochondrial oxidative phosphorylation, pyruvate dehydrogenase (PDH) activation and downregulation of malic enzymes catalyzing the conversion of malic acid into pyruvate (Hutter et al. 2004; Kaplon et al. 2013).

The senescence-associated secretory phenotype (SASP) - Cellular senescence frequently entails the secretion of numerous factors including cytokines, chemokines, and proteases, being the ensemble globally termed senescence-associated secretory phenotype (SASP) (Coppé et al. 2008) or senescence messaging secretome (SMS) (Kuilman & Peeper 2009). Such diversity of secreted molecules allow to discriminate senescence from other cellular events resulting in cycle arrest. Moreover, the SASP modifies the cellular microenvironment and, in such a way, it can influence other cellular processes such as EMT, wound healing, tissue repair, inflammation, cell proliferation, cell motility and cell differentiation (Krtolica et al. 2001; Ohuchida et al. 2004; Tsai et al. 2005). In this sense, SASP may have either a pro- or anti-tumor role and is able to act both in an autocrine or a paracrine fashion depending on the tissue milieu.

SASP composition varies along the course of the senescent response and depending on the cell type and senescence inducer, although some factors such as cytokines and chemokines appear ubiquitous among different senescence responses (Coppé et al. 2010). SASP factors can be divided into four groups: soluble signaling factors, secreted proteases, ECM components and non-proteinaceous secretions. Soluble factors include interleukins, mainly interleukin 6 (IL-6) (Rodier et al. 2009) and Interleukin 1 (IL-1) (Mantovani et al. 2001); chemokines and members of the insulin growth factor (IGF) family. Extracellular proteases comprise different metalloproteases (MMPs), serine proteases and their inhibitors. Other components of the ECM such as fibronectin, collagens and laminin also belong to the SASP. Non-proteinaceous signaling molecules including reactive oxygen species (ROS) and transported ions (such as nitric oxide) are also included.

SASP promotes the reinforcement of the senescent phenotype by engaging positive feedback loops of DDR that help to maintain the cell cycle arrest. However, this reinforcing effect does not imply that SASP is sufficient to allow the irreversibility of the cell cycle arrest since it has been reported that such secreted factors are maintained in cells in which cycle arrest is reversed by p53 inactivation (Coppé et al. 2008). Another striking effect of SASP is its ability to attract the immune system for the elimination of senescent cells, in a process called immune clearance. As discussed below, the clearance of senescent cells will influence whether senescence will be beneficial or detrimental for tumor progression (Davalos et al. 2010; Muñoz-Espín & Serrano 2014).

Table 1. List of senescence markers from (Salama et al. 2014)**.**

SENESCENCE MARKERS
PHENOTYPIC MARKERS
Lack of cell proliferation
Large and flat cell morphology
Lack of response to growth factors
Senescence-associated β-galactosidase activity (sa-β-gal)
Senescence-associated heterochromatic foci (SAHF)
Senescence-associated secretory phenotype (SASP)
DNA segments with chromatin alterations reinforcing senescence (dna scars)
MOLECULAR MARKERS
CDKIS (p16, p21, etc.)
p53
DDR (ATM, 53BP1, γ-h2AX, MBS1, Chk2, etc.)
SASP factors and receptors (IL6, IL8, IL1, MMPs, etc.)
heterochromatin markers (HP1, H3K9ME3, etc.)

1.2.3. Senescence Effectors

Several classes of stimuli, both exogenous and endogenous, can trigger cellular senescence when the level of damage achieves a certain threshold (**Figure 1**).

In general terms, senescence can be classified into two broad forms: replicative and premature.

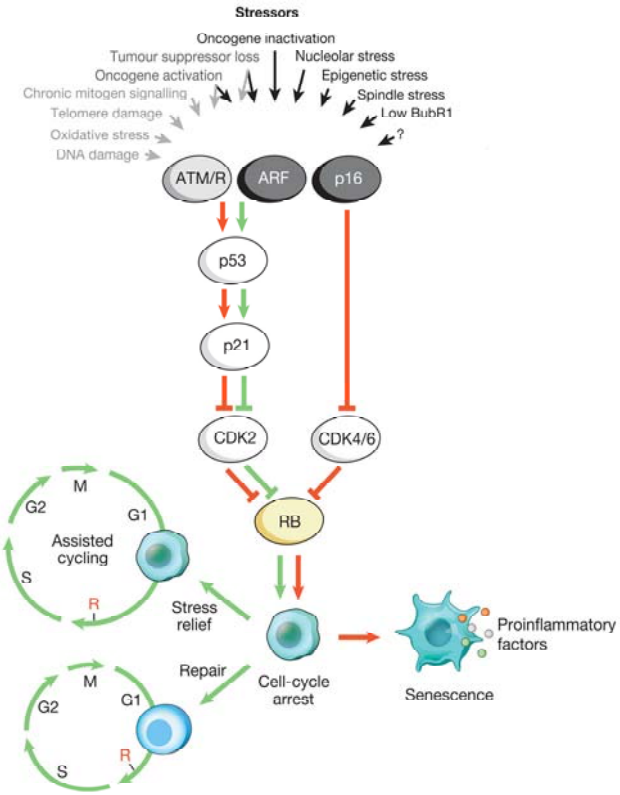

Figure 1. Representation of senescence-inducing stimuli and main effector pathways. Several stressors, both intrinsic extrinsic, can activate the cellular senescence program. These stressors engage different cellular signaling cascades. Types of stress that activate p53 via DDR signaling are indicated with grey text and arrows. Activated p53 induces p21, promoting a temporal cell cycle arrest by inhibiting Cdk2. p16 inhibits cell-cycle progression by inhibiting cyclin D-Cdk4 and cyclin D-Cdk6 complexes. In turn, both p21 and $p16^{Ink4a}$ prevent the inactivation of Rb. Severe stress (red arrows) leads to a senescent growth arrest. Cells exposed to mild damage can resume cell growth either successfully repairing the damage or by stress support pathways in a process termed assisted cycling (green arrows). Cells undergoing senescence induce the secretion of SASP factors (colored dots). Red and green connectors indicate 'senescence-promoting' and 'senescence-preventing' activities, respectively. Adapted from (van Deursen 2014) with permission of Macmillan Publishers Ltd.

1.2.3.1. Replicative Senescence: Telomere Shortening

In the 1970s Olovnikov and Watson described that telomeres, the repetitive sequences located at the end of chromosomes, undergo a gradual shortening after each round of cell division. This effect is known as the "end replication problem" and, according to this model, telomeres represent a sort of "mitotic clocks" that would recapitulate the replicative history of a cell and dictate the maximum number of divisions (Watson 1972; Olovnikov 1973; Harley et al. 1990; Olovnikov 1996).

Telomeres are protective structures that prevent chromosome ends from being recognized as DSBs and processed by the DNA repair machinery. Human telomeric DNA is composed of double-stranded (ds) 5′-TTAGGG-3′ repeats, ending in a single-stranded (ss) G-rich 3′ overhang (Moyzis et al. 1988). Telomere attrition stems from the inability of DNA polymerase to replicate the ends of chromosomes resulting in a loss of around 50-200 base pairs in each cell cycle division (Shay & Wright 2005). To counteract this replicative constraint, cells might deploy a reverse transcriptase activity, which is exerted by telomerase, to maintain the length of these capping structures. However, telomerase is not expressed by all human cells; rather, the repertoire of human cells expressing telomerase only includes embryonic stem cells, some adult stem cells, few types of somatic cells and cells cancer cells (Campisi 2013). Telomerase comprises the reverse transcriptase catalytic subunit hTERT and the RNA component hTR, serving as template for RNA extension (Feng et al. 1995; Nakamura et al. 1997; Bennett & Medrano 2002), being the levels of both units determinant for telomerase activity. Interestingly, most human cancers have developed mechanisms to overcome telomere attrition, enabling cancer cells to perpetuate proliferation, which include the expression of high levels of telomerase and/or the activation of the alternative lengthening of telomeres (ALT) mechanism (Pickett et al. 2009).

Three different structures can be distinguished according to the three-state model of telomere end protection: closed, capped or telomere-state (t-loop), intermediate state, and uncapped state. These different structures adopted by the telomeres might, in turn, influence different cellular outcomes. In the t-loop state, telomeres adopt a highly ordered stable structure in which the shelterin complex is involved. Such t-loops are formed by the invasion of the 3’ overhang into a proximal region located on the same chromosome end (Griffith et al. 1999). This state prevents telomere end terminus from being recognized as DSB, therefore protecting from DDR activation and inhibiting non-homologous end joining (NHEJ) repair. The intermediate state is only partly protective, since chromosomal ends activate DDR but NHEJ is not engaged due to sufficient shelterin levels. In this state, when telomeres reach a critical length, the senescence program can be activated (Pickett et al. 2009; Cesare & Karlseder 2012). Remarkably, telomeres in senescent cells adopt a structure highly similar to that of the intermediate state (Kaul et al. 2012). In the uncapped state, NHEJ cannot be avoided due to the low levels of shelterin and the repair machinery is activated, thus facilitating chromosome end fusions. In turn, this leads to chromosomal translocations and genome instability, both characteristic of malignant transformation (**Figure 2**) (Riha et al. 2006).

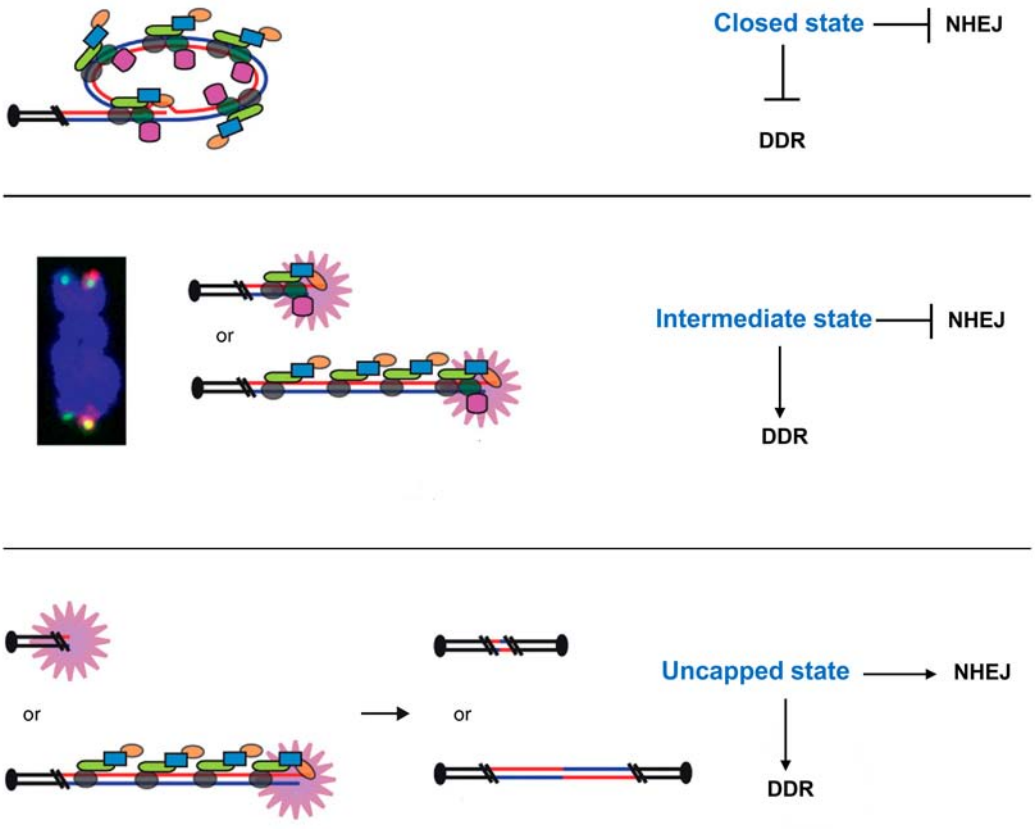

Figure 2. Schematic representation of closed-state, intermediate-state and uncapped-state of human telomeres. The closed-state forms a t-loop and does not activate either DDR or NHEJ. Intermediate-state telomeres are the result of excessive telomere shortening and characterized by the activation of DDR signaling. Uncapped-state telomeres are the result of excessive shortening, consistent with spontaneous fusions at crisis. In this state, both DDR and NHEJ are activated. Adapted from (Cesare & Karlseder 2012) with permission of Elsevier Ltd.

1.2.3.2. Premature Senescence

The initial view that senescence arises strictly upon telomere shortening has evolved from the realization that cells can also senesce prematurely (i.e. before telomere shortening) in response to several stimuli. These cells are indistinguishable from those resulting from replicative senescence. This type of senescent response can be triggered upon different cellular stressors or stress conditions such as oxidative stress, sub-optimal cell culture conditions, oncogenic activation, chemotherapeutic drugs, etc. Accordingly, such senescent triggers can be classified into four groups within premature senescence: DNA damage-induced, cell culture-induced, oncogene-induced and tumor suppressor loss-induced (Sherr & DePinho 2000; Kuilman et al. 2010).

1.2.3.2.1. DNA Damage-induced Senescence

DSB is one of the major sources of DNA damage which can be triggered by chemotherapeutic agents, ROS or ionizing radiation, among other stresses (d'Adda di Fagagna 2008). Cells respond to DNA damage by activating the DDR, thus regulating a myriad of cellular responses. Accordingly, low DNA damage levels

activates the repair machinery while at higher levels, such machinery is overwhelmed leading to the activation of different cell fates including apoptosis, necrosis, senescence or autophagy. The decision between these cell fates will depend on the intensity of DNA damage, the status of key DDR proteins, the regulation of DNA repair genes and the persistence of the DNA lesion (Campisi & d'Adda di Fagagna 2007; Di Micco et al. 2011). Concretely, in senescent cells DDR is involved in both the initiation and the maintenance of the senescent phenotypes (Olivieri et al. 2015).

The initial steps of DDR comprise the recruitment and activation of the damage sensor kinases ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) at DSB sites. These kinases phosphorylate histone H2AX (whose phosphorylated form is γH2AX) and other downstream substrates like 53BP1, and the checkpoint kinases Chk1 and Chk2. At DDR-foci, Chk1 and Chk2 control the activity of cell cycle regulators including, among other targets, cell division cycle 25 (CDC25) phosphatase, whose phosphorylation causes cell cycle arrest. Alternatively, they can also phosphorylate and activate p53, therefore inducing p21-mediated senescence (Wang et al. 2015).

1.2.3.2.2. Cell Culture-induced Senescence

Several studies have revealed that sub-optimal culture conditions can drive cells to senesce. In line with this, fibroblasts and keratinocytes arrested cell growth when cultured in plastic plates and low serum levels whereas its cultivation on feeder layers avoided premature senescence (Ramirez et al. 2001; Fu et al. 2003). Oxygen levels have also influence this cellular response, since mouse embryonic fibroblasts (MEFS) senesce under culture conditions of 20% oxygen whereas physiological oxygen levels of 3% avoided unleashing the senescent program (Parrinello et al. 2003).

1.2.3.2.3. Oncogene-Induced Senescence

Besides promoting cell proliferation, oncogenes also induce a particular form of cellular senescence. In 1997, Serrano and colleagues described that in human fibroblasts, the exogenous expression of the oncogene Ras induced an initial phase of cell hyperproliferation which was then followed by a stable senescent cell cycle arrest (Serrano et al. 1997). Since then, several oncogenes have been demonstrated to trigger this cellular response, known as oncogene-induced senescence (OIS) (**Table 2**) (Salama et al. 2014). Remarkably, mounting evidence indicates that, like other forms of senescence, OIS represents an important safeguard that cells should bypass for malignant progression. This idea is further reinforced by OIS being frequently activated in precancerous lesions but rarely found in advanced tumors (**Figure 3**) (Courtois-Cox et al. 2008; Suram et al. 2012).

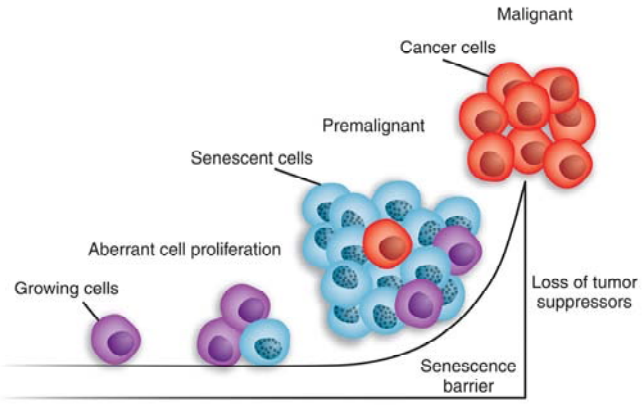

Figure 3. Oncogene-induced senescence in vivo. Abnormal activation of oncogenes or loss of tumor suppressors can promote aberrant cell proliferation (purple cells), which can promote the activation of cellular senescence response. If senescence program remains intact, neoplastic growth can be maintained as benign. However, several mutations can impair cellular senescence (such those affecting p53 or p16; orange cells) and promote tumor progression. Reproduced from (Narita & Lowe 2005) with permission of Macmillan Publishers Ltd.

Although OIS encompasses responses mediated by a wide diversity of signaling pathways and may result in different cellular outcomes, some common mechanisms can be still distinguished in this cellular response. Most of oncogenes triggering OIS activate p53 which, in turn, induces p21 and/or p16 expression (Xu et al. 2014). Yet another common trait is the activation of DDR, often triggered by the initial hyperproliferative phase (Colavitti & Finkel 2005; Di Micco et al. 2011). Other frequent hallmarks of OIS comprise the presence of SAHF (Bartkova et al. 2006) or the expression of SASP (Freund et al. 2010), being both important determinants for the stability of the senescent response. Indeed, OIS is not an irreversible event itself but mechanisms like DDR, chromatin remodeling or SASP contribute to reinforce the irreversibility of cell cycle arrest.

Table 2. Oncogenes triggering senescence.

GENE	PATHWAY-FUNCTION
TGFB	Smad Signaling
H-RAS^{V12}	Ras Signaling
K-RAS^{G12V}	Ras Signaling
N-RAS^{G12D}	Ras Signaling
BRAF ^{R600}	Ras signaling
C-MYC^{A,C}	Ras signaling-transcription and chromatin remodeling factor
B-CATENIN	Wnt signaling
AKT	PI3K/Akt signaling
RHEB	PI3K/Akt/mTOR signaling
E2F	G1 to S phase-transcription factor

P53	Various signaling pathways-transcription factor
INFB	STAT signaling-activates p53
CXCR2	Angiogenic CXC chemokine receptor
RAC1	Rho signaling
SMURF2	p53/pRb
RUNX	Transcription and chromatin remodeling factors
PTEN	Downregulates PI3K/Akt/mTOR signaling
SPROUTY 2	Downregulates Ras signaling
EGFR	Ras signaling
RAF	Ras signaling
MOS	Ras signaling
MEK	Ras signaling
IGFBP3, IGFBP5, IGFBP7	IGF1 signaling pathway
P38A-D176A, P38G-D179A	p38MAPK signaling
STAT5	JAK-STAT signaling
CYCLIN E	G1 to S phase
CDC6	Promotes S phase progression
CDT1	Promotes S phase progression
$P16^{INK4A}$	Inhibits G1 progression
PML	Ras signaling effector; induces p53
DEC1	p53 effector
PAI-1	p53 effector

Adapted from (Gorgoulis & Halazonetis 2010)

PI3K/Akt Pathway

The phosphoinositide 3-kinase/protein kinase-B (PI3K/Akt) axis governs important processes such as cell proliferation, cell differentiation, metabolism and cytoskeletal reorganization (Datta et al. 1999). Studies on the regulation of this signaling axis is one of the objectives of this thesis.

The phosphoinositide 3-kinase (PI3Ks) is a lipid kinase that phosphorylates phosphatidylinositol (PtdIns) lipids in the plasma membrane. PI3Ks are divided into three classes based on structural characteristics and substrate preferences. Among them, the best studied is class I which are composed of heterodimers of a catalytic subunit (p110) and a regulatory subunit (p85). The latter regulates receptor binding, activation, and localization of the enzyme (Vanhaesebroeck et al. 2010).

Class I PI3K are activated by receptor tyrosine kinases (RTKs) and G protein-coupled receptors. Upon growth factor stimulation, these receptors are autophosphorylated and subsequently activated. PI3K is therefore

recruited to the plasma membrane and its activation leads to the conversion of phosphatidylinositol-4,5-bisphosphate (PtdIns(3,5)P_2) to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (Thorpe et al. 2014).

The generation of PtdIns(3,4,5)P_3 activate 3-phosphoinositide-dependent kinase 1 (PDK1) which phosphorylates and activates different proteins kinases including the serine-threonine protein kinase Akt (also known as protein kinase B, PKB). PDK1 phosphorylates Akt in the catalytic domain at Thr308 whereas the phosphorylation of the hydrophobic motif at Ser473 is mediated by mTORC2. The phosphorylation of Akt at Thr308 is sufficient to activate mTORC1 by phosphorylating and inhibiting two substrates, namely Proline-Rich AKT1 Substrate 1 40 (PRAS40) and Tuberous Sclerosis 2 protein (TSC2, also known as tuberin) (Zurashvili et al. 2013). However, both phosphorylation events, at Thr308 and Ser473, are needed for the full activation of Akt.

Akt regulates multiple functions including cell proliferation, cell death or cell differentiation by phosphorylating different substrates. Among them, this kinase inhibits the pro-apoptotic proteins Bax and Bad. As well, Akt also inhibits members of the forkhead box class O (FoxO) transcription factor which regulate processes like cell cycle arrest, apoptosis, glucose metabolism and oxidative stress. Akt positively regulates cell survival by controlling the NF-κB pathway and promotes cell cycle progression through phosphorylation of CDKs. In neurons, this kinase regulates axon specification through the inhibition of glycogen synthase kinase 3 β (GSK3β) (Yamaguchi & Wang 2001; Zhang et al. 2011).

The PI3K/Akt pathway appears frequently deregulated in many types of human malignancies. One of the mechanisms explaining the deregulation of this pathway is the loss of the phosphatase and tensin homolog (PTEN). PTEN is a lipid phosphatase that dephosphorylates PtdIns(3,4,5)P_3 to PtdIns(3,5)P_2 leading to the repression of the PI3K/AKT pathway. However, PTEN is disrupted in multiple types of human cancer and its loss results in hyperactivation of the PI3K/AKT pathway (Carracedo & Pandolfi 2008; Song et al. 2012).

Aside from the canonical proliferative effect associated with the hyperactivation of the PI3K/Akt pathway, the activation of this axis can also result in cellular senescence. The involvement of this pathway in senescence was initially reported in human endothelial cells where Akt activation induced cell cycle arrest through activating the p53/p21 pathway. In this cellular response, the inhibition of FOXO3a was essential for Akt-mediated cell growth arrest. More concretely, FOXO3a inhibition led to the inactivation of radical scavenger genes such as the manganese superoxide dismutase (MnSOD), resulting in increased ROS levels (Miyauchi et al. 2004). These findings were further supported by Nogueira and co-workers showing that inhibition of FoxO transcription factors increased ROS levels by raising the degree of oxygen consumption and decreasing ROS scavenging (Nogueira et al. 2008). Recent studies have demonstrated that Akt-mediated induction of p53 is mTORC1-dependent. Interestingly, unlike Ras-mediated senescence, Akt-induced senescence does not require the initial hyperproliferation phase and is, therefore, DDR-independent. In spite of that, the SASP is determinant to reinforce the senescent phenotype in this type of response (Astle et al. 2012).

1.2.3.2.4. Loss of Tumor Suppressors-induced senescence

The loss of tumor suppressors such as PTEN, Retinoblastoma 1 (Rb1), Neurofibromin 1 (NF1) and Von Hippel-Lindau (VHL) is also associated to the senescence response. Of particular importance is the loss of PTEN, inducing the so-called PTEN-loss induced cellular senescence (PICS). This particular form of senescence is independent of either the hyperproliferative phase or the DDR, being triggered by the activation of the PI3K/Akt pathway leading to the activation of p16 and p53 (Di Mitri & Alimonti 2015).

1.2.4. Senescence in Cancer and Aging

Senescence increases with age and, besides, a sharp correlation is observed between cancer incidence and age. Moreover, senescent cells are detected at sites of age-related pathologies, suggesting a common link between senescence, aging, cancer and degenerative diseases. Aging is characterized by the onset of degenerative and hyperplastic diseases, conditions thought to be influenced by cellular senescence (Campisi et al. 2011).

1.2.4.1. Senescence and Aging

There is mounting evidence supporting the link between senescence, aging and age-related pathologies. Likewise, accumulation of senescent cells over the lifespan has been observed *in vivo* in humans, primates and rodent tissues. Interestingly, senescent cells are also detected at sites of age-associated diseases such as osteoarthritis, pulmonary fibrosis, atherosclerosis, and Alzheimer's disease (Coppé et al. 2010). The relationship between senescence and degenerative pathologies is exemplified by the disruption of alveolar and branching morphogenesis in mammary epithelial cells induced by senescent fibroblasts (Parrinello et al. 2005). Moreover, senescent pulmonary smooth muscle cells contribute to the hypertrophy of pulmonary arteries which, in turn, results in hypertension (Noureddine et al. 2011). In addition, the presence of senescent cells is associated with epidermal thinning in mice skin, a common age-related feature (Ressler et al. 2006; Velarde et al. 2012). An important evidence for the association between cellular senescence and age-related pathologies was obtained from the BubR1 progeroid mouse, which exhibits premature aging and age-associated disorders. In this model, the elimination of p16-positive senescent cells results in a substantial protection from age-related pathologies such as cataracts or sarcopenia (Baker et al. 2011).

The mechanisms by which senescent cells can promote age-related pathologies are not completely understood. One plausible explanation is that the age-related deterioration of the immune system prevents the elimination of senescent cells by immune clearance. This fact, together with the ability of senescent cells to amplify the senescent response through the SASP, leads to an accumulation of these cells resulting in chronic inflammation. This chronic inflammation is postulated to fuel chronic disease progression. Another possible scenario is that senescent cells can target stem cells, thus declining the regenerative potential associated with aging (**Figure 4**) (Krtolica & Campisi 2002; Collado et al. 2007; Campisi 2011).

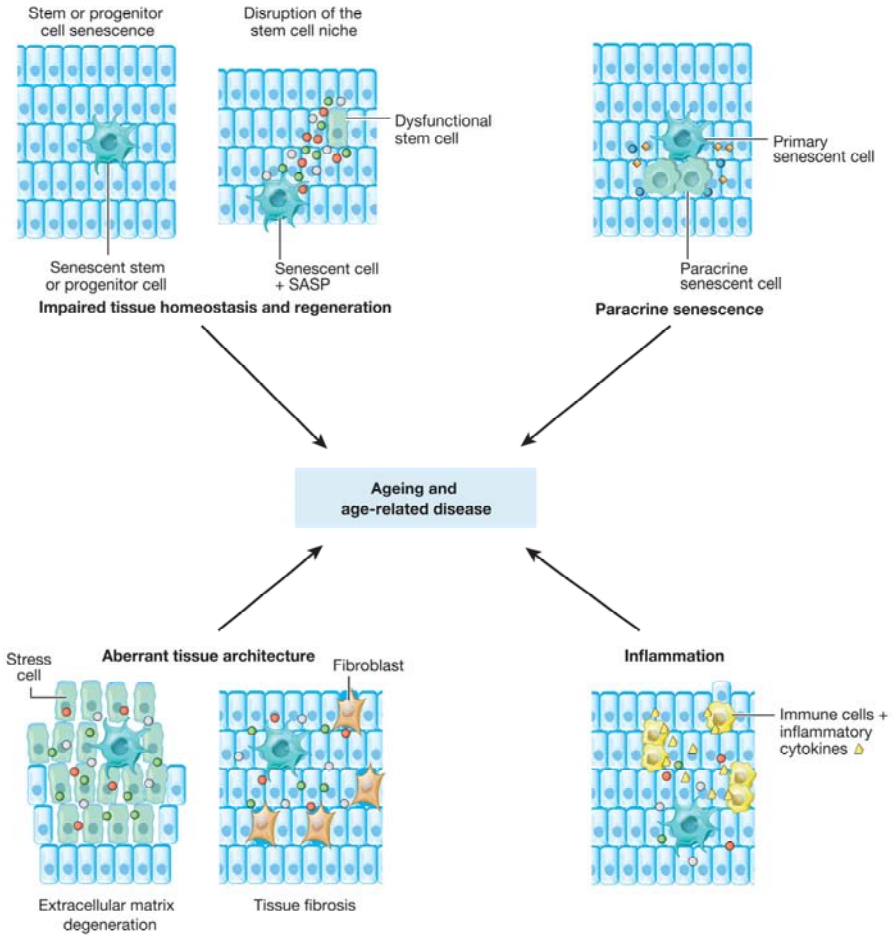

Figure 4. Contribution of senescence to age-related disease. Cellular senescence is thought to contribute to organ dysfunction and age-related diseases by different mechanisms. Senescence interferes with tissue homeostasis and regeneration, by depleting the pools of cycling cells, including stem and progenitor cells through the secretion of SASP factors. There are different paracrine mechanisms by which senescent cells could promote tissue dysfunction, namely perturbation of the stem cell niche, alteration of the extracellular matrix, stimulation of tissue inflammation, and induction of senescence in neighboring cells (paracrine senescence). Adapted from (van Deursen 2014) with permission of Macmillan Publishers Ltd.

1.2.4.2. Senescence and Tumor Progression

Senescent cells can alter the tissue microenvironment creating a permissive milieu for the expansion of neoplastic cells. SASP is one of the major players involved in this tumor-promoting role of senescence (Campisi et al. 2011; Campisi 2013). This is illustrated by the conversion of mammary epithelial cells into a more invasive and undifferentiated phenotype when co-injected with senescent fibroblasts in mice (Krtolica et al. 2001; Parrinello et al. 2005). Moreover, in patients with chronic obstructive pulmonary disease, senescent cells induced the proliferation and migration of non-senescent neighboring pulmonary artery smooth muscle cells (Noureddine et al. 2011). One of the postulated mechanisms of senescence-induced tumor progression is that senescent cells may increase microvascular permeability of surrounding tissues, enabling adjacent cells exposed to serum factors to increase cell growth (Liu & Hornsby 2007).

1.2.4.3. Senescence and Tumor Suppression

The main anti-tumor function of senescence resides in its capacity to arrest cell proliferation in cells at risk of malignant transformation (Campisi 2001). In this regard, senescence acts as failsafe mechanism that avoids the essential steps towards malignant transformation. Several pieces of evidence support the tumor suppressive potential of the senescent phenotype. First, senescent cells accumulate in pre-malignant lesions but they are rare in the developed malignant lesions (Castro et al. 2003; Braig et al. 2005; Wajapeyee et al. 2008). In this context, senescence could represent the key mechanism inducing cell cycle arrest in benign neoplasms (Braig et al. 2005; Collado et al. 2005; Collado & Serrano 2010). Second, senescence is mediated by the two major tumor suppressor pathways, p53 and p16/Rb, whose inactivation often results in impairment of the senescent response and increased cancer propensity (Rodier & Campisi 2011). Finally, the reversal of the senescence response by, for example, p53 inactivation often results in malignant transformation (Chen et al. 2005).

Whether these senescent cells are removed or not by the immune system will be determinant for the anti- or pro-tumorigenic effect of senescence, respectively. As mentioned before, SASP plays an essential role in this process. In this regard, pre-malignant senescent hepatocytes, by secreting chemokines and cytokines, undergo immune-clearance whereas the impairment of this process gives rise to murine hepatocellular carcinoma (Kang et al. 2011; Lujambio et al. 2013).

1.2.5. Tissue Repair

Consistent with the fact that many constituent factors of the SASP are proteins involved in wound healing and, also with the accumulation of senescent cells in tissues after injury, the involvement of senescence in tissue repair is well-established (**Figure 5**) (Rodier & Campisi 2011).

This effect is well-exemplified in a model of acute liver injury in which senescent hepatic stellate cells are able to dissolve the fibrotic scar likely through the secretion of MMPs, contrasting with the severe fibrosis observed in senescence-deficient mice (Krizhanovsky et al. 2008; Jun & Lau 2010).

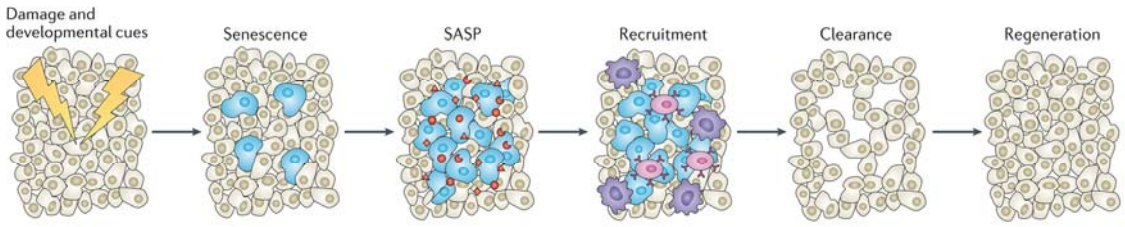

Figure 5. Contribution of senescence to tissue remodeling. Senescence recruits immune cells through the induction of SASP, initiating tissue remodeling processes. Senescent cells are cleared by the immune system, and progenitor cells can regenerate the damaged tissue. Reproduced from (Muñoz-Espín & Serrano 2014) with permission of Macmillan Publishers Ltd.

1.2.6. Senescence in Clinics

The chemicals employed targeting proliferating (cancerous) cells are a relevant drawback in current chemotherapies as they also affect healthy organs with active proliferation. Senescence induction arises as an interesting alternative therapy strategy to avoid these side effects. However, as explained before, senescence also entails several tumor promoting effects that should be counteracted (Nardella et al. 2011).

Nonetheless, promising pharmacological agents able to activate cellular senescence are already under clinical trials. The most relevant ones include drugs enhancing p53 activity like nutlins (Vassilev et al. 2004), or the recently discovered PRIMA-1^{MET}, that allows for the restoration of p53 wild type activity. The latter compound is in phase II clinical trials for the treatment of refractory hematological malignancies and prostate cancer (Bykov et al. 2002). In addition, a NEDD8-activating enzyme inhibitor (MLN4924), with pro-senescence potential, is currently in Phase I clinical trials (Lin et al. 2010). GRN163L is also currently in phase II trials due to its ability to inhibit the enzymatic activity of telomerase (**Table 3**) (Djojosubroto et al. 2005).

Table 3. Senescence-induced molecules in clinical trials.

FUNCTION	COMPOUND
P53 STABILIZATION	R7112 (nutlin-3 analogue)
MUTANT P53 REACTIVATION	PRIMA-1MET (APR-246)
	Ellipticine
SCF–SKP2 COMPLEX INHIBITOR	MLN4924
CDK-INHIBITORS	Flavopiridol
	UCN-01
	CYC202 (seliciclib)
	SNS-032 (BMS-387032)
MYC INHIBITORS	10058-F4 and its derivatives
PTEN INHIBITORS	VO-OHpic
TELOMERASE INHIBITOR	GRN163L (Imetelstat)

CDK, cyclin-dependent kinase; SCF, SKP1–CUL1–F-box protein; SKP2, S phase kinase-associated protein 2. Adapted from (Nardella et al. 2011; Tchkonia et al. 2013).

An appealing therapeutic avenue worth to explore is the potential elimination of already-induced senescent cells on the basis that, once damaged cells arrest their cell cycle, tumor-promoting effects could be avoided by removing them. A first, although not very successful approach, consisted in antibodies recognizing specific

epitopes of senescent cells coupled to cytolytic agents. A more promising strategy relied on the identification by high-throughput screenings of small molecules, known as senolytic drugs, targeting senescent cells (Zhu et al. 2015). Within this class of agents is ABT263, a specific inhibitor of the anti-apoptotic proteins BCL-2 and BCL-xL, that selectively eliminates senescent cells (Chang et al. 2015). Yet another strategy consists in preventing the SASP and, therefore, its detrimental associated effects (Laberge et al. 2012; Tchkonia et al. 2013). In line with this approach, senescence induction by p16 activation could also be of therapeutic interest since, in this type of cellular response, cell cycle is arrested without SASP production (Coppé et al. 2011).

1.2.7. Acute and Chronic Senescence

As mentioned before, senescence can have both dual pro- and anti-tumorigenic roles depending on the cellular context whose specific requirements will determine the persistence of this response, the SASP composition and the removal efficiency of senescent cells (van Deursen 2014). Local and specific processes such as tissue repair, wound healing and embryonic development engage a tightly programmed and temporally controlled senescence response which is characterized by the presence of acute senescent cells (Roninson 2003; Jun & Lau 2010). Acute senescence is triggered by a specific inducer and results in a homogeneous response and SASP composition. These features lead to the efficient removal of acute senescent cells by the immune system (Matheu et al. 2007). Conversely, the progressive accumulation of cellular stresses, as happens in the context of aging, leads to the gradual increase of senescent cells when the damage does not reach the apoptosis-inducing threshold (Finkel et al. 2007). Thus, a broad repertoire of effectors and signaling pathways can trigger chronic senescence, giving rise to heterogeneous cellular responses and SASP composition. Chronic senescence represents, however, a stochastic process where its heterogeneity, together with the progressive deterioration of the immune system with age, contribute to the inefficient clearance of senescent cells. The progressive accumulation of senescent cells is accompanied by chronic inflammation which ultimately culminates in the development of both malignant and degenerative age-related pathologies (**Figure 6**) (Childs et al. 2014; Roos et al. 2016). Remarkably, senescence can either suppress tumorigenesis in young organisms or promote it in old ones (Gorgoulis & Halazonetis 2010). This idea fits with the evolutionary concept of antagonistic pleiotropy, which refers to a trait that, although being beneficial early in life, becomes detrimental over the life span. Consequently, the desirable function of cellular senescence is the growth arrest, being a safeguard to ensure that damaged cells do not proliferate. However, there is also an unselected trait associated with this process, as senescence can bring about many detrimental consequences that are only manifested as life span increases (Krtolica & Campisi 2002; Giaimo & d'Adda di Fagagna 2012).

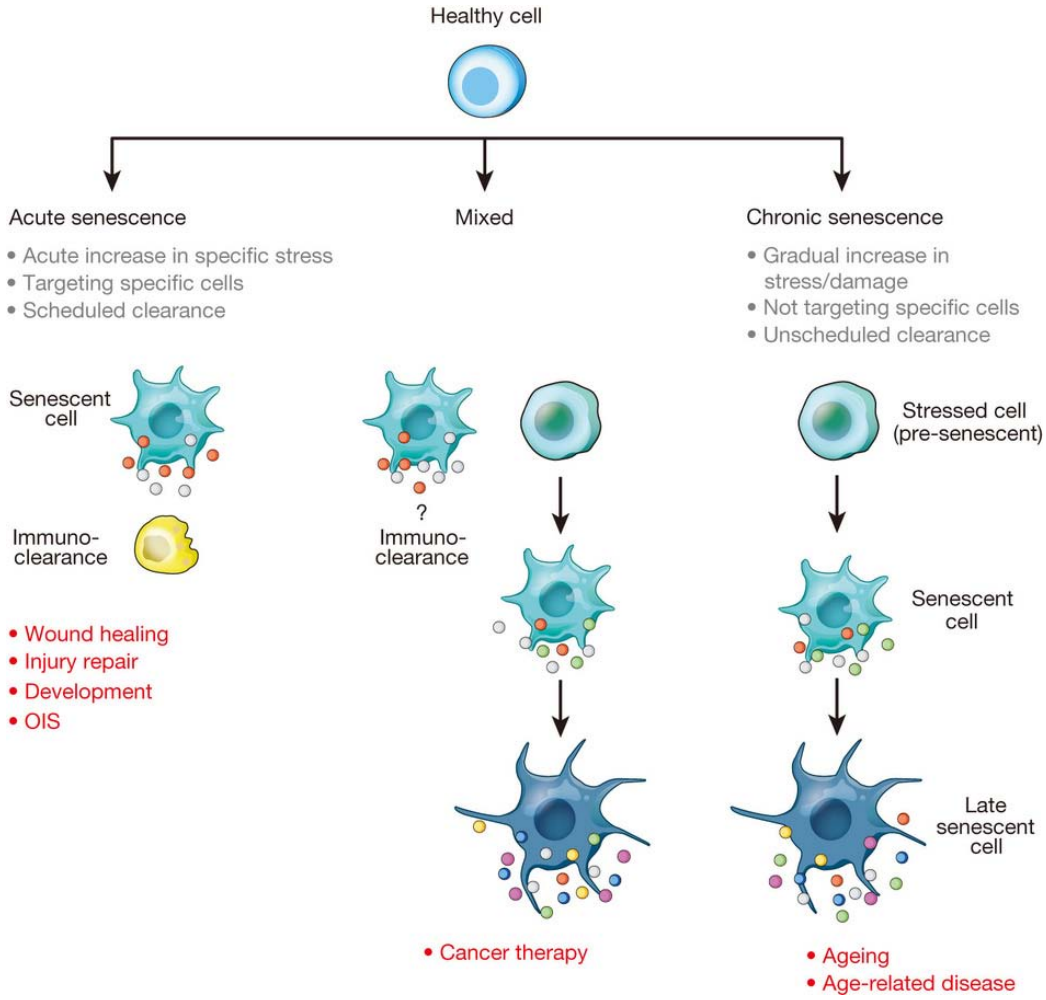

Figure 6. Schematic representation of acute and chronic senescent cells. Acute senescent cells are involved in programmed biological processes, namely wound healing, tissue repair or embryonic development. Acute senescence is induced through a stimuli that target a specific population of cells in the tissue and produce a SASP with defined paracrine functions. Acute senescent are then eliminated by attracting the immune system. Chronic senescence occurs after the accumulation of progressive cellular stress. Chronic senescence is not a programmed process and does not target specific populations of cells. Immune cells inefficiently eliminate chronic senescent cells, leading to their gradual accumulation. Senescence induced during cancer therapy may initially be acute and later chronic. Adapted from (van Deursen 2014) with perimission of Macmillan Publishers Ltd.

1.3. CELL DIFFERENTIATION

Cell differentiation is an essential mechanism for development that instructs cells to progress along the differential stages. This multifactorial process requires a complex coordination of genes and signaling pathways to orchestrate the direction towards specific cell states or functions. Cell differentiation is organized according to a hierarchical structure, where stem cells represent the apex with the highest multipotential ability. As cells are committed to differentiate into specific lineages, their differentiation potential becomes more restricted and the possible cell fate choices available are reduced (**Figure 7**) (Rossi et al. 2008; Valent et al. 2012). In this regard, the stem cell niche describes the environment where stem cells reside and dictate differentiation towards a specific lineage. Examples of these niches include hematopoietic stem cells (HSCs)

in the bone marrow, the neural stem cell niche in the brain or the epithelial stem cell niche in the skin (Loh et al. 2006; Lo Celso & Scadden 2011; Font de Mora & Díez Juan 2013).

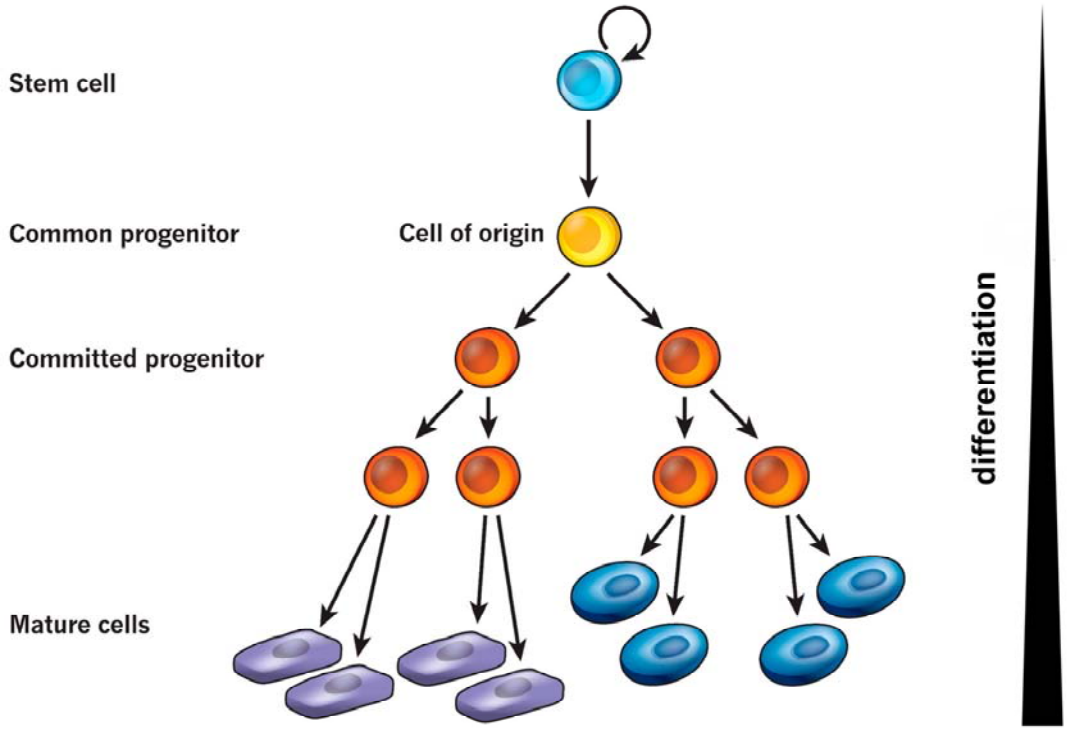

Figure 7. Representation of cellular hierarchy along the process of differentiation. Stem cells progressively generate more restricted progenitor cells, gradually narrowing the differentiation choices along the process of differentiation. Adapted from (Visvader 2011) with permission of Macmillan Publishers Ltd.

Tissues are composed by different populations with a broad repertoire of differentiation states. However, undifferentiated states are a common characteristic of cell populations giving rise to malignant neoplasms. Aberrant differentiation also leads to malignant progression, similarly to deregulated proliferation. In line with this idea, pioneering work conducted by Pierce and collaborators already demonstrated that malignant cells can differentiate into nonmalignant populations corresponding to more advanced stages along the specific lineage (Pierce & Wallace 1971; Pierce 1983). Altogether, these findings provided the basis for the differentiation therapy aiming to direct malignant cells into more advanced differentiation stages with lower tumorigenic potential. This therapeutic avenue is regarded as a targeted-therapy and often compares favorably to cytotoxic therapy, since it normally requires mildly cytotoxic concentrations of the drugs employed to induce differentiation (Hansen et al. 2000; Spira & Carducci 2003; Sell 2004).

Following this rationale, many compounds have emerged for clinical use due to their anti-proliferative and pro-differentiation potential, such as vitamin D (Chiang & Chen 2009), dimethyl sulfoxide (DMSO) (Collins et al. 1978), anthracyclines (Morceau et al. 1996), histone deacetylase inhibitors (Newmark et al. 1994), retinoids (Breitman et al. 1980) and peroxisome proliferator-activated receptor-γ (PPARγ) agonists (Koeffler 2003).

Among them, retinoids represent one of the best examples of differentiation therapy. In the next section, an overview of the variety of retinoids, differentiation pathways they are involved in, and their potential applicability in cancer therapeutics is presented.

1.3.1. Retinoids Overview

Retinoids are signaling molecules chemically related to Vitamin A (or all-*trans* retinol). Such molecules bear a β-ionone ring, a polyene side chain, and a polar end group. Variations in the polyene chain stereoisomerism and in the oxidation state of the polar group give rise to the different existing retinoid metabolites (Noy 2010). Biologically-active retinoids govern a wide range of functions including vision, neural function, immune function, organogenesis, cell growth, cell proliferation, cell differentiation and cell death (Altucci & Gronemeyer 2001; Tang & Gudas 2011).

1.3.2. Retinoids Processing

Vitamin A is obtained from dietary sources in the form of retinyl esters and carotenoids (mostly β-carotene). Retinyl esters are stored intracellularly in the stellate cells of the liver. Alternatively, retinyl esters are hydrolyzed by retinyl ester hydrolases (REHs) into retinol, which can be secreted from the liver. Retinol is transported in the blood by the retinol binding protein (RBP) and is delivered to target cells. Once in the target cells, retinol is oxidized to retinaldehyde (retinal) by retinol dehydrogenases (ADHs), being further oxidized by retinaldehyde dehydrogenases (ALDHs) to yield retinoic acid (RA). Intracellular concentrations of retinoids are highly variable, depending on both the particular cell type and the differentiation stage, as a function of the expression levels of the metabolic enzymes controlling retinoid processing (Mongan & Gudas 2007; Theodosiou et al. 2010).

Several retinoid metabolites are detected endogenously having different affinities for the nuclear receptors and, therefore, affording different biological actions (Buck et al. 1991; Achkar et al. 1996; Napoli 1996). Among them, all-*trans* RA (ATRA) is the most physiologically abundant, widely studied and represents the first of this family of compounds that has been used with clinical purposes. ATRA can be isomerized to 9-*cis* or 13-*cis* RA (Altucci & Gronemeyer, 2001; Garattini et al., 2014). Moreover, several retinoid derivatives with different degrees of physiological activity have been synthesized including fenretinide, tazarotene, bexarotene, adapalene, and benzoic acid (Barnard et al. 2009).

1.3.3. Canonical Receptors

RA exerts a plethora of biological activities by regulating the expression of RA-responsive genes upon binding to retinoid receptors, which are nuclear receptors that function as ligand-dependent transcription factors and regulate the expression of genes involved in cell growth, cell differentiation, cell survival and cell death. These receptors can be divided into two groups: RA receptor (RARs) and retinoic X receptors (RXRs), each one

comprising 3 different subtypes, namely α, β and γ (Leid et al. 1992; Chambon 1996; Robinson-Rechavi et al. 2003). RARs bind ATRA, fenretinide and 9-*cis* RA whereas 13-*cis* RA, bexarotene and 9-*cis* RA preferentially activate RXRs. RAR and RXR form heterodimers that recognize a specific consensus sequence present in promoter regions of target genes, termed RA response elements (RAREs). RAREs consist of two direct repeats of a core hexameric motif 5'-RGKTCA-3' (where R is A or G and K is G or T) separated by 1, 2 or 5 nucleotides organized in direct repeats or palindromes (Durand et al. 1992; Rochette-Egly 2014). In order to bind these nuclear receptors, RA requires being shuttled from the cytosol to the nucleus, a process carried out by the cytosolic retinoic acid binding protein 2 (CRABP2), which controls RA delivery to RAR (Budhu & Noy 2002).

RAR-RXR heterodimers are also regulated by different activators and repressors that regulate gene transcription. In absence of the ligand, RXR-RAR heterodimer binds corepressor complexes which, in turn, allow the recruitment of histone deacetylase-containing complexes (HDAC). Gene transcription is therefore prevented through chromatin condensation resulting from histone deacetylation. However, upon RA binding, the heterodimer binds, instead, coactivator complexes including histone acetyltransferase complexes that activate the transcriptional machinery through acetylating lysine residues (Perissi et al. 2010).

1.3.4. Non-canonical Receptors

RARs can also form heterodimers with different other nuclear receptors, including the estrogen receptor-α, the AP-1 receptor, the PPAR, liver X receptors, and the vitamin D receptor (Connolly et al. 2013). RA is also able to modulate the expression of different genes, aside from those controlled by the canonical mechanism based on the RAR-RXR binding to RAREs. Of particular relevance is the nuclear receptor PPAR which can form heterodimers with RXRs and bind RA with high affinity. In this case, RA is transferred to PPARs by the fatty acid binding protein 5 (FABP5), transporting this organic compound from the cytosol to the nucleus, similarly to CRABP2 function. Remarkably, the FABP5/CRABP2 ratio determines which nuclear receptor RA will preferentially bind to, with higher levels of FABP5 over CRABP2 resulting in increased binding to PPARs whereas the opposite ratio favors binding to RARs. This balance between binding to different nuclear receptors is further translated in a differential outcome. While RA binding to RARs following the classical mechanism leads to the canonical anti-proliferative function, enhanced binding to PPARs gives rise to a proliferative and anti-apoptotic effect via activation the Akt/PDK1 pathway (Schug et al. 2007; Schug et al. 2008).

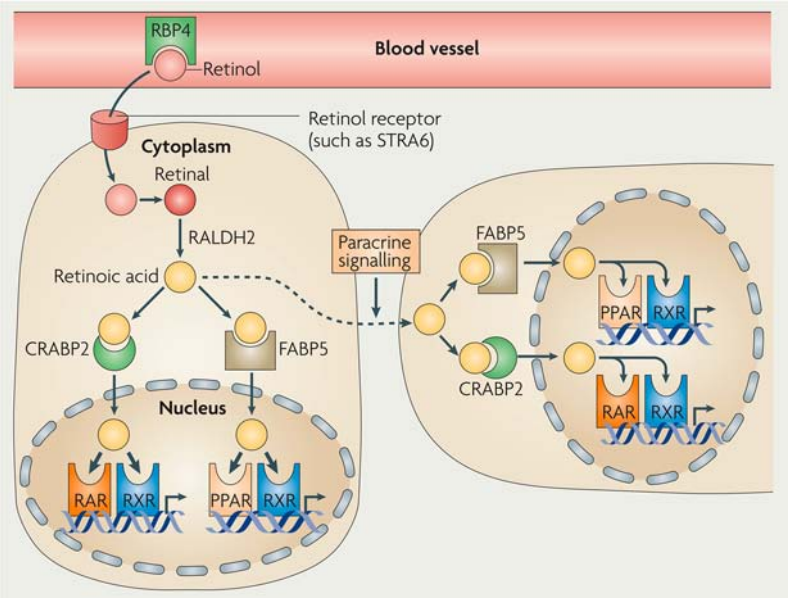

Figure 8. Retinoic acid signaling. Retinoic acid is metabolized from vitamin A and stored in the liver. In the liver, retinol forms a complex with retinol-binding protein 4 (RBP4), which allows its transport through the blood. Once in the cell, retinol is transformed into retinal to be subsequently metabolized into retinoic acid by retinal dehydrogenases (RALDH1, RALDH2, RALDH3). Cellular retinoic acid-binding protein 2 (CRABP2) delivers retinoic acid to DNA-binding retinoic acid receptors (RARs), while fatty acid-binding protein 5 (FABP5) transfers it to DNA-binding peroxisome proliferator activated receptor-β (PPARβ) or PPARδ, which, in turn, results in cell cycle arrest or proliferation, respectively. Reproduced from (van de Pavert & Mebius 2010) with permission of Macmillan Publishers Ltd.

1.3.5. Non-genomic Pathways

RA also mediates cellular functions without regulating gene transcription. Specifically, RA can activate several pathways, such as the p38 mitogen activated kinase (MAPK) (Piskunov & Rochette-Egly 2012), the p42/p44MAPK (Chen & Napoli 2008), the PI3K/Akt (Fanjul et al. 1994), the Wingless (Wnt) (Mulholland et al. 2005) or the NF-κB (Austenaa et al. 2004).

1.3.6. Retinoids in Development

Retinoids are important regulators of embryogenesis and, as such, constitute essential molecules for life. These compounds play key roles in tissue development, differentiation, organogenesis and embryonic patterning. The importance of RA during development is well documented by animal models of vitamin A deficiency (VAD) giving rise to a variety of congenital malformations in the ocular, cardiac, respiratory and urogenital systems. Moreover, RA is also important for the normal development of heart, kidney, urinary tract diaphragm, lung and upper respiratory tract, skeleton, pancreas, and limb (Das et al. 2014). Nonetheless, its morphogen potential makes RA a double-edged sword: whereas VAD causes the above-mentioned malformations, excessive concentrations of vitamin A are also toxic for the liver, central nervous system, musculo-skeletal

system, internal organs, and skin. Accordingly, RA is regarded as a teratogen (McCaffery et al. 2003; Lee et al. 2012).

In the context of nervous system development, RA controls tissue patterning and neuronal differentiation. More specifically, RA regulates anteroposterior and dorsoventral patterning in the neural plate and in the neural tube of the developing nervous system. RA also decreases stem cells or progenitor cells numbers, and induces differentiation of neurons and glia by regulating the transcription of many genes involved in developmental processes. In addition, RA also modulates signaling pathways involved in development such as the Fibroblast Growth Factor (FGF), Wnt, Bone Morphogenetic Protein (BMP), and Sonic Hedgehog (Shh). Notably, by virtue of its ability to activate differentiation programs, RA is also essential in neural regeneration after damage (Mark et al. 2006; Niederreither & Dollé 2008; Mark et al. 2009).

On the opposite side, several pieces of evidence also support a connection between RA and the process of aging. In this regard, vitamin A-deprived rodents exhibit an impairment in spatial memory tests and depression of long-term potentiation, two features that correlate with aging (Etchamendy et al. 2003). In addition, vitamin A-deprived rats present increased levels of the amyloid-β (Aβ) peptide, downregulated levels of RARα and loss of choline acetyltransferase expression in their forebrain cortical neurons and in their cerebral vessels, being all features of Alzheimer's disease (Corcoran et al. 2004; Maden 2007).

1.3.7. Retinoids in Cancer

In general terms, RA is a pro-differentiation, anti-proliferative, anti-oxidant, and pro-apoptotic compound. These properties, coupled with the fact that RA can activate several tumor suppressor genes, and that RARs are frequently downregulated in several cancer types, suggest a potential antitumor role for this agent. In fact, an emerging body of studies show the correlation between Vitamin A deficiency and the appearance of different types of cancer including breast, head and neck and lung cancers (Wolbach & Howe 1925; Niles 2004). Likewise, retinoids (including RA stereoisomers and their natural or synthetic derivatives) have also been tested for their potential applicability in the prevention and treatment of cancer (**Table 4**) (Connolly et al. 2013).

Table 4. Clinical trials of retinoids.

NAME	PHASE/INDICATION
TRETINOIN (ATRA)	Launched: acne, APL, warts Phase II: brain, breast, renal cancers, SCLC, Kaposi's sarcoma, Wilms' tumor, malignant melanoma
ALITRETINOIN (9-*CIS* RA)	Launched: Kaposi's sarcoma

ISOTRETINOIN (13-*CIS* RA)	Launched: acne Phase III: isotretinoin plus IFNα plus vitamin E in III or stage IV head and neck cancer Phase III: high-grade glioma Phase III: combination therapy with isotretinoin in neuroblastoma Phase II:T-cell malignancies Phase II: IFNα plus isotretinoin plus paclitaxel recurrent SCLC Phase II combination chemotherapy in juvenile myelomonocytic leukaemia
TRETINOIN TOCOFERIL	Launched: skin ulcer
TAZAROTENE	Launched: acne, psoriasis, photodamage
FENRETIDINE	Phase III: early breast cancer
BEXAROTENE	Phase II: metastatic breast cancer

Adapted from (Altucci & Gronemeyer 2001; Altucci et al. 2007; Connolly et al. 2013; Garattini et al. 2014).

Perhaps the major potential of RA for cancer treatment resides on its ability to cease cell proliferation and to arrest the cell cycle in certain cellular contexts. This effect is achieved via the RARβ-mediated regulation of p21 and p27 levels, and also by RA inducing a greater posttranslational stability of these proteins. Furthermore, RA increases the rate of proteolytic turnover of both cyclin D1 and cyclin E, leading to cell cycle arrest (Langenfeld et al. 1997; Dimberg et al. 2002).

1.3.7.1. Retinoids in Cancer Prevention and Therapy

Compelling evidence reports the ability of RA to prevent the conversion from pre-malignant to malignant stages, thus reducing cancer risk. Accordingly, RA is effectively employed in the treatment of several pre-cancerous lesions including leukoplakia, actinic keratosis and cervical dysplasia (Langenfeld et al. 1997; Freemantle et al. 2003; Niles 2004).

The use of retinoids with therapeutic purposes, known as retinoid therapy, has already been proven successful in some preclinical and clinical trials for the management of different types of cancers (Tang & Gudas 2011). The US Food and Drug Administration (FDA) has already approved its use for the treatment of cutaneous T-cell lymphoma (Duvic et al. 2001) and acute promyelocytic leukemia (APL) (Degos & Wang 2001). In fact,

treatment of APL is the prototypical example of RA-based therapy. APL is caused by a translocation of RARα leading to its fusion with the promyelocytic leukemia (PML) gene. The resulting PML-RARα fusion binds tightly co-repressors, impairing the transcription of RA-regulated genes. However, the PML-RARα fusion is still RA-responsive at high ATRA doses which are able to promote the dissociation of co-repressor complexes from PML-RARα, allowing the transcription of RA-responsive genes. These pharmacological doses of ATRA suffice to induce the differentiation of leukemic cells into normal granulocytes. The combination of ATRA with chemotherapy has substantially changed the course of this disease, resulting in a dramatic increase of the overall survival (Fenaux et al. 2000; Tallman et al. 2002). On the other hand, 13-*cis* RA is currently employed as maintenance therapy in standard high risk protocols of neuroblastoma treatment to eradicate the minimal residual disease remaining after chemotherapy (Matthay et al. 2009). Besides, synthetic retinoids have also been tested against neuroblastoma. These synthetic agents are of particular interests because of their ability to regulate cellular functions independently of RARs, thus having activity in cells resistant to RA because of RARs downregulation. More specifically, fenretinide, which is currently under several clinical trials is able to induce cytotoxicity in neuroblastoma tumors (Lovat et al. 2004).

A major limitation of retinoid therapy is the resistance exhibited by several tumors. Such resistance mainly arises from the deregulation of RA-dependent pathways, the presence of stem cells, the inactivation of co-activators and the activation of co-repressors, changes in RA metabolism and availability, and downregulation of RARs (Noy 2010). The latter source is of special relevance; particularly, RA-resistance is highly influenced by the expression levels of the RARβ receptor, which is found downregulated in many pre-malignant and malignant cancers via promoter methylation and histone deacetylation. This receptor promotes apoptosis and growth arrest and its expression correlates with better clinical outcome, being classified as a tumor suppressor (Xu 2007). In this sense, RARβ represents an important biomarker of breast cancer, and the relevance of its expression expands to other types of cancer such as lung, ovarian, prostate, neuroblastoma, renal cell carcinoma, liver, head and neck, and pancreatic cancers (Toulouse et al. 2000; Sirchia et al. 2002; Ren et al. 2005). Such a wide distribution highlights the importance of assessing RARβ expression status prior to the application of RA-based therapy, and can also explain the failure of many RA-based therapeutic attempts to date. Interestingly, the employment of demethylating agents or HDAC inhibitors allows to recover RARβ expression , thus restoring responsiveness in front of RA treatment (Liu et al. 1996).

2. NEUROBLASTOMA

Neuroblastoma is the most common solid tumor in childhood, accounting for 8-10% of all pediatric malignancies. This tumor occurs almost exclusively during infancy or early childhood, and represents the most common cause of cancer diagnosed at one year of life (Kaatsch 2010). Neuroblastoma is responsible for about 15% of childhood deaths and around 90% of the cases are diagnosed during the first 5 years of life (Øra & Eggert 2011; Owens & Irwin 2012).

Neuroblostoma are embryonic tumors arising from the developing neural crest (NC) and harboring a great heterogeneity reflecting the diversity of differentiation, migration and growth stages taking place along the developing sympathetic lineage from where it emerges (Takahashi et al. 2013). Consistent with its origin from multipotent NCs, tumors often display a great propensity to differentiate either spontaneously or after induction with certain chemical agents, being therefore suitable and well-studied models of differentiation (Garrett M. Brodeur 2003; Marshall et al. 2014).

2.1. CELL OF ORIGIN

The NC is a transient structure comprising different populations of multipotent cells with the ability to generate a wide spectrum of cell lineages. The plasticity observed in neuroblastoma tumors derives from the NC origin blocked at the different stages of the sympathetic nervous system development. The NC originates during neurulation at the borders between the neural plate and the non-neuronal ectoderm, when the neural plate bends and closes by bringing the borders together to form both the NC and the neural tube (**Figure 9**). NC cells then undergo EMT, allowing them to migrate within the embryonic tissues. Once at their final location, they can generate a wide spectrum of different cell lineages, including, among others, those of the peripheral nervous system (giving rise to neurons, sensory ganglia, sympathetic and parasympathetic ganglia, neuroglial cells, and Schwann cells), of the endocrine and paraendocrine systems (adrenal medulla), and also those resulting in pigment cells, facial cartilage and bone, and connective tissue (Gammill & Bronner-Fraser 2003; Huang & Saint-Jeannet 2004).

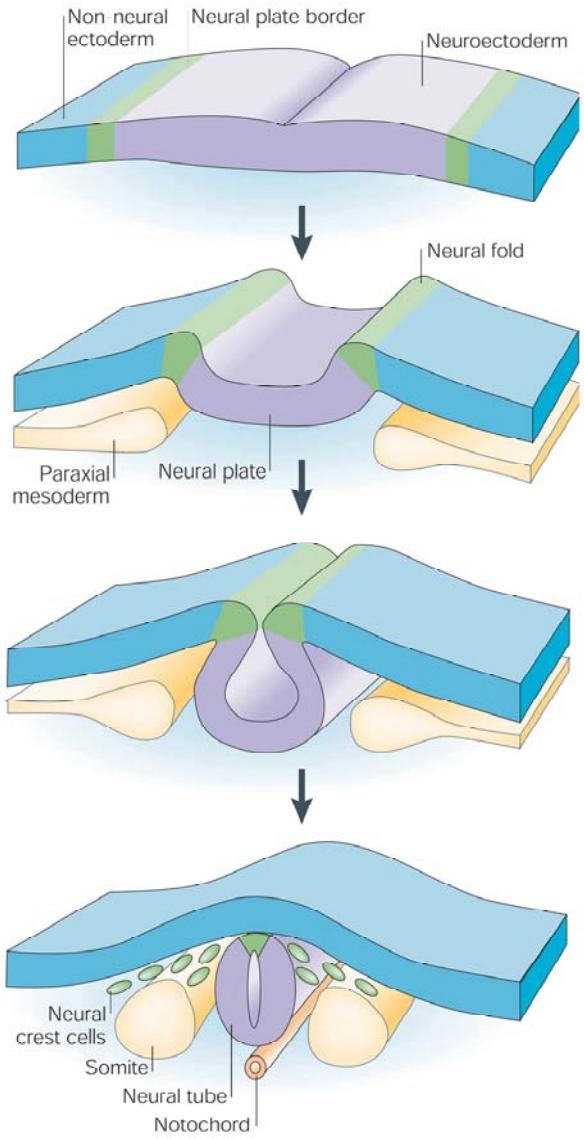

Figure 9. Representation of Neural Crest formation. During neurulation, the neural plate borders (neural folds) close together to form the neural tube. Neural crest cells (green) delaminate from the neural folds or the dorsal neural tube. Reproduced from (Gammill & Bronner-Fraser 2003) with permission of Macmillan Publishers Ltd.

To orchestrate this process, a fine balance between cell death, cell proliferation and cell differentiation must be achieved. In this sense, diseases such as neuroblastoma result from the alteration of these processes during the migration of cellular populations. Consistently, the vast majority of the genetic and epigenetic alterations found in neuroblastoma tumors normally affect genes controlling development and differentiation including MYCN, paired-like homeobox 2B gene (PHOX2B) or Neurotrophic Tyrosine Kinase, Receptor, Type 2 (NTRK2) (Hallbook et al. 1995; Nakagawara 2001; Grimmer & Weiss 2006).

2.2. CLINICAL PRESENTATION AND TREATMENT

Due to its embryonic origin, neuroblastoma tumors can be located in any region along the sympathetic nervous system. They arise frequently in the abdomen, with about 50% found in the adrenal medulla. Less abundant cases are detected in the paraspinal ganglia in the neck (5%), chest (20%) or pelvis (5%) (Maris et al. 2007). Likewise, the clinical manifestation may vary widely depending on the specific location where it emerges. Around 40% are locoregional tumors with favorable characteristics, being successfully treated with surgery. However, over 50% correspond to metastatic tumors that spread the disease usually to bone tissues, the bone marrow, liver, lymph nodes, or the skin, and, less frequently, to lung and the central nervous system (Owens & Irwin 2012).

Clinical manifestations are very heterogeneous, ranging from tumors that spontaneously regress or differentiate to metastatic and highly resistant ones. This heterogeneity in clinical presentation requires an adjustment of neuroblastoma treatment according to the risk classification of the tumor. Very low-risk patients often undergo spontaneous regression (85% are event free at 5 years) and need no intervention. In low-risk patients, the common treatment consists on surgical resection and, in some cases, chemotherapy. Intermediate-risk patients are subjected to surgery and chemotherapy (Maris et al. 2007). Finally, multimodal approaches are most frequently undertaken in high-risk patients whose treatment remains a major challenge in the management of neuroblastoma. Current standard therapies for this group consist of four different phases: induction of remission, local control, consolidation of the remission, and maintenance phase (Owens & Irwin 2012).

The aim of the induction therapy is to reduce tumor burden via administration of cyclic doses of cisplatin, vincristine, doxorubicin, cyclophosphamide and topotecan. After induction, the local control phase consists on the surgical resection of the tumor combined with radiotherapy (Modak & Cheung 2010). Next, the consolidation stage comprises chemotherapy followed by myeloablative therapy with autologous stem-cell transplantation (Adkins et al. 2004). The last maintenance phase aims to reduce the risk of relapse, which is mostly attempted through the induction of differentiation employing retinoids or via immunotherapy using anti-GD2 antibodies (Matthay et al. 1999; Yu et al. 2010).

Additionally, extensive effort has been made for the development of novel therapies targeting single molecules selectively, usually referred to as targeted-therapies, which aim to increase the therapeutic potential against neuroblastoma while avoiding the toxicity of current standard treatments. These therapies include inhibitors of Aurora A kinase (AURKA), anaplastic lymphoma kinase (ALK) inhibitors, the norepinephrine analogue metaiodobenzylguanidine (^{131}I-labeled MIBG) or immunotherapy approaches with anti-GD2 antibodies. Additionally, other proteins have been proposed for this class of therapy like the insulin-like growth factor 1 receptor (IGF1R), the tropomyosin receptor kinase (TRK), or mTOR (Maris 2010; Verissimo et al. 2011; Matthay et al. 2012).

2.3. GENETIC AND MOLECULAR ALTERATIONS ASSOCIATED TO UNFAVORABLE CLINICAL OUTCOME

Several genetic abnormalities play a key role in the development of the disease and represent important predictive factors of neuroblastoma outcome.

DNA content- DNA index (ploidy) is a relevant biomarker that allows the prediction of neuroblastoma outcome and, consequently, was adopted as a biomarker by the International Neuroblastoma Risk Group (INRG). Lower stages associated with favorable outcome often present near-triploid nuclear DNA content which is characterized by whole chromosome gains or losses without structural aberrations. However, more advanced tumors are often near-diploid or near-tretraploid and are characterized by segmental chromosomal translocations (Garrett M. Brodeur 2003; Ambros et al. 2009).

MYCN amplification- The v-Myc Avian Myelocytomatosis viral oncogene neuroblastoma-derived homolog (MYCN) is a transcription factor involved in embryonic development. More specifically, it regulates functions such as cell invasion and motility, cell cycle, immune surveillance, self-renewal and apoptosis. Accordingly, when its expression is altered by amplification or overexpression, these processes are impaired resulting in undifferentiated cellular stages that, in turn, are associated to greater tumor aggressiveness. MYCN is amplified in different types of cancers, generally of embryonic origin. Concretely, MYCN amplification accounts for about 22% of neuroblastoma tumors and is normally associated with a survival rate of 15-35%, being an important prediction marker of poor outcome (Schwab et al. 1984; Westermark et al. 2011). MYCN maps to chromosome 2p24.1 and, in amplified regions, is also located in double minute chromatin regions (DM) and homogenously staining regions (HSR) (Reiter & Brodeur 1998). MYCN amplification represents the most important genetic signature in neuroblastoma despite other genetic alterations such as chromosome 1p deletion or ALK activation are concomitantly observed to this amplification (Garrett M. Brodeur 2003).

TERT activation- Recent reports have shown that telomerase reverse transcriptase (TERT) activity is enhanced in high-risk neuroblastoma. This results from rearrangements in a proximal region of the TERT gene, which are only found in high-risk cases, leading to its increased expression. Accordingly, telomere lengthening represents also a hallmark of high-risk neuroblastoma tumors (Peifer et al. 2015).

ALK activation- ALK oncogene encodes a tyrosine kinase transmembrane receptor that regulates proliferation and differentiation along the sympathoadrenal lineage during development through the activation of the JAK-STAT, PI3K/AKT or RAS/MAPK pathway. ALK activity may be aberrantly enhanced by different mutations or by chromosome rearrangements resulting in increased aggressiveness. These alterations lead to the autophosphorylation of the ALK tyrosine kinase domain, resulting in a permanent activation of the receptor. Alterations in the ALK oncogene are indicators of familial predisposition to neuroblastoma, accounting for 50% of hereditary neuroblastoma cases (Mossé et al. 2008; Yan et al. 2011).

PHOX2B inactivation- The paired-like homeobox 2B gene (PHOX2B) is a transcription factor that modulates sympathoadrenal differentiation. Concretely, it promotes cell cycle exit and differentiation into noradrenergic sympathetic neurons by activating the transcription of noradrenergic markers such as tyrosine hydroxylase and dopamine-β-hydroxylase. Mutations in this gene are found in 2-6% of familial cases and rarely in sporadic neuroblastoma events (Øra & Eggert 2011).

Other chromosome deletion/gains- Chromosome copy number alterations commonly found in neuroblastoma mainly comprise deletions, such as 1p, 3p, and 11q, and gains like 1q, 2p, and 17q (Øra & Eggert 2011). Deletion of the short arm of chromosome 1 (1p) is identified in 25-35% of neuroblastoma tumors and correlates with advanced stages of the disease. Another common deletion is the allelic loss at 11q23 chromosomal region, detected in 35-40% of tumors and inversely correlated with MYCN amplification, thus becoming a suitable biomarker of MYC-non-amplified tumors. Moreover, unbalanced gain of the long arm of chromosome 17 is associated with near-triploid lesions and occurs in more than 50% of neuroblastoma cases, whereas gains of the whole chromosome are observed in 40% of hyperploid cases (Cheung & Dyer 2013; Berbegall et al. 2014).

2.4. NEUROBLASTOMA CLASSIFICATION

Given the wide repertoire of clinical manifestations existing in neurbolastoma, strong efforts have been conducted to classify these tumors into different risk groups. The systems proposed for the classification of neuroblastoma incorporate international consensus criteria that helped to improve current therapies and clinical outcome.

Shimada and co-workers initially classified neuroblastoma tumors as favorable or unfavorable based on the grade of differentiation, Schwannian stroma content, age at diagnosis, and the mitosis-karyorrhexis index (Shimada et al. 1985). This system was subsequently modified by the International Neuroblastoma Pathology Committee (INPC) that classified these tumors into four categories: neuroblastoma (Schwannian stroma-poor); ganglioneuroblastoma intermixed (Schwannian stroma-rich); ganglioneuroma (Schwannian stroma-dominant); and ganglioneuroblastoma nodular sub-type (Schwannian stroma-rich/stroma-dominant and stroma-poor) (Shimada et al. 2001; Peuchmaur et al. 2003).

The International Neuroblastoma Staging System (INSS) identified distinct prognostic stages (namely 1, 2A, 2B, 3, 4 and 4S) based on age at diagnosis, the extent of disease and postsurgical evaluation. For several years, the INSS stage, group age and MYCN amplification were considered the three major prognostic factors for tumor stratification. Within this frame, patients can be classified as low-, intermediate- or high-risk based on the INSS stage, group age and tumor biology (MYCN status, DNA index, etc) (Brodeur et al. 1988; Brodeur et al. 1993).

In 2004, the International Neuroblastoma Risk Group Staging System (INRGSS) established a new classification system based on age, histologic category, degree of tumor differentiation, MYCN amplification, presence or absence of 11q aberrations, and tumor cell ploidy to classify patients at the time of diagnosis. Based on this system, 4 stages, L1, L2, M and MS were defined (Cohn et al. 2009; Monclair et al. 2009).

2.5. DIFFERENTIATION OF NEUROBLASTOMA TUMORS

Neuroblastoma is an extremely heterogeneous disease and such variety is translated into the biological and clinical behavior it displays. In biological terms, a broad spectrum of cellular phenotypes co-exists within a single tumor, each one presenting different morphological and biochemical characteristics. This diversity ranges from tumors with undifferentiated neuroblasts to those predominantly consisting fully differentiated neurons surrounded by a dense stroma. Remarkably, such differences tightly correlate with the clinical outcome. Indeed, neuroblastoma can differentiate into ganglioneuroblastomas or benign ganglioneuromas with a favorable prognosis. Likewise, Schwann cell content increases along neuroblastoma maturation and remarkably correlates with a better clinical outcome (Biedler et al. 1973; Ciccarone et al. 1989).

Two different cell populations were initially identified in mature neuroblastoma tumors: neuronal and Schwannian. Furthermore, studies conducted by Biedler and colleagues also identified the presence of different phenotypes in cell lines derived from neuroblastoma tumors. Initially, the neuroblastic (N-type) and the substrate adherent or stromal (S-type) sub-types were identified in cell lines. Subsequently, a phenotype sharing characteristics of both the N- and S-phenotype was discovered, hence termed intermediate subtype (I-type). Different biochemical studies revealed that N-type cells express markers of immature sympathoblasts whereas S-type cells exhibited Schwannian, melanocyte, and/or smooth muscle cells features. Collectively, these findings support the hypothesis that such phenotypic variants represent different neural crest cell lineages (Biedler et al. 1973; Piacentini et al. 1996; Ross et al. 1983).

Two different theories have emerged in the literature regarding the origin of Schwann cells that are present in neuroblastoma tumors. First, Schwann cells are recruited from surrounding non-malignant tissues. Second, Schwann cells arise from the same precursor than N-type cells. Supporting the first postulation, Ambros and colleagues demonstrated that, in a cohort of neuroblastoma tumors, neuroblasts and ganglionic cells exhibited chromosomal alterations whereas Schwann cells displayed a normal DNA content, thus suggesting that this cell type does not have a neoplastic origin. Considering these findings, they proposed a model in which undifferentiated neuroblasts secrete mitogens, chemotactic factors or growth factors that influence the proliferation of Schwann cells which are subsequently recruited to the tumor and favor the differentiation of neuroblasts by secreting neurotrophins and other factors (**Figure 10**) (Ambros et al. 1996; Cohen et al. 2003). On the contrary, in line with the idea of common a precursor, Mora and co-workers analyzed different neuroblastoma tumors and evidenced that neuroblasts and Schwann cells share the same chromosomal alterations (Mora et al. 2001).

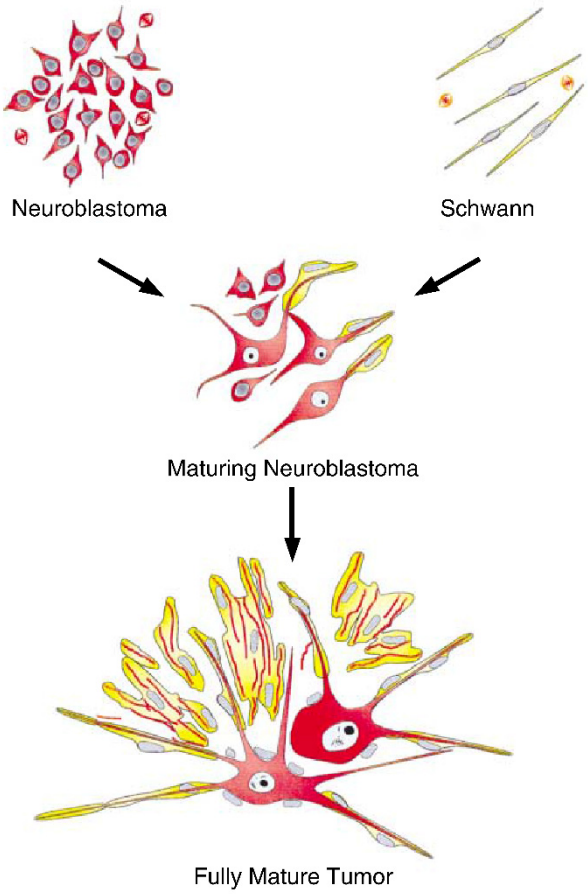

Figure 10. Schematic representation of the model proposed by Ambros. Undifferentiated neuroblastoma cells recruit Schwann cells and induce their proliferation. Therefore, Schwann cells migrate into the tumor, where they suppress the proliferation of neuroblastoma cells and induce their differentiation. Reproduced from (Ambros et al. 1996) with permission of Massachusetts Medical Society.

The three sub-types display several differences in the morphology, growth pattern and biochemical properties which are summarized below (**Table 5**).

N-type- The morphology of these cells is characterized by round cell bodies, scant cytoplasm and several, often numerous, short neuritic-like processes. They adhere weakly to substrate, attaching better to each other than to the substrate and, accordingly, use to form cell aggregates, which are recognized as clumps of floating spheres. Moreover, they express markers of noradrenergic neurons such as tyrosine hydroxylase and dopamine-β-hydroxylase (Acosta et al. 2009; Han et al. 2011).

S-type- These cells grow slowly, show contact inhibition, adhere tightly to substrate and lack neuritic-like processes. Accordingly, they do not exhibit neuronal markers but, instead, they present markers of menalocytes (tyrosinase), of glial and Schwann cells (collagen) as well as markers common to all these three type of cells (fibronectin and vimentin) (Ciccarone et al. 1989). S-type cells tend to form monolayers in culture and exhibit

a higher resistance to cytotoxic treatments than the other sub-types (Bian et al. 2002; Hopkins-Donaldson et al. 2002; Bian et al. 2004; Biagiotti et al. 2006).

I-type- Termed I because this subtype harbors an intermediate morphology between N and S cells. I-type cells present low cytoplasm/nuclei ratio with more cytoplasm than N-type cells and short neurite process formation. This sub-type displays weak adherence to the substrate and, similarly to N-type cells, they grow as multilayers forming aggregates. They have bipotential differentiation capacity towards both phenotypes. This fact, together with the presence of stem cell markers (such as CD133 or c-kit), suggests that they could be cancer stem cells (Ross et al. 1995; Ross & Spengler 2007). However, it was also suggested that the I-type is an intermediate phenotype in the transdifferentiation process between N and S sub-types, although it has been observed that both phenotypes can also interconvert without any intermediate cell type (Ross et al. 2003).

Table 5. Markers of N- S- and I-type phenotypes.

N-TYPE	S-TYPE	I-TYPE
Tyrosine Hydroxylase	CD44	c-kit
Dopamine-β-hydroxylase	Vimentin	CD133
Growth Associated Protein (GAP43)	Actinin (ACTN)	Peripherin
Neurofilaments (NF68, NF150, NF200)	S100A6	Growth Associated Protein (GAP43)
HU	Calcyclin	Neural Cell Adhesion Molecule (NCAM)
Nerve Growth Factor Receptor (NGFR)	Epidermal Growth Factor (EGF)	MYCN
Chomogranin A	Fibronectin	
Secretogranin II	Laminin	
βiii Tubulin	Colagen Type I	
Norepinephrine	Colagen Type III	
Peripherin		
Neural Cell Adhesion Molecule (NCAM)		

Adapted from (Walton et al. 2004; Ross & Spengler 2007; Acosta et al. 2009).

A correlation between malignant potential and these phenotypic variants has been demonstrated. Indeed, S-type cells did not form tumors when injected in athymic mice and they showed few or no colony formation in soft agar. The N sub-type was weakly tumorigenic and exhibited low-to-moderate colony-forming efficiency. By contrast, I-type cells had the greatest percentage of colony forming efficiency and tumor growth in athymic mice (Ross et al. 2003; Walton et al. 2004).

A great number of differentiation agents have been tested for its ability to induce neuroblastoma differentiation including forskolin, dibutyryl cyclic AMP, phorbol esters, staurosporine or 5-bromo-2'-deoxyuridine (BrdU). Among them, RA is one of the most effective at inducing differentiation. The differentiation agent BrdU is also of special relevance, being able to specifically differentiate N-type cells into the S-type phenotype (Frey et al. 1993; Jalava et al. 1993; Avraham et al. 1994).

2.5.1. BRDU as S-type Inducer

BrdU is a halogenated thymidine analog that incorporates into newly synthetized DNA of replicating cells. Thus, the measurement of its incorporation indicates the cell proliferative index. Moreover, this agent also presents anti-proliferative and pro-senescence activity in different cancer models (Suzuki et al. 2001; Levkoff et al. 2008; Ross et al. 2008; Ross et al. 2011).

In neuroblastoma cells, BrdU induces morphological and biochemical changes that were suggestive of Schwannian cell differentiation. In this regard, this thymidine analog induced a flat epithelial-like morphology and increased the expression of the S100 protein and CNPase Shwannian markers (Sugimoto et al. 1988).

In embryonic neural stem cells, BrdU stimulation promoted cell cycle exit and cells acquired a morphology reminiscent of astrocytes and induced the expression the astrocyte marker GFAP and decreased expression of the stem cell markers Nestin, Sox2 and Pax6 (Schneider & d'Adda di Fagagna 2012).

3. TUMOR SUPPRESSORS

Cancer arises because of the accumulation of mutations, which are normally somatic, although germ-line mutations are also responsible for heritable cancers. Such mutations typically affect tumor suppressors and oncogenes which govern the initiation and development of cancer (Evan & Vousden 2001). Oncogenes control diverse processes including cell proliferation, differentiation or apoptosis. They can be activated by different mechanisms that confer a selective advantage towards the progression of cancer (Stéhelin 1995). In contrast, tumor suppressors counteract the action of oncogenes. They are involved in cellular processes such as proliferation, differentiation, cell cycle, metabolism or cell death, among others. These genes, when inactivated, promote cell proliferation, genomic instability and evasion of apoptosis. The inactivation of tumor suppressor mainly results from genetic alterations (mutations) or epigenetic changes, leading to the silencing or downregulation of their expression. Therefore, a proper balance between both, oncogenes and tumor

suppressor genes, guarantees the correct cell homeostasis, whereas their deregulated function entail diseases such as cancer (Lowe et al. 2004; Sherr 2004; Yang & Karin 2014).

Based on their biological function, tumor suppressors are classified as caretakers and gatekeepers. Caretaker genes protect cells from genomic alterations that may cause genomic instability. Their impaired function can increase the susceptibility towards more alterations and, as a final step, can drive tumorigenesis. On the other hand, gatekeepers inactivate or remove already damaged cells by promoting cell death or cellular senescence responses. In this regard, their functional failure fosters tumorigenesis (Kirkwood & Austad 2000; Krtolica & Campisi 2002; Sherr 2004).

Knudson postulated that tumor suppressor genes require the inactivation of both alleles to drive tumorogenesis in the so-called two-hit model. From that standpoint, loss of one allele causes susceptibility whereas loss of both induces cancer. A prototypical example of the two-hit hypothesis is the Rb gene. However, alternative mechanisms have been subsequently described. The haploinsufficiency concept implies that the loss of one allele is sufficient to induce cancer, and is best exemplified by the p53 tumor suppressor gene. On the other hand, a recent model defines obligate happloinsufficiency when the partial loss (haploinsufficiency) is more tumorigenic than the complete loss of both alleles, being the case of the tumor suppressor PTEN (Knudson & G 1971; Paige 2003; Berger et al. 2011; Song et al. 2012).

3.1. DNA METHYLATION

Activation of oncogenes and inactivation of tumor suppressors can also arise from epigenetic mechanisms including acetylation, DNA methylation, chromatin remodeling or microRNAs (Peltomäki 2012). Among them, DNA methylation is a well-known epigenetic modification associated with transcriptional silencing of tumor suppressor genes. It involves the addition of a methyl group to the 5’ of a cytosine residue preferentially at cytosine and guanine (CpG) dinucleotides, which is catalyzed by DNAmethyltransferases (DNMTs) (Garinis et al. 2002). In non-malignant cells, most human CpG are methylated but this event is rare in the CpG islands (regions of DNA with CG content higher than 50%). On the contrary, cancer cells often exhibit an aberrant pattern of methylation characterized by a global hypomethylated pattern and hypermethylation of CpG islands located in the promoter regions of several genes. Interestingly, most of these hypermethylated genes are indeed tumor suppressor genes. Based on the two-hit hypothesis, it was postulated that epimutations such as hypermethylation may act as a one of the cancer-predisposing hits (Ghosh et al. 2010; Baylin & Jones 2011)**.**

Consistently, demethylating agents such as azacytidine, 5-Aza-2’-Deoxycytidine (5-Aza-dC) and decitabine have been employed for the reactivation of tumor suppressor genes, yielding successful results in hematologic malignancies (Wongtrakoongate 2015).

4. LATEXIN

Among the wide repertoire of tumor suppressor genes reported, latexin is a recently discovered and poorly known putative tumor suppressor gene, whose mechanism of action remains elusive.

4.1. LATEXIN IN CANCER

As aforementioned, epigenetic events such as hypermethylation represent common hallmarks of cancer, leading to the inactivation of several tumor suppressor genes. Accordingly, there exists a correlation between promoter hypermethylation at CpG sites and decreased latexin expression in several cancer cell lines. Latexin silencing by promoter hypermethylation is evidenced in medulloblastoma cell lines (Anderton et al. 2008), gastric carcinomas (Yong Li et al. 2011), melanoma (Muthusamy et al. 2013), prostate cancer (Kloth et al. 2012), and hepatocellular carcinoma (Ni et al. 2014). Concomitant hypermethylation of both, latexin and its familial related gene Retinoid Acid Receptor Responder 1 (RARRES1), occurs in prostate cancer, as well (Yong Li et al. 2011).

In line with this idea, a wide body of evidence suggests that the levels of latexin expression significantly influence cancer outcome, supporting its putative role as a tumor suppressor. In melanoma cell lines, latexin dowregulation could be reverted by the demethylating agent 5-aza-dC. In addition, ectopic expression of latexin resulted in slower tumor growth, decreased colony formation ability and reduced size of melanoma spheres (Muthusamy et al. 2006; Muthusamy et al. 2013). The relationship between latexin expression and tumor development has also been observed in gastric cell lines, in such a way that, while normal human gastric cell lines exhibited elevated levels of latexin expression, notably decreased levels were found in their malignant counterparts. In this study, the expression of this protein attenuated colony formation ability and restrained tumor growth in nude mice. (Yong Li et al. 2011). Hematopoietic cells were also employed to ascertain latexin involvement on related cancers showing that its ectopic expression in mouse BALB/c-derived A20 B lymphoma cell line strikingly reduced cancerous cell growth and tumor size. Moreover, latexin was absent or strongly downregulated at both mRNA and protein levels in lymphoma, leukemia and primary cells from patients, while being highly expressed in non-tumoral hematopoietic cells (Liu et al. 2012). Further evidence comes from hepatocellular carcinoma models, where the overexpression of latexin resulted in a decline in cell proliferation and colony formation while its knockdown promoted this activity. Latexin expression also influenced cell cycle since its overexpression induced arrest at G0/G1 phase but the knockdown subsequently resulted in a cell cycle transition from G0/G1 to S phase (Ni et al. 2014).

Interestingly, the study conducted by Oldridge and colleagues highlighted that both, latexin and RARRES1, are RA-responsive genes. Likewise, the expression of both genes was substantially lower in stem cells benign prostatic hyperplasia than in more differentiated cells (Oldridge et al. 2013). In agreement with this, a

proteomic study succeeded in identifying latexin as one of the main upregulated proteins in response to RA treatment in breast cancer cells (Kamal et al. 2014).

Latexin was also demonstrated to reduce cell growth in radio- and chemotherapy-injured cells in a murine leukemogenic cell line. In this model, latexin hindered DNA repair machinery by decreasing ATR and phosphorylated Chk1. Cells with stable latexin overexpression presented a higher percentage of aberrant chromosomes leading to cell cycle G2/M arrest (You et al. 2014).

4.1. LATEXIN IN BRAIN

Latexin was initially identified in the lateral neocortex of the rat nervous system (Arimatsu, Kojima, et al. 1999). It was primarily described as an antigen detected in a subset of cortical neurons and its expression was reported to increase gradually over the course of the development (Arimatsu et al. 1992; Arimatsu et al. 2003). These findings suggest a significant involvement of latexin in cellular processes associated to postnatal development.

Latexin-positive neurons are restricted to layer VI and, to a lesser extent, to layer V of lateral neocortical areas. Thus, latexin immunoreactivity in neurons exhibits a regional and laminar pattern of distribution, with high expression in cortical regions, moderate in laterodorsal cortical regions, and low in dorsal cortical regions (Arimatsu et al. 2009). These latexin-positive neurons present pyramidal-like morphologies, are glutatame-immunoreactive and exhibit long-projection neurons (Arimatsu, Ishida, et al. 1999).

4.2. OTHER FUNCTIONS OF LATEXIN

Besides its putative role as a tumor suppressor in a variety of cancers and its involvement in neuronal development, latexin has benn associated to other cellular functions. This protein has been reported to promote BMP-2-driven chondrocyte differentiation, and its expression increases dramatically during bone fracture (Kadouchi et al. 2009). Most notably, compelling evidence suggest that latexin functions as negative regulator of stem cell numbers in the hematopoietic system. In this elegant study, two mice strains significantly differing in the stem cell numbers were compared, with the aim to identify a quantitative trait loci associated to this process. They discovered a region associated to the regulation of stem cell numbers in the chromosome 3, which corresponded to the latexin gene. In addition, latexin overexpression resulted in a decrease of HSC stem cell numbers in young mice. Furthermore, the analysis of latexin-knockout mice unveiled an attenuated expression of N-cadherin, Tie2, and Roundabout 4 (Robo4), which are proteins governing the interaction of HSCs with the bone marrow niche (Mitsunaga et al. 2012).

Latexin is also highly expressed in macrophage-enriched tissues, being upregulated upon lipopolysaccharide (LPS) stimulation, which suggests a potential role in inflammation (Aagaard et al. 2005). Furthermore, latexin-deficient mice exhibited reduced sensitivity to thermal pain (Jin et al. 2006).

4.3. RARRES1/TIG1

The only gene with a significant sequence homology to latexin known to date is the RARRES1, exhibiting around 30% identity. Both genes are located in the chromosome 3, thus suggesting they could be genetically or functionally related genes. RARRES1, also known as Tazarotene-Induced Gene 1 (TIG1), was originally identified as one of the genes whose expression was upregulated upon treatment with the synthetic retinoid tazarotene in human skin raft cultures (Nagpal 2004). Further studies demonstrated that RARRES1 is RA-responsive gene (Oldridge et al. 2013) and that was also regulated by pro-differentiation agent 1,25-dihydroxyvitamin D3 (Wood et al. 2004). RARRES1 appears silenced through promoter hypermethylation in several tumors and cell lines such as prostate, endometrial, and head and neck cancers. Consistently, increased expression of RARRES1 reduces invasiveness and tumor size in nasopharyngeal carcinoma cells and prostate cells. Therefore, RARRES1 appears as a potential tumor suppressor gene. In spite of that, RARRES1 behaves as an oncogene in breast cancer cells, contributing to tumor growth and invasion promotion (Youssef et al. 2004; Wang et al. 2013).

4.4. CARBOXYPEPTIDASE INHIBITORY ACTIVITY OF LATEXIN

Latexin was initially identified in the rat brain because of its carboxypeptidase (CP) inhibitory activity. It was purified from cerebral rat homogenates and subsequently cloned. However, its expression is not merely restricted to the brain, being also detected in colon, stomach, pancreas and lung tissues (Normant et al. 1995).

The human latexin protein consists of 222 amino acids, and has a molecular weight of 29 kDa. It represents the only endogenous protein known with inhibitory CP activity, its size is considerably larger than that of any other carboxypeptidase inhibitor (CPi) and it does not share sequence homology with any of them. The crystal structure of human latexin in complex with human carboxypeptidase A4 (hCPA4) revealed that the latexin mechanism of inhibition is far different from that of other CPis. While in the rest of CPis their polypeptide termini establishes interactions with and within the active site cleft of the CP, latexin forms a complex with the enzyme that occludes the entrance to the catalytic site. This complex consists of a large interaction surface that is solely maintained by specific contacts between the molecular surfaces involving residues outside the active site, without any specific interaction with catalytic or ligand-binding residues. Recombinant latexin inhibits hCPA4 in a non-competitive fashion and in the nanomolar range. The resolved complex with hCPA4 also showed that latexin structure comprises two topologically equivalent subdomains with a high structural homology to the cysteine protease inhibitor cystatin, each of them being composed of a twisted β-sheet that clasps an α-helix. The two subdomains pack against each other through their extended α-helix, and are also connected by a third helix that links both β-sheets (Arolas et al. 2005; Pallarès et al. 2005).

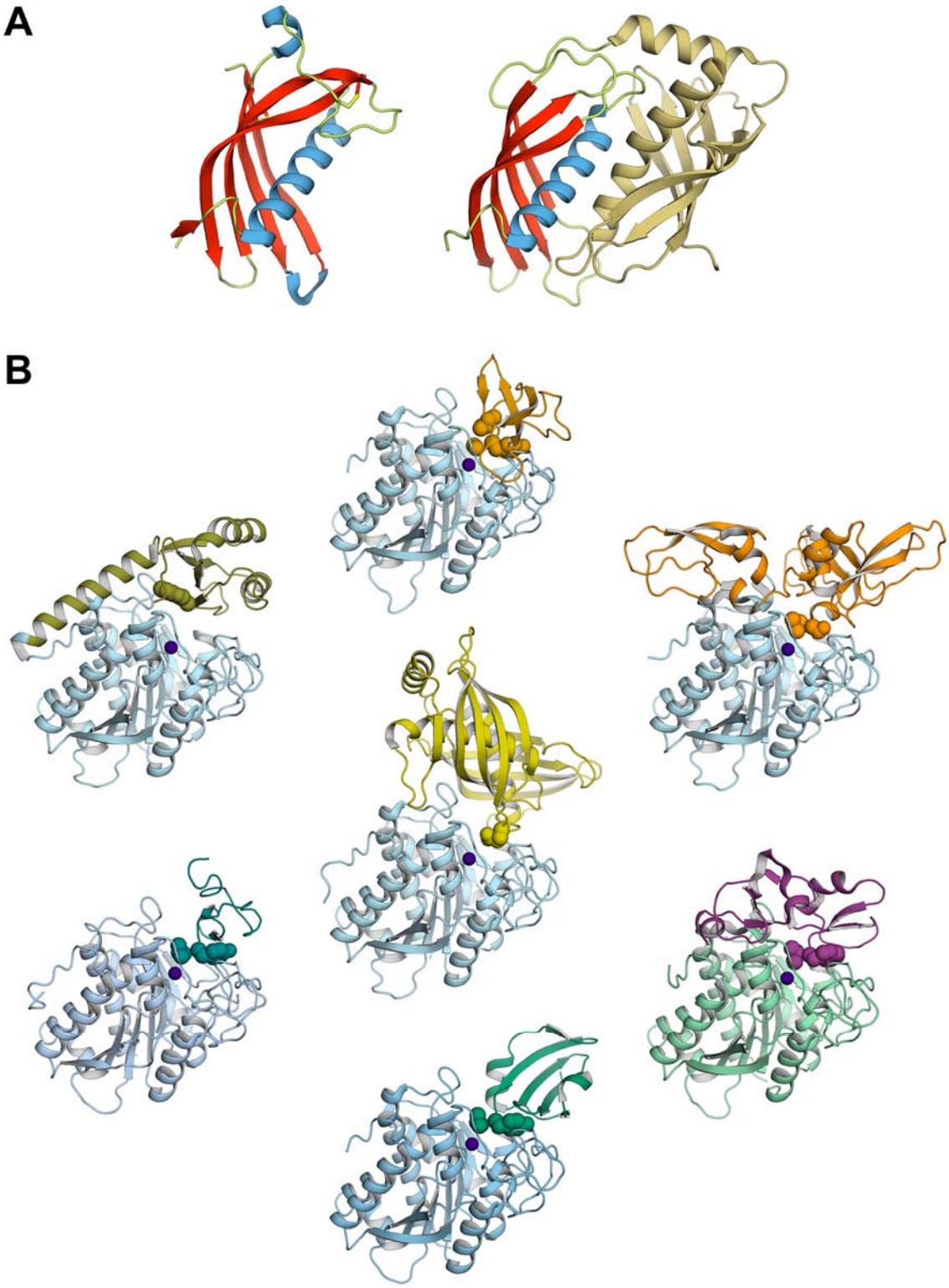

Figure 11. Latexin structural features and mechanism of carboxypeptidase inhibition. *A,* Human latexin is composed of two identical domains which adopt the α+ β cystatin-like fold: the canonical cystatin-like fold populated by the human V83N Cystatin-C variant (left, PDB 3nx0) consists of an antiparallel β-sheet bending to wrap an extended α-helix; the first cystatin-like domain of latexin (right, PDB 2bo9) is highlighted, while the connecting α-helix and the second domain are shown in sand color**.** α-helical secondary structure is shown in blue, β-strands in red and unstructured regions in green; disulfide bonds are depicted as yellow sticks. *B*, latexin inhibits carboxypeptidase activity by occluding the access of polypeptide substrates to the active site cleft, similarly as the carboxypeptidase propeptide autoinhibits the enzyme before its activation through the proteolytic cleavage of this region: complex

(center, PDB 2bo9) of human Carboxypeptidase A4 (cyan) and human latexin (gold) and structure of human proCarboxypeptidase A4 (top-left, PDB 2boa) with its activation propeptide highlighted (olive). Conversely, proteinaceous carboxypeptidase inhibitors more commonly act by protruding into the carboxypeptidase catalytic pocket: complex (top-middle, PDB 4a94) of human Carboxypeptidase A4 (cyan) with Nerita versicolor carboxypeptidase inhibitor (ochre), complex (top-right, PDB 4bd9) of human Carboxypeptidase A4 (cyan) with Sabellastarte magnifica carboxypeptidase inhibitor (orange), complex (bottom-left, PDB 4cpa) of bovine Carboxypeptidase A1 (light blue) with Potato carboxypeptidase inhibitor (bluish green), complex (bottom-middle, PDB 1dtd) of human Carboxypeptidase A2 (blue) with Leech carboxypeptidase inhibitor (green), and complex (bottom-right, PDB 1zli) of human Carboxypeptidase B (light green) with Tick carboxypeptidase inhibitor (purple). The catalytic zinc ion complexed by carboxypeptidase is represented as a dark violet sphere. Residue side chains of the carboxypeptidase inhibitors, or the carboxypeptidase propeptide, with atoms within 8Å of the catalytic zinc atom are shown as spheres.

5. METALLOCARBOXYPEPTIDASES

Carboxypeptidases (CPs) are exopeptidases that catalyze the hydrolysis of the last peptide bond at the C-terminus of peptides and proteins. They are broadly distributed in the organism and perform a wide repertoire of functions and may display different catalytic mechanisms. Metallocarboxypeptidases (MCPs) are the largest family of CPs, all of them containing a coordinated divalent cation in their active site necessary to catalyze the hydrolysis of peptide bonds. They are classified under clan MC, family M14 and subfamilies M14A, B, C and D according to the MEROPS classification (Rawlings et al. 2014). The most widely studied subfamily M14A comprises a wide number of variants with different specificities and cellular localizations including CPA1, CPA2, CPA3 (mast cell carboxypeptidase), CPA4, CPA5, CPA6, CPB, CPU (known as thrombin-activatable fibrinolysis inhibitor or TAFI). These enzymes are synthesized in the form of zymogens shaped by a catalytic domain and a massive, about 100 residues long, activation domain which is lost by limited proteolysis upon activation (Vendrell et al. 2000). Most of the enzymes belonging to this subfamily are only activated after secretion, although there are some exceptions. Concretely, CPA6 is activated in the secretory pathway, being secreted as an active enzyme that bounds to the ECM (Lyons et al. 2008; Lyons et al. 2010; Sapio & Fricker 2014).

Table 6. Classification of Carboxypeptidases.

CATALYTIC TYPE	MEROPS FAMILY	NAME
	M2	ACE2
		CPA1
		CPA2
		CPA3
		CPA4
	M14 subfamily A	CPA5

		CPA6
		CPB
		CPU or TAFI
		CPO
		CPE
		CPD
		CPM
METALLO	**M14 subfamily B**	CPN
		CPZ
		CPX1
		CPX2
	M14 subfamily C	Gamma-D-glutammyl-(L)-meso-diaminopimelate peptidase I
	M15 D	Zinc D-Ala-D-Ala carboxypeptidase
	M20 subfamily A	Glutamate carboxypeptidase
	M20 subfamily D	Carboxypeptidase Ss1
	M28 subfamily B	Glutamate carboxypeptidase II NAALADASE L peptidase
	M32	Carboxypeptidase taq TcCP1
		TcCP2
SERINE	**S10**	Serine carboxypeptidase A Vitellogenic carboxypeptidase-like RISC peptidase
	S28	Lysosomal Pro-X carboxypeptidase
CYSTEINE	**C1 subfamily A**	Cathepsin X

Adapted from (Petrera et al. 2014; Rawlings et al. 2014).

5.1. CPA4

This enzyme is a prototypical MCP enzyme and also the one whose structure was resolved in complex with latexin. This enzyme functions as an extracellular peptidase being activated only upon secretion. In contrast with cystatin, which inhibits cysteine proteases extracellularly, latexin does not seem to be secreted to the extracellular space and neither presents a membrane specific signal peptide sequence (Tanco et al. 2010). In addition, latexin only inhibits CPA4 in its mature form, thus considerably limiting the possibility of a physiological interaction between them. Indeed, there are no studies disclosing the interaction between CPA4 and latexin under physiological conditions.

CPA4 was found to be widely expressed in different tissues including placenta, testis, uterus, heart, brain, intestine, kidney, and muscle. In the rat brain, mRNA levels of this enzyme are detected at the olfactory bulb, specifically in the granular and mitral layers. CPA4 was initially discovered as one of the upregulated genes in response to the differentiation agent sodium butyrate in prostate cancer cells and its expression is also induced by the histone deacetylase inhibitor trichostatin A (Cells et al. 1999; Kayashima et al. 2003).

This enzyme participates in the processing of neuropeptides such as neurotensin, granins like chromogranin A and B and secretogranin II, and opioid peptides. Furthermore, CPA4 also takes part in the inactivation of peptides involved in cell proliferation and in aggressiveness of prostate cancer (Tanco et al. 2010).

II. OBJECTIVES

1. OBJECTIVES

The Chapters that compose the present thesis all share a common purpose, namely unraveling the biological role of latexin, a recently discovered RA-responsive and putative tumor suppressor gene. Because the potential involvement of latexin in cancer has been largely supported by a growing number of studies but, at the same time, the precise mechanism of action of latexin in malignancies remains obscure, we focused on tumor-derived cell lines to discern the functional role of latexin. The work presented in this thesis comprises a structure that ranges from a general screen of cellular processes latexin could be implicated in towards deeper and more specific understanding of the functional role of this protein. The insights extracted from each Chapter give rise to a comprehensive characterization of the biological role of latexin in different cellular models. The purpose of this thesis is addressed by the following objectives:

1. To explore the potential involvement of latexin in archetypical cellular processes that are deregulated in cancer namely cell proliferation, cell death or cell differentiation in human neuroblastoma-derived SH-SY5Y cells. For this purpose, the following specific objectives were set out:

   To analyze whether the overexpression of latexin could influence cell proliferation in SH-SY5Y cells.

   To elucidate if enhanced expression of latexin could modulate cell death response in SH-SY5Y cells.

   To study the potential link between latexin expression and RA-promoted differentiation in SH-SY5Y cells.

2. To characterize the specific cellular outcome that the overexpression of latexin promotes in RA-induced SH-SY5Y cells and the specific pathways behind this effect. The specific objectives were:

   To analyze the potential alteration of neuroblastoma cell lineage markers by latexin overexpression.

   To explore signaling pathways potentially associated with latexin-mediated effects.

   To assess the final cellular outcome promoted by latexin upon RA induction.

   To extend the results by employing other differentiation agents.

3. To explore the molecular landscape of gene expression responsible for latexin effects in RA-induced SH-SY5Y cells. This purpose involved the following objectives:

To characterize the gene expression profile of cells overexpressing latexin

To assess the transcriptome that RA promotes in cells with enhanced expression of latexin.

4. To extend our results to other cellular paradigms. The main objectives supporting this purpose were:

To study whether the observed behavior could be extended to different neuroblastoma cells.

To extrapolate, if possible, these results to a different cellular model, namely human-derived glioblastoma multiforme cells.

To analyze whether the expression of latexin could be translated in differences in overall patient survival in both neuroblastoma and glioblastoma cellular models by exploiting publicly available tumor datasets.

III. MATERIALS AND METHODS

1. REAGENTS

All chemicals were obtained from Sigma-Aldrich unless otherwise indicated. PI-103 (528100) and U0126 (662005) inhibitors were purchased from Merck Millipore. MK-2206 inhibitor (S1078) was obtained from Selleckchem. GSK2334470 (SML0217) inhibitor, Fibronectin (F0895), Laminin (L2020) and Collagen (C8919) were purchased from Sigma-Aldrich. Mitotracker Red CMXRos was obtained from Thermofisher (M7512).

2. CELL LINES AND CULTURE PROCEDURES

All human neuroblastoma-derived cells employed (LAN-1, SH-SY5Y, SK-N-AS, SK-N-BE, SK-N-LP, SK-N-SH), human GBM-derived cell lines (A172, LN-18, LN-229, U87-MG, U373) and the cervical carinoma Hela cell line were routinely grown in 100-mm culture dishes (BD Biosciences) containing 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin/streptomycin (100 units/ml and 100 µg/ml, respectively) and 10% of heat-inactivated fetal bovine serum (FBS). Cells were maintained at 37°C in a saturating humidity atmosphere containing 95% air and 5% CO_2. For the different experiments, cells were grown at the adequate cell densities in culture dishes or multiwell plates (BD Biosciences).

3. CELL DEATH ASSAYS

3.1. LDH RELEASE

Extracellular lactate dehydrogenase (LDH) measurements were performed by a modified protocol from Decker and Lohmann-Matthes (Decker & Lohmann-Matthes 1988). Cells were seeded in a 96-multiwell plate at an initial density of 3.5×10^5 cells/ml the day before treatment. Afterwards, cells were treated with the indicated concentrations of staurosporine (STP). After 24 hours, 20 µl of lactate solution (14.38 mg/ml in 10 mm Tris buffer, pH 8.5) was added, followed by the addition of 20 µl of INT solution (0.9 mg/ml INT in PBS prepared from a 20 mg/ml stock solution in dimethyl sulfoxide (DMSO)). The enzymatic reaction was started by addition of 20 µl of a PBS solution containing NAD+ (1.65 mg/ml), diaphorase (7·10−7 units/ml), BSA (0.015%), and sucrose (0.6%) and allowed to proceed for 20 min. The reaction was stopped by adding 20 µl of the LDH inhibitor oxamate (5.6 mg/ml in PBS). Absorbance was measured at 490 nm in a BIO-TEK FL800 fluorometer (Izasa). The percentage of LDH activity in the culture medium was calculated considering that the LDH activity obtained from non-treated cells permeabilized with a solution containing 1% Triton X-100 represented 100% of LDH activity (Sánchez-Osuna et al. 2014).

4. CELL SURVIVAL ASSAYS

4.1. MTT REDUCTION

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) is a water-soluble tetrazolium salt which can be reduced by metabolically viable cells to give a colored water-insoluble formazan salt. Cells were seeded in 96-well plates at an initial density of 3.5×10^5 cells/ml. The day after, cells were treated and MTT was performed. MTT was added at a final concentration of 0.5 mg/mL to the culture medium. Plates were incubated at 37 °C for 30 minutes and MTT-containing medium was replaced with 100 µL DMSO plus 0.05% triton X-100. Formazan salts are allowed to dissolve by shaking for 10 minutes at room temperature. Absorbance was quantified by BIO-TEK Power Wave XS. Final values were calculated as the result of subtracting 620 nm from 590 nm lectures. Data is represented as the means of the percentage of viability ±SEM being 100% the viability of untreated cells (adapted from (Boix et al. 1997)).

4.2. TRYPAN BLUE EXCLUSION ASSAY

This assay is based on the principle that live cells possessing intact cell membranes can exclude certain dyes, such as trypan blue, whereas dead cells do not. Cells were seeded at initial density of 3.5×10^5 cells/ml and treated with the staurosporine (STP) for the specified times. Cells were detached and dissolved in PBS and mixed with trypan blue solution (final concentration 0.4%). Then, 10 µl of the resulting mixture were counted using a hemocytometer. Cell death is expressed as percentages of trypan-blue stained over the total number of cells (Gozzelino et al. 2008).

5. DIFFERENTIATION METHODS

For differentiation assays, SH-SY5Y cells were seeded at an initial density of 10^4 cells/cm^2 , SK-N-LP cells at an initial density of 10^4 cells/cm^2 , LN-18 cells at an initial density of 7×10^3 cells/cm^2 and U87MG cells at an initial density of 5×10^3 in multiwell plates (BD Biosciences) in DMEM supplemented with 10% FBS. Forty-eight hours after seeding, all-*trans*-retinoic acid (RA) at a final concentration of 10 µM or at the indicated concentrations, 5-Bromo-2'-deoxyuridine (BrdU) at a final concentration of 10 µM, doxorubicin at a final concentration of 0.25 µM or berberine at a final concentration of 10 µM were added to the cell culture media in DMEM supplemented with 15% FBS.

For optimization of culture media conditions, SH-SY5Y cells were seeded at an initial density of 10^4 cells/cm^2 in Neurobasal Medium (Gibco) supplemented with 2% B27 (Gibco), DMEM supplemented with 0% FBS, DMEM supplemented with 1% FBS or DMEM supplemented with 15% FBS for the indicated times. Forty-eight hours after seeding, RA was added at a final concentration of 10 µM.

For the preparation of coating surfaces, fibronectin, laminin and collagen were thawed at 4°C to prevent the formation of a gel. The solutions were then diluted in PBS to a final concentration of 10 µg/mL and incubated for 2 hours at room temperature. Afterwards, the solutions were aspirated carefully and the plates were washed 3 times with PBS. Plates were used immediately or stored at 4°C for up to one month (Timpl et al. 1979). Cells were seeded onto coated plates as described before.

For inhibition assays, SH-SY5Y cells and SK-N-LP cells were seeded at an initial density of 10^4 cells/cm^2 in multiwell plates (BD Biosciences) in DMEM supplemented with 10% FBS. Forty-eight hours after seeding, RA or BrdU were added to the cell culture media at a final concentration of 10 µM in DMEM supplemented with 15% FBS. Cells were pretreated for 1 h with U0126, PI-103, GSK2334470 or MK-2206 before treated with RA or BrdU.

All the different compounds were dissolved in DMSO. The final DMSO concentration never exceeded 0.05% to avoid unspecific effects in either neurite outgrowth or cell survival.

6. SENESCENCE METHODS

6.1. EDU ASSAY

The 5-Ethynyl-2′-deoxyuridine (EdU) incorporation assay was performed as previously described (Salic & Mitchison 2008), with minor modifications. This method is based on the detection of labeled DNA precursors whose incorporation indicates the rate of cell proliferation. Traditional assays to measure the level of DNA synthesis were based on the incorporation of nucleotide analogs such as [^{3}H] thymidine or 5-bromo-2'-deoxyuridine (BrdU) which are usually detected by autoradiography or using specific BrdU antibodies, respectively. These methods present several disadvantages such as is time consumption and complex handling, in the case of autoradiography, or the strong denaturing conditions needed to expose BrdU epitopes. EdU is thymidine analogue bearing a terminal alkyne group, which is incorporated into DNA during the S phase of the cell cycle. This terminal alkyne group can be detected via reacting with fluorescent azides, in a Cu(I)-catalyzed reaction (Rostovtsev et al. 2002). Accordingly, the size of the fluorescent azide results in its rapid penetration without the requirement of the denaturing step (Itahana et al. 2007).

Briefly, cells were seeded in 24-multiwell plates (2 x 10^4 cells/well), incubated at 37°C for 48 h and treated with the specific drugs. Cells were then incubated with 10 µM EdU in complete growth media at 37°C for 24 h. Afterwards, cells were rinsed twice with PBS and fixed using 2% paraformaldehyde EM grade (Electron Microscopy Sciences (EMS)) at 4ºC for 24 h. Cells were washed twice with PBS and 1 µM Azide-fluor 488 (Sigma-Aldrich) was added in a reaction buffer containing 100 mM Tris-HCl, pH 8.5, 1 mM $CuSO_4$ and 50 mM ascorbic acid. After 4 h, cells were washed three times with PBS containing 0.5% triton and then counterstained with 0.05 µg/ml Hoechst 33258 for 30 min at room temperature (Gozzelino et al. 2008). Cell nuclei were visualized with a Nikon ECLIPSE TE2000-E microscope equipped with epifluorescence optics

under UV illumination and photographed with a Hamamatsu ORCA-ER photographic camera (software Metamorph).

6.2. SA-β-GAL ASSAY

The senescence-associated β-gal (SA-β-gal) assay measures the residual activity of β-galactosidase at suboptimal pH of 6.0 resulting from the raise in the β-galactosidase activity that occurs during cellular senescence. This assay is based on the detection of the blue color developed after cleavage of the chromogenic substrate 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal) by β-galactosidase (Debacq-Chainiaux et al. 2009).

Although SA-β-gal is the most extensive used method to characterize cellular senescence, it presents some drawbacks associated with the positivity observed in some experimental settings such as confluent conditions (Dimri et al. 1995). For this reason, this assay should be carried out on sub-confluent cell culture conditions and corroborated by alternative assays or markers of cellular senescence.

Cells were seeded in 24-multiwell plates (2 x 10^4 cells/well), incubated at 37°C for 48 hours and treated with the specific drugs. After 15 days, cells were washed with PBS and then fixed using 2% fomaldehyde and 0.2% glutaraldehyde in PBS, for 5 minutes at room temperature. Fixation time was optimized since longer times interfered with the development of blue color. It is reported that longer fixation times can destroy the enzymatic activity of β-galactosidase, thus hindering its detection (Debacq-Chainiaux et al. 2009). Under our experimental settings, fixation during 5 minutes was enough for SA-β-gal detection. Cytochemical detection of SA-β-gal was performed using the Senescence β-galactosidase Staining Kit (Cell Signaling Technology). After fixation, cells were washed 3 times with PBS and then incubated with the freshly prepared staining solution composed of 5mM potassium ferrocyanide, 5mMpotassium ferricyanide, 2 mM magnesium chloride, 150 mM sodium chloride, 40 mM citric acid/phosphate buffer (pH 6) with 1 mg/ml Xgal for 12 hours at 37°C without CO_2. SA-β-gal-positive cells were visualized under a Nikon ECLIPSE TE2000-E microscope equipped with epifluorescence optics under UV illumination and photographed with a Nikon DS-2Mv color camera (software NIS-Elements). SA-β-gal positive cells were determined by counting the number of blue cells over the total population.

6.3. LIVE-CELL MITOCHONDRIA IMAGING

SH-SY5Y cells were seeded in 35-mm culture dishes (9, 5 x 10^4 cells/well), incubated at 37°C for 48 hours and treated with the specific drugs. After the indicated time, cells were incubated with 50 nM of MitoTracker Red for 15 minutes. Images were captured using a laser confocal microscope (Zeiss LSM 700, Carl Zeiss, Oberkochen, Germany).

7. CELL TRANSFECTION

7.1. LATEXIN CLONING AND TRANSFECTION

The human latexin cDNA was amplified from pGAT2-LXN vector (Pallarès et al. 2005) by PCR. The amplified product was verified by automated sequencing at the "Servei de Genòmica i Bioinformàtica" (Universitat Autònoma de Barcelona). Latexin insert was subcloned under the control of a CMV constitutive promoter in the pcDNA3 expression vector. Then, SH-SY5Y cells were transfected using the Lipofectamine LTX & PLUS transfection reagents according to manufacturer's instructions. To obtain stably transfected cells, 24 h after transfection, cells were grown in selective medium containing geneticine (G-418): 0.5 mg/ml for SH-SY5Y cells, 0.7 mg/ml for SK-N-LP cells or 1 mg/ml for LN-18 cells, until non-transfected cells died in the selective medium.

7.2. siRNA TRANSFECTION

U87-MG cells were seeded at an initial density of 5 x 10^4 cells/cm^2 in DMEM supplemented with 10% FBS. After 24 hours, small interfering RNA (siRNA) transfection was performed by using the DharmaFECT 1siRNA transfection Reagent (Dharmacon) according to manufacturer's instructions. Briefly, siRNAs were incubated for 5 minutes in DMEM 0% FBS. Next, siRNA suspensions were mixed with DharmaFECT 1 reagent in DMEM 0% FBS to achieve a final siRNA concentration of 35 nM and subsequently incubated for 20 minutes. The resulting mixtures were then added to cell culture wells. After 5-6 hours, the appropriate FBS volume was added to achieve a final 10% concentration of FBS in culture. The following siRNA sequences were employed:

Table 7. siRNA sequences

siRNA	Sequence
Non-rellevant	5'-AUAUGCGAUCGAGAUAUCG-3'
Latexin	5'-GCACAAAAUAUUCCAGACA-3'
Latexin_1	5'-GAGAUUAUUCCCUGGCAAA-3

8. PROTEIN EXTRACTION AND WESTERN BLOT

Cells were detached, pelleted at 500 x *g* for 5 min and washed once with PBS. Then, cells were lysed with Igepal buffer (50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 150 mM NaCl, 1% Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride (PMSF)) incubated on ice for 10 minutes and then centrifuged for 5 min at 16,000 x *g* at 4 °C. The protein concentration was quantified by a modified Lowry assay (DC protein assay, Bio-Rad). Protein extracts were loaded in SDS-polyacrylamide gels. The proteins were electrophoresed and

electrotransferred onto Protran nitrocellulose transfer membrane (Whatman GmbH). After blocking with Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% nonfat dry milk, the membranes were probed with the specific primary antibodies for 1 hour at room temperature or overnight at 4 °C, and incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. Finally, detection was performed using Clarity western ECL substrate kit (Bio-Rad).

Table 8. List of antibodies used in this thesis.

Antibody	Reference	Dilution	Producer Host	Supplier
Latexin		1:5000	Rabbit	*Homemade**
p-Akt Ser473	4060	1:1000	Rabbit	Cell Signaling
p-Akt Thr308	13038	1:1000	Rabbit	Cell Signaling
Akt (total)	9272	1: 1000	Rabbit	Cell Signaling
p-ERK 1/2	9101	1:1000	Rabbit	Cell Signaling
ERK 1/2 (total)	9102	1:1000	Rabbit	Cell Signaling
p-PRAS40	2997	1:1000	Rabbit	Cell signaling
PRAS40 (total)	05-988	1:1000	Mouse	Merck Millipore
Id1	sc-488	1:2000	Rabbit	Santa Cruz
NSE	10171	1:5000	Mouse	Abcam
Vimentin	V6389	1:5000	Mouse	Sigma
β-actin	5316	1:20,000		
Mouse HRP conjugated	A9044	1:20,000	Rabbit	Sigma
Rabbit HRP conjugated	A0545	1:20,000	goat	Sigma

*The polyclonal antibody against latexin was produced at the "Servei de Producció d'Anticossos" (Universitat Autonòma de Barcelona) by immunizing rabbits with the recombinantly obtained full-length protein. Antibodies were purified by affinity chromatography on a Protein A-Sepharose resin (GE Healthcare, Barcelona, Spain). Bound antibodies were eluted with Glycine-HCl, pH 2.7. Protein concentrations were determined with the BCA Protein Assay Kit (Pierce, Barcelona, Spain) and the specificity and selectivity was probed by *Western blotting*.

9. CELL PROLIFERATION ASSAY

SH-SY5Y cells were seeded at an initial density of 2 x 10^4 cells/well in 24-multiwell plates. Cells were detached at the indicated times and counted by the Scepter ^{TM} Handheld Automated cell counter.

10. CYTOTOXIC CELL TREATMENTS

SH-SY5Y cells were seeded at an initial density of 3.5 × 10^5 cells/ml the day before treatment. Cells were treated with the following cytotoxic drugs: staurosporine (STP) (1 μM or from 1 nM to 1000 nM), camptothecin (CPT) (20 μM), etoposide (100 μM), oxaliplatin (100 μM), MG132 (25 μM), paclitaxel (50 μM), cisplatin (50 μM), doxorubicin (100 μM), irinotecan (500 μM).

11. CELL DETACHMENT PROTOCOL

N-type cells were removed on the basis of their differential substrate adherence compared with S-type cells (Piacentini et al. 1996). Then, weakly adhered cells (mostly N-type cells) were knocked off with PBS by gentle agitation during 5 min (Bell et al. 2013). Adhered cells (mostly S-type cells) were fixed with 2% paraformaldehyde and stained with 0.5% crystal violet. Crystals were dissolved in 10% acetic acid and the absorbance was measured at 590 nm wavelength under a BIOTEK Powerwave HT Microplate Spectrophotometer.

12. RNAseq ANALYSIS

12.1. STRANDED RNAseq LIBRARY PREPARATION AND SEQUENCING

Libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit v2 according to the manufacturer's protocol. Briefly, 1 μg of total RNA were used for poly(A)-mRNA selection using streptavidin-coated magnetic beads and were subsequently fragmented to approximately 300bp. cDNA was synthesized using reverse transcriptase (SuperScript II, Invitrogen) and random primers. The second strand of the cDNA

incorporates dUTP in place of dTTP. Double-stranded DNA (dsDNA) was further used for library preparation and dsDNA was subjected to A-tailing and ligation of the barcoded Truseq adapters. All purification steps were performed using AMPure beads. Library amplification was performed by PCR using the primer cocktail supplied by the kit.

Final libraries were analyzed using Agilent DNA 1000 chip to estimate the quantity and check size distribution, and were then quantified by qPCR using the KAPA Library Quantification Kit (KapaBiosystems).

12.2. RNAseq ANALYSIS AND DIFFERENTIAL EXPRESSION

Quality of the raw sequencing data was checked using the FastQC tool (S. Andrews: FastQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Mapping of the raw data to the Human genome (Ensembl Homo sapiens release 75) was performed using STAR (Dobin et al. 2013), followed by the quantification of raw reads per gene using htseq-count (Anders et al. 2015). Differential expression analysis at gene-level was carried out on raw read counts using DESeq2 (Love et al. 2014) R/Bioconductor package (Huber et al. 2015), which method fits negative binomial generalized linear models for each gene and uses the Wald statistical test for significance testing across experimental conditions.

13. BIOINFORMATIC TOOLS

13.1. FUNCTIONAL ENRICHMENT ANALYSIS

Gene Ontology (GO): is a defined and well-established specific vocabulary designed to provide information about a gene or gene product (Ashburner et al. 2000). GOs can be classified into 3 classes:

Biological Process: including chemical or physical transformation that is attributed to a gene. They can display different levels of specificity.

Molecular Function is related to a biochemical activity performed by a gene or gene product.

Cell Component is the cellular place where the gene perform its action.

For GO annotations, we employed the Database for Annotation, Visualization and Integrated Discovery (DAVID). DAVID is a functional annotation tool for large gene lists from high-throughput experiments. This webserver associates lists of genes to GO terms, indicating the most statistically over-represented (enriched) (Huang et al. 2007). Annotation algorithms are based on the DAVID Knowledgebase, a gene-annotation database with highly data coverage. This database integrates well-known annotation resources with improved cross-reference capability across redundant genes (Sherman et al. 2007).

The mRNAs obtained from RNA-seq analysis for each pairwise comparison were divided into up and downregulated genes to indicate the direction of the change. Therefore, gene clusters were associated to GO terms by employing the DAVID database. We used FDR < 0.05 as the cut-off to determine whether a GO term was significantly enriched.

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a bioinformatic tool that links a set of genes with cellular processes based on networks of interacting molecules. KEGG contains three databases: PATHWAY, GENES and LIGAND. The PATHWAY database associates gene clusters to reference metabolic pathways based on their Enzyme Commission (EC) numbers (Kanehisa & Goto 2000). Gene clusters were associated to signaling pathways by employing the KEGG database. To this end, FDR < 0.05 was the cut-off for determining significantly enriched pathways.

13.2. PUBLIC WEBSITES

We employed the R2: microarray analysis and visualization platform (http://r2.amc.nl) to obtain Kaplan Meier survival curves. This platform contains information from datasets of tumor sample patients. Within each dataset, patients are sorted in two groups (high or low levels) based on a cut-off value of the expression of the gene of interest. The platform determines the optimal cut-off of expression to calculate the survival probability for each group. Survival curves associated with each group of expression are plotted together to analyze the potential differences. The graph shows the p-value corrected for the multiple testing (Bonferroni correction) of cut-off levels (Bewick et al. 2004).

14. RNA EXTRACTION AND GENE EXPRESSION VALIDATION

SH-SY5Y cells were seeded at an initial density of 10^4 cells/cm^2 in 6-multiwell plates in DMEM supplemented with 10% FBS. After 48 h, cells were treated with RA during 5 days at a final concentration of 10 µM in DMEM supplemented with 15% FBS, or left untreated. Cells were then detached, pelleted at 500 x g for 5 minutes and washed once with PBS. RNA was subsequently extracted by using the RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. This protocol is based on guanidine-isothiocyanate sample lysis.

Genes were quantified by quantitative real-time PCR (qRT-PCR). The qRT-PCR was performed by real-time PCR system using SYBR green real-time PCR mix (Bio-Rad), primers were also purchased from Bio-Rad. The PCR amplification was performed in triplicate, using the following parameters: 94°C for 2 minutes, followed by 40 cycles of 15 seconds at 94°C, 15 seconds at 60°C, and 34 s at 72°C. The quantification cycle (Cq) values were normalized according to the ΔΔCq method for calculating fold changes of mRNA levels, based on the following equation: $\Delta\Delta Cq=2^{-(\Delta Cq\ sample-\Delta Cq\ control)}$ (Pfaffl 2001).

IV. RESULTS

1. CHAPTER 1. THE ROLE OF LATEXIN IN RA-INDUCED DIFFERENTIATION IN SH-SY5Y CELLS

Cancer arises as the result of the accumulation of diverse genetic mutations in somatic cells, leading to deregulated cell growth (Evan & Vousden 2001). When cells acquire the ability to adapt and proliferate in different environments, even in unfavorable ones, cancer ultimately develops. In line with this, mutations or epimutations activating or inactivating oncogenes and tumor suppressors, respectively, result in the evasion of proliferative constraints and other barriers avoiding the spread of cellular damage (Evan & Vousden 2001; Lowe et al. 2004). In particular, tumor suppressors are genes that, when inactivated, promote cell proliferation, genomic instability and evasion of apoptosis, among other cellular responses (Berger et al. 2011). For this reason, therapeutic approaches aiming to restore the expression of tumor suppressor genes are of special relevance (Garinis et al. 2002). Among them, latexin and Retinoic Acid Receptor Responder 1 (RARRES1) are two putative tumor suppressors and RA-responsive genes belonging to the same family whose cellular functions are still scarcely known (Oldridge et al. 2013). Interestingly, both genes appear downregulated through promoter hypermethylation in several types of cancer, suggesting that they could exert a putative tumor suppressor role (Youssef et al. 2004; Ni et al. 2014). However, the precise mechanism of action of latexin and/or RARRES1 as tumor suppressor genes is largely unknown. Therefore, the aim of this study was to unveil the cellular process latexin could be involved in neuroblastoma cells.

1.1. HUMAN NEUROBLASTOMA-DERIVED CELLS LACK LATEXIN EXPRESSION

Since latexin and RARRES1 are downregulated in several cancer cells, we first screened for their protein expression in several human neuroblastoma-derived cells including LAN-1, SH-SY5Y, SK-N-AS, SK-N-BE, SK-N-LP and SK-N-SH; Hela, a cervical carcinoma cell line, where high latexin expression has been previously reported (Yong Li et al. 2011), served as a positive control of expression. Unlike RARRES1, whose expression was detectable in SH-SY5Y, SK-N-BE and SK-N-LP cells, latexin expression was commonly absent in all the cell lines screened (**Figure 12A**). Then, the lack of latexin expression appeared as a common molecular trait among neuroblastoma cells. Accordingly, we selected the human neuroblastoma-derived SH-SY5Y cell line for further experiments because it represents a canonical model of apoptosis and cell differentiation (Yuste et al. 2002; Cheung et al. 2009). Since latexin expression appears downregulated by promoter hypermethylation in several types of cancers (Muthusamy et al. 2006; Kloth et al. 2012; Liu et al. 2012; Muthusamy et al. 2013; Oldridge et al. 2013)(Muthusamy et al. 2013; Muthusamy et al. 2006; Liu et al. 2012; Kloth et al. 2012; Oldridge et al. 2013), we explored whether its protein expression could be induced by employing a demethylating agent. As shown in **Figure 12B**, SH-SY5Y cells upregulated latexin when treated with the DNA demethylating agent 5-Aza-2'-deoxycytidine (5-aza-dC). Therefore, promoter

hypermethylation seemed to be the potential underlying mechanism explaining the loss of latexin expression observed in neuroblastoma cells. We also analyzed whether latexin expression was upregulated in response to the differentiation agent retinoic acid (RA), as previously observed in other cellular models (Oldridge et al. 2013; Kamal et al. 2014). As shown in **Figure 12C**, SH-SY5Y cells upregulated latexin in response to RA. Taken altogether, latexin expression is commonly downregulated in neuroblastoma cells and such downregulation can be pharmacologically modulated. This fact largely excludes the possibility of other genetic alterations, such as mutations or translocations, being responsible of its lack of expression in neuroblastoma cells.

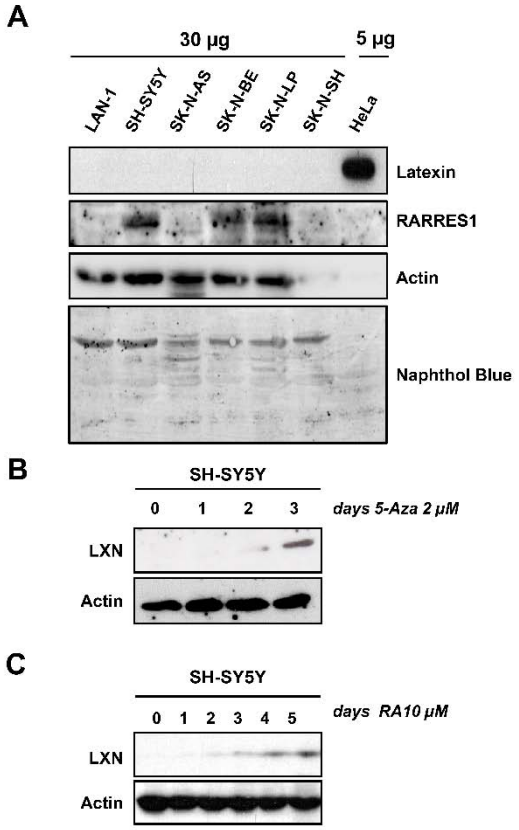

Figure 12. Latexin is commonly downregulated in human neuroblastoma-derived cell lines and can be upregulated upon RA or 5-aza-dC treatment in SH-SY5Y cells. *A,* Protein extracts from different neuroblastoma cell lines were obtained and *Western blotting* against latexin and RARRES1 were performed. Cervical carcinoma HeLa cells were used as a positive control of expression. Either 30 µg or 5 µg of protein were loaded for neuroblastoma cell lines extracts or HeLa cell line extract, respectively. The membranes were reprobed with β-actin antibody to check equal loading. *B,* Wild type SH-SY5Y cells were treated with 2 µM 5-Aza-2'-deoxycytidine (5-aza-dC) for up to 3 days and protein extracts were obtained before (0) or after treatment at the indicated times (1, 2 and 3 days). Latexin expression was analyzed by *Western blotting*. β-actin was used as loading control. *C,* Wild type SH-SY5Y cells were treated with 10 µM RA for up to 5 days and protein extracts were obtained before (0) or after treatment at the indicated times (1, 2, 3, 4, 5 days). Latexin expression was analyzed by *Western blotting*. β-actin served as loading control.

1.2. LATEXIN IS NOT DIRECTLY INVOLVED IN CELL PROLIFERATION OR CELL DEATH PROCESSES

At first, we wanted to explore if the overexpression of latexin was sufficient to alter neuroblastoma behavior, since both RA and 5-aza-dC do not specifically modulate latexin expression but also all the genes regulated by these mechanisms. To this end, SH-SY5Y cells were constitutively and stably transfected with either empty pcDNA3 or pcDNA3-latexin plasmids. First, we evaluated if latexin expression could affect cell growth or cell viability, two well-characterized hallmarks of tumor suppression. Cells transiently transfected with empty pcDNA3 or pcDNA3-latexin plasmids for up to 72 hours (**Figure 13B**) exhibited comparable percentages of cell death as detected by trypan blue exclusion assay (**Figure 13A**).

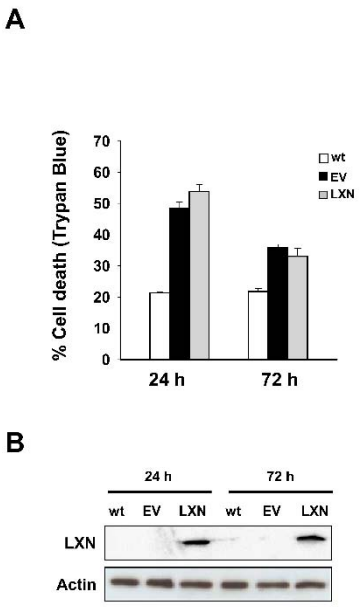

Figure 13. Cell death is not substantially altered by transiently transfection of latexin. SH-SY5Y cells were transiently transfected with empty pcDNA3 or pcDNA3-latexin plasmids for up to 72 hours. *A*, Cell death percentage was calculated for the indicated times in wild type (wt), empty vector-transfected (EV) or latexin-overexpressing (LXN) cells by trypan blue exclusion assay as detailed in *Materials and Methods* section. *B*, Latexin transient overexpression was analyzed by *Western blotting*. β-actin was used as loading control.

Therefore, stable cell lines transfected with empty vector or overexpressing latexin were employed henceforth (**Figure 14B**). Moreover, cell counts performed over 5 days in culture revealed that latexin did not have a significant effect on cell proliferation either (**Figure 14A**).

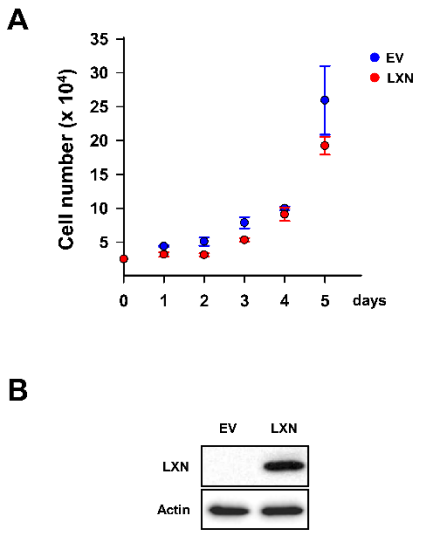

Figure 14. Stable overexpression of latexin does not significantly influence cell proliferation. SH-SY5Y cells were stably transfected with empty pcDNA3 or pcDNA3-latexin plasmids. *A*, Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were detached at the indicated times and counted by Scepter automated cell counter during 5 days. The graph represents cell counts as mean count values ± S.D. (n=3). *B*, Protein extracts from EV or LXN cells were analyzed by *Western blotting* to corroborate the overexpression of latexin. The membranes were reprobed with β-actin antibody to check equal loading.

Then, we assessed whether latexin expression could modify the cytotoxic profile of cells exposed to different apoptotic stimuli. MTT reduction assay evidenced no substantial differences between empty vector-transfected and latexin-overexpressing cells in terms of cell viability in response to a broad battery of different cytotoxic insults (**Figure 15A**). We selected staurosporine (STP), a broad kinase inhibitor and well-known apoptosis inducer (Iglesias-Guimarais et al. 2012) to analyze if, by modulating the cytotoxic response, we could observe differential effects in latexin-overexpressing cells compared to empty vector-transfected cells. The presence of 1 μM STP in the culture medium for up 24 hours resulted in comparable percentages of cell death in empty vector-transfected and latexin-overexpressing cells as measured by trypan blue exclusion assay (**Figure 15B**). This result indicated that latexin was neither modifying the temporary activation of the apoptotic cell death induced by this kinase inhibitor. Furthermore, by using sub-lethal concentrations of STP we did not observe any significant differences in the percentage of cell death promoted by this apoptosis inducer as evidenced by MTT and lactate dehydrogenase (LDH) assays (**Figures 15C and 15D**). Taken together, these findings suggest that latexin is not primarily promoting cell growth inhibition or cell death in SH-SY5Y cells.

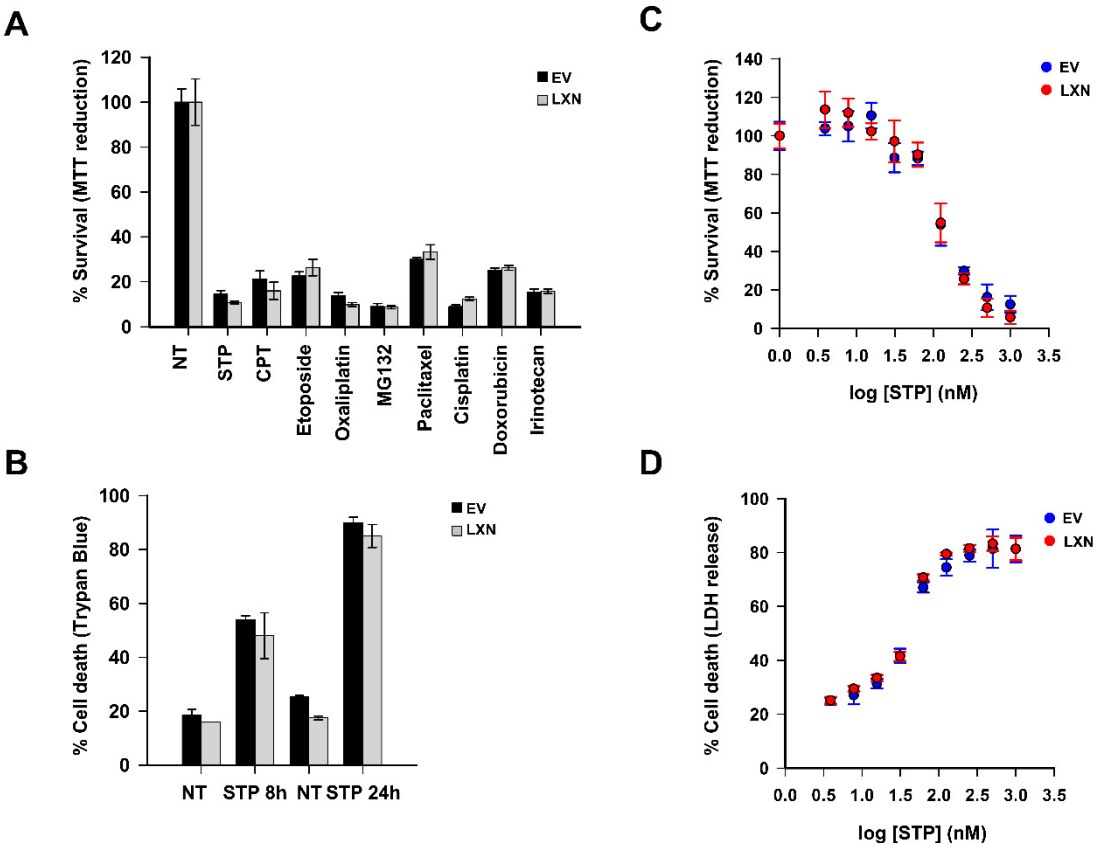

Figure 15. Latexin overexpression does not markedly affect the cytotoxic profile of cells treated with different apoptotic stimuli. *A*, Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were treated for 24 h according to "Cell treatments" in *Materials and Methods* or left untreated (Non-treated, NT). Cell viability was determined by MTT reduction assay and was expressed as a percentage of control cells. The values are represented as mean ± S.D. (n=3). *B,* EV and LXN cells were treated with 1 μM staurosporine (STP) or left untreated (NT) for 8 and 24 hours and cell death was assessed by trypan blue exclusion assay. *C* and *D*, EV and LXN cells were treated with the indicated concentrations of STP for 24 hours. Cell viability was measured by MTT reduction assay (*C*) and cell death was measured by lactate dehydrogenase (LDH) release (*D*). The values are represented as mean ± S.D. (n=3).

1.3. LATEXIN FACILITATES RA-PROMOTED S-TYPE APPEARANCE

Differentiation is also a tumor suppressive mechanism, having a particular relevance in the development of neuroblastoma tumors (Garrett M Brodeur 2003). Therefore, we explored whether latexin expression could influence this cellular response. As shown in **Figure 16**, stable overexpression of latexin did not bring about any apparent sign of neuronal differentiation.

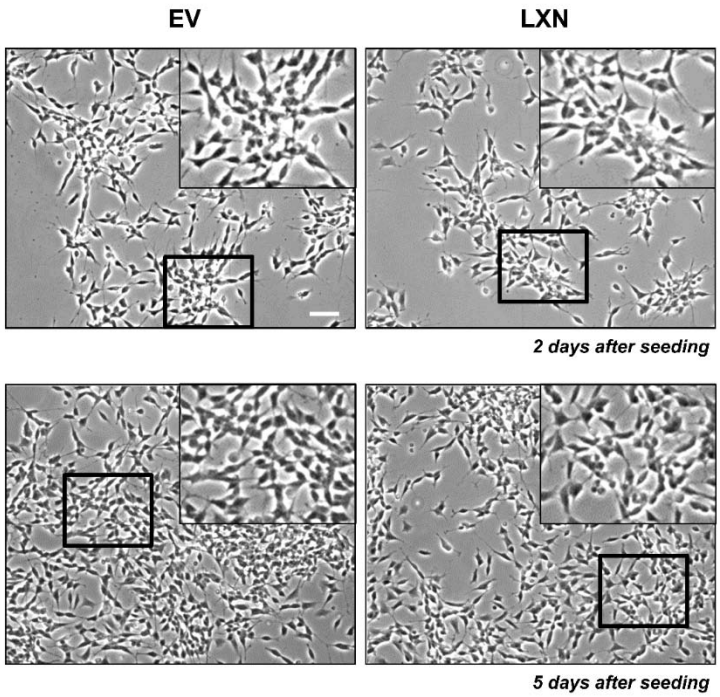

Figure 16. Latexin overexpression does not promote noticeable cell morphology changes up to 5 days in culture. Representative phase contrast microphotographs showing cell morphology of empty vector-transfected (EV) and latexin-overexpressing (LXN) cells for 2 and 5 days after seeding. The insets in each condition are higher magnifications of the framed cells. Scale bar = 70 μm.

Likewise, we sought to determine if cells overexpressing latexin could respond better to chemically-induced neuronal differentiation. We selected RA because is the best-characterized and widely-employed differentiation agent and also because latexin is regarded as an RA-responsive gene (Oldridge et al. 2013; Kamal et al. 2014). To this end, we employed different established cell differentiation conditions. As a first approach, cells were cultured in neurobasal medium supplemented with B27, DMEM supplemented with 0% FBS, DMEM supplemented with 1% FBS or DMEM supplemented with 15% FBS. As shown in **Figure 17**, cells cultured in the presence of 10 μM RA exhibited different morphologies depending on the cell culture medium employed. However, in all the conditions surveyed, RA-induced neuronal-like differentiation was characterized by the gradual extension of neurites and the decrease in cell proliferation, as previously established (Cheung et al., 2009) **(Figure 17)**. Remarkably, neuronal differentiation promoted by RA was apparently similar in both empty vector-transfected and latexin-overexpressing cells, regardless of the cell culture medium employed (**Figure 17**). However, the most striking difference between empty vector-transfected and latexin-overexpressing cells consisted in the early appearance of a population of flattened cells, in the latter subset, resembling the S-type phenotype, that was only detected under high serum conditions (15% FBS) (**Figure 17**). Accordingly, the appearance of S-type cells depended on the presence of FBS in the cell culture medium as previously established (Encinas et al. 2000).

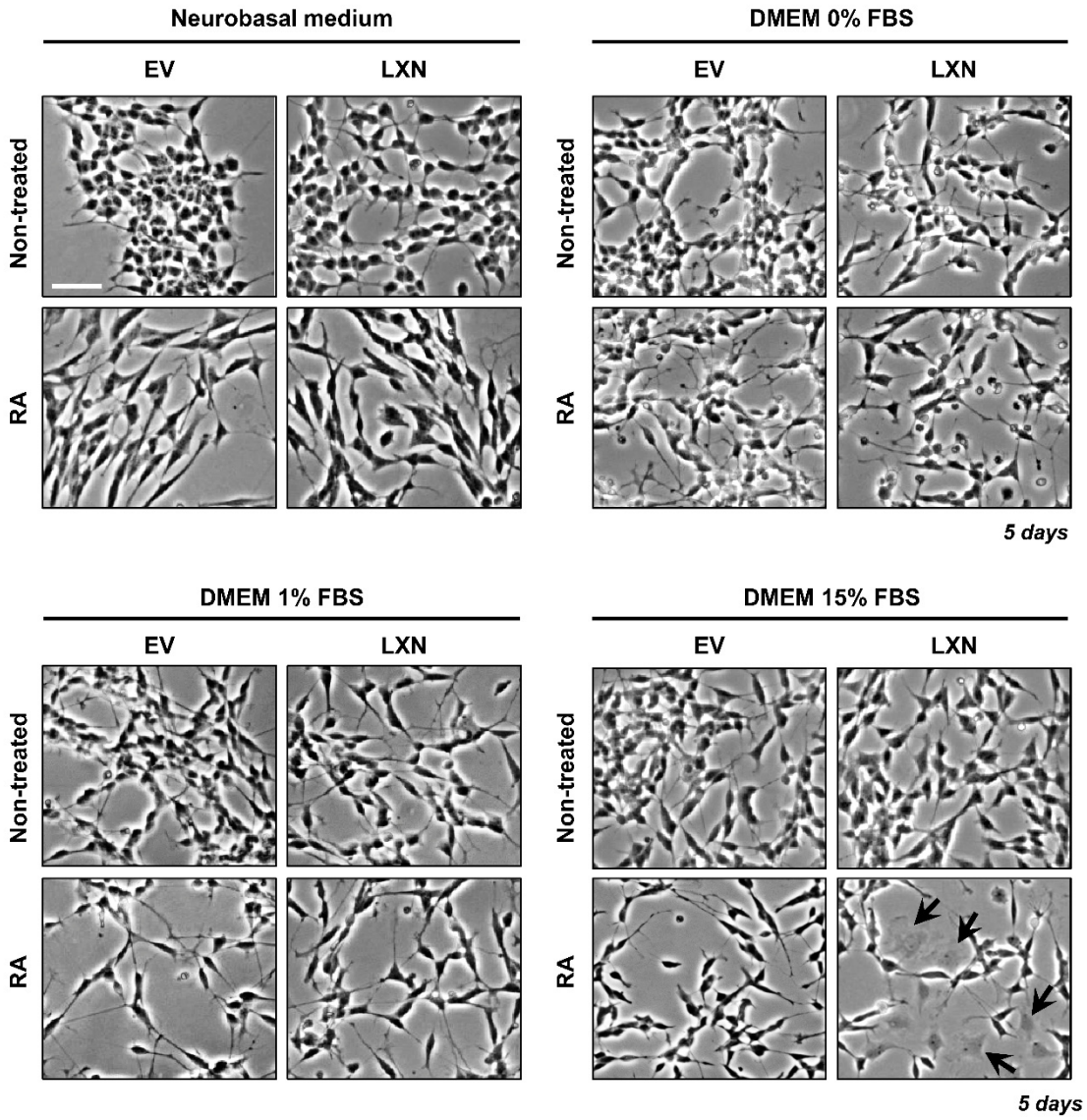

Figure 17. Latexin overexpression facilitates RA-induced S-type phenotype in cells cultured in DMEM supplemented with 15% FBS. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were cultured in Neurobasal Medium supplemented with B27, DMEM supplemented with 0 % FBS, DMEM supplemented with 1% FBS or DMEM supplemented with 15% FBS. After 48 hours, cells were treated for 5 days with 10 μM RA or left untreated (Non-treated). S-type cells are indicated by arrows. Representative phase contrast microphotographs for each condition are shown. Scale bar = 70 μm.

We further explored if a maximization of the above-mentioned differences was attainable through optimization of the cell culture parameters known to promote neuronal differentiation. Since cell adhesion is a relevant factor influencing differentiation outcome, we sought to determine whether proteins of the extracellular matrix (ECM) could influence the differentiation status in empty vector-transfected cells or latexin-overexpressing cells. To address this point, cells were grown on fibronectin-, laminin- or collagen-coated plates in DMEM supplemented with 15% FBS and cell morphology was monitored microscopically (**Figure 18**). As shown in **Figure 18**, cells cultured on ECM coated conditions displayed enhanced neuronal features compared with cells

grown in non-coated plates. However, neuritic-like processes in empty vector-transfected and latexin-overexpressing cells were still similar regardless of the coating condition tested. Remarkably, in both, empty vector-transfected and latexin-overexpressing cells, RA-induced S-type cells appeared earlier than when cells were cultured on uncoated plates. Notably, S-type cells still apparently represented a higher fraction in latexin-overexpressing cells. However, differences in the S-type induction between empty vector-transfected and latexin-overexpressing cells were lower than when cells were cultured on non-coated conditions, since ECM conditions also favored the appearance of RA-induced S-type phenotype in empty vector-transfected cells (**Figure 18**).

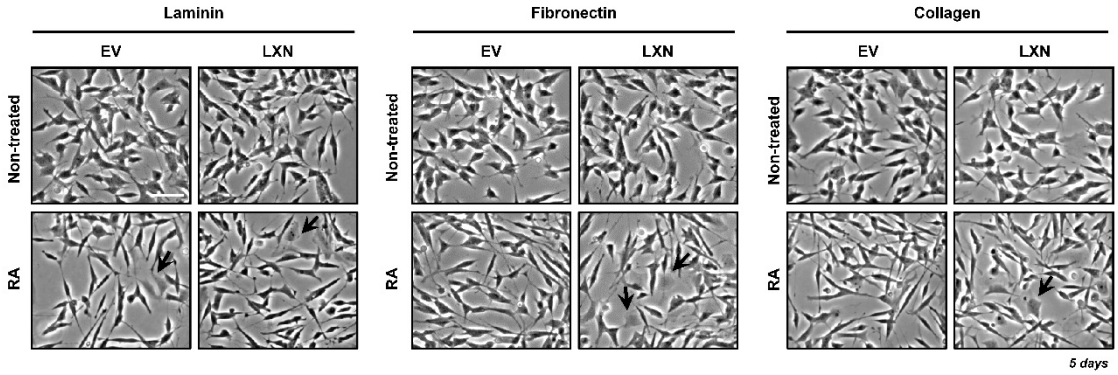

Figure 18. The S-type phenotype is promoted in cells cultured on extracellular matrix-coated plates. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were seeded on plates coated with 10 µg/ml of laminin, collagen or fibronectin. After 48 h, cells were treated for 5 days with 10 µM RA or left untreated (Non-treated). Representative phase contrast microphotographs for each condition are shown. S-type cells are indicated by arrows. Scale bar = 70 µm.

We first employed 10 µM RA since it is the optimal concentration reported for differentiation in SH-SY5Y cells (Cheung et al. 2009). However, we sought to determine if, by varying the concentration of this agent, we could modulate the differentiation outcome. As shown in **Figure 19**, the anti-proliferative effect of RA was already evident in latexin-overexpressing cells treated with 5 µM RA, whereas empty vector-transfected cells required at least 10 µM RA to start ceasing cell proliferation. Moreover, although concentrations of RA beyond 30 µM resulted cytotoxic in empty vector-transfected cells, latexin-overexpressing cells exhibited an enhanced resistance towards high concentrations of RA (**Figure 19**).

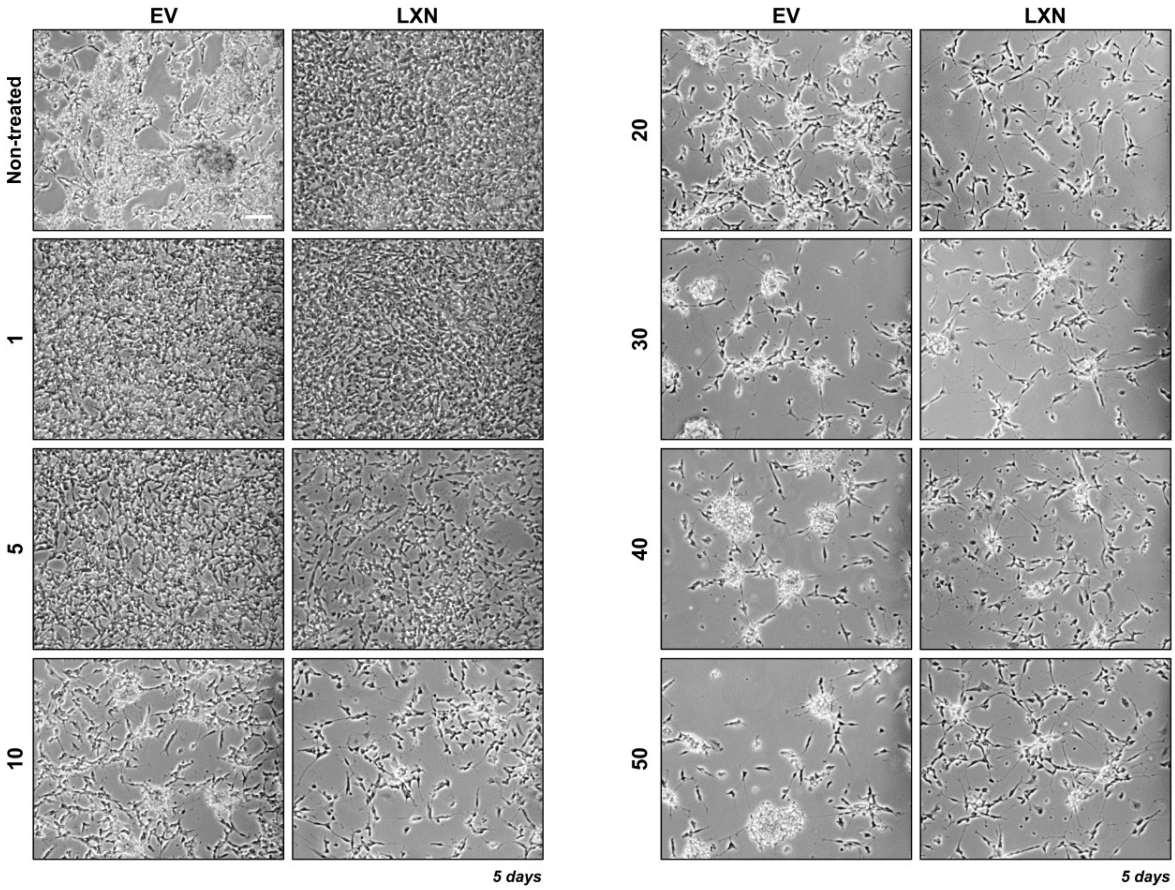

Figure 19. Latexin-overexpressing cells are more resistant to the cytotoxicity promoted by high concentrations of RA. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were treated with different concentrations of RA (0, 1, 5, 10, 30, 40 or 50 μM) or left untreated (Non-treated) for 5 days, as indicated. Representative phase contrast microphotographs for each condition are shown. *Scale bar* = 70 μm.

Furthermore, it has been reported that S-type cells exhibit a high substrate adherence potential and, consequently, they tend to grow below the neuritic-like cells (Ross et al. 2003; Ross & Spengler 2007), thus hampering the visualization of S-type cells. To overcome this limitation, we gently removed the weakly adherent N-type cells with PBS (see *Materials and Methods*), while maintaining the strongly adhered S-type cells, as previously reported (Bell et al. 2013). This assay allowed us to corroborate that latexin is facilitating RA-promoted S-type phenotype emergence (**Figure 20**). As shown in **Figure 20**, there is an inverse correlation between S-type cells appearance and RA concentration in latexin-overexpressing cells, being 5 μM and 10 μM the optimal RA concentrations to promote the emergence of S-type cells (**Figure 20**). However, neuronal-like differentiation is better promoted at 10 μM (**Figure 20**). According to these results, and unless otherwise stated, we proceeded to culture cells in 10 μM RA in DMEM 15% FBS henceforth since, under these conditions, both S-type appearance and N-type differentiation were promoted.

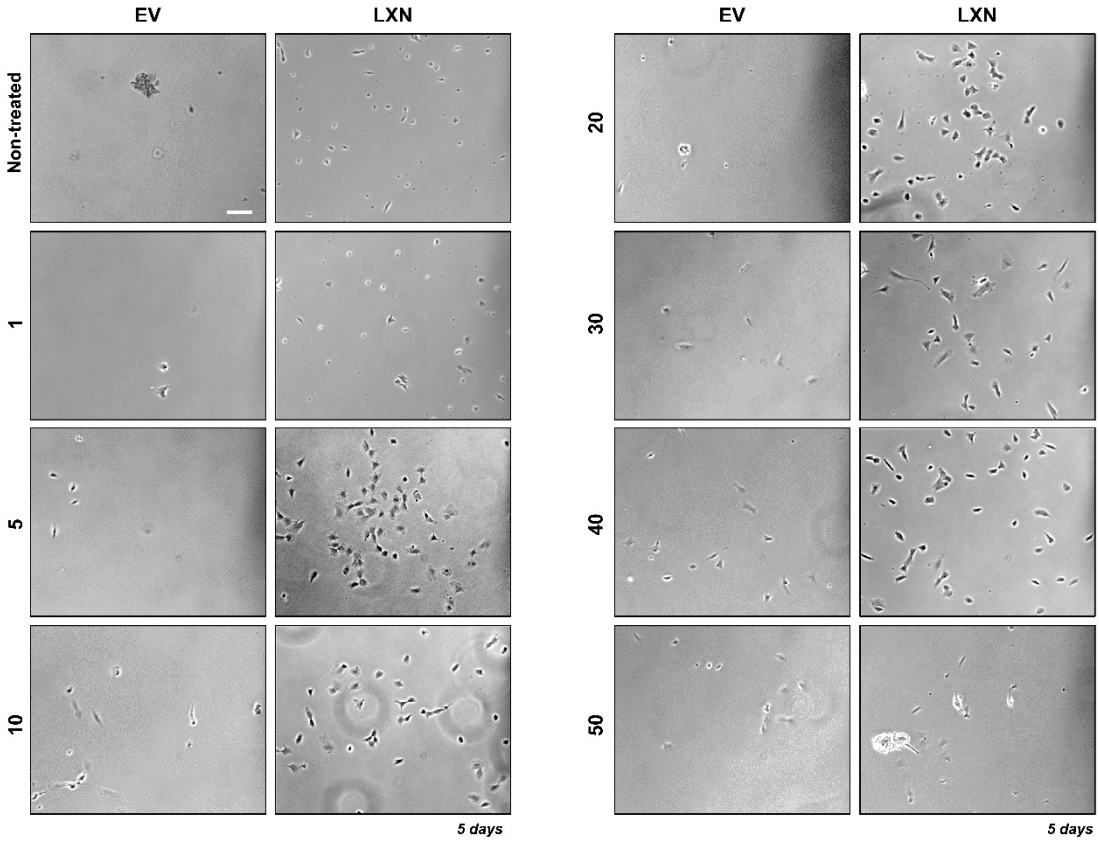

Figure 20. S-type phenotype promoted by RA in latexin-overexpressing cells is concentration-dependent. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were treated with different concentrations of RA (0, 1, 5, 10, 30, 40 or 50 μM) or left untreated (Non-treated) for 5 days as indicated. Representative phase contrast microphotographs of the remaining adhered cells after performing the "*Detachment protocol*" described in the *Materials and Methods* section are shown. *Scale bar* = 70 μm.

Taken altogether, latexin expression appeared downregulated in several neuroblastoma-derived cells and could be upregulated in response to different agents. Moreover, the stable overexpression of latexin did not apparently result in changes in either cell proliferation or cell death but markedly affected RA-mediated cell differentiation.

2. CHAPTER 2. LATEXIN FACILITATES LONG-TERM CELL SURVIVAL AND SENESCENCE VIA PDK1/AKT PATHWAY

Neuroblastoma tumors exhibit a large heterogeneity since they are composed of a mixture of divergent cell populations resulting from the multipotent nature of the neural crest (Garrett M. Brodeur 2003). Cell lines established from neuroblastoma tumors reflect such diversity and three morphologically different cellular sub-types have been subsequently defined: the N-type (neuroblastic or neuronal-like cells), the S-type (substrate adherent, or glial/Schwann-like or melanocytic-like cells) and the I-type (intermediate cells) (Biedler et al. 1973; Ciccarone et al. 1989; Piacentini et al. 1996). Such pediatric tumors can easily differentiate either spontaneously or upon induction with certain differentiation agents, being RA the most extensively studied. The potentiality of RA in cancer therapy resides in its ability to cease cell proliferation and promote differentiation by activating the expression of genes involved in cell proliferation, cell death and cell differentiation (Noy 2010; Connolly et al. 2013). RA carries out its function also by activating signaling cascades involved in neurite outgrowth, differentiation and survival including the PI3K/PDK1/Akt and p42/p44MAPK pathways (Encinas et al. 2002; López-Carballo et al. 2002; Crowe et al. 2003).

2.1. PI3K/PDK1/AKT PATHWAY PLAYS A KEY ROLE IN THE EMERGENCE OF RA-INDUCED S-TYPE CELLS FACILITATED BY LATEXIN

In the previous Chapter, we revealed that, upon RA treatment, the most notable difference between empty vector-transfected and latexin-overexpressing cells consisted in the early appearance in the latter of a population of flattened cells, resembling the S-type phenotype. This effect was clearly observed at 5 days of RA treatment (**Figure 21A**) whereas in empty vector-transfected cells S-type phenotype was perceptible later and represented a systematically lower fraction of the overall population. To gain more insight in such differential effect, we first analyzed the expression of differentiation markers characteristic of both N and S phenotypes. As shown in **Figure 21B**, differentiation was accompanied by an increase in the expression of the neuron specific enolase (NSE) differentiation marker, and a decrease in the levels of the DNA-binding protein inhibitor (ID1) de-differentiation marker. Indeed, differentiation markers supported the phenotypic changes observed, since both cell lines expressed comparable levels of these neuronal markers after RA treatment (**Figure 21B**). Interestingly, ID1 levels seemed slightly attenuated in RA-treated latexin-overexpressing cells (**Figure 21B**). On the other hand, latexin overexpressing cells showed a much higher expression of the S-type marker vimentin than empty vector-transfected cells after 5 days of RA treatment (**Figure 21B**). Strikingly, the expression of vimentin was already detected in latexin-overexpressing cells without the requirement of RA (**Figure 21B**). **Figure 21C** shows the detailed morphologic features of S-type and N-type cells; the S-type phenotype exhibits an enlarged and flattened phenotype whereas N-type cells display scant cytoplasm and neuritic-like extensions.

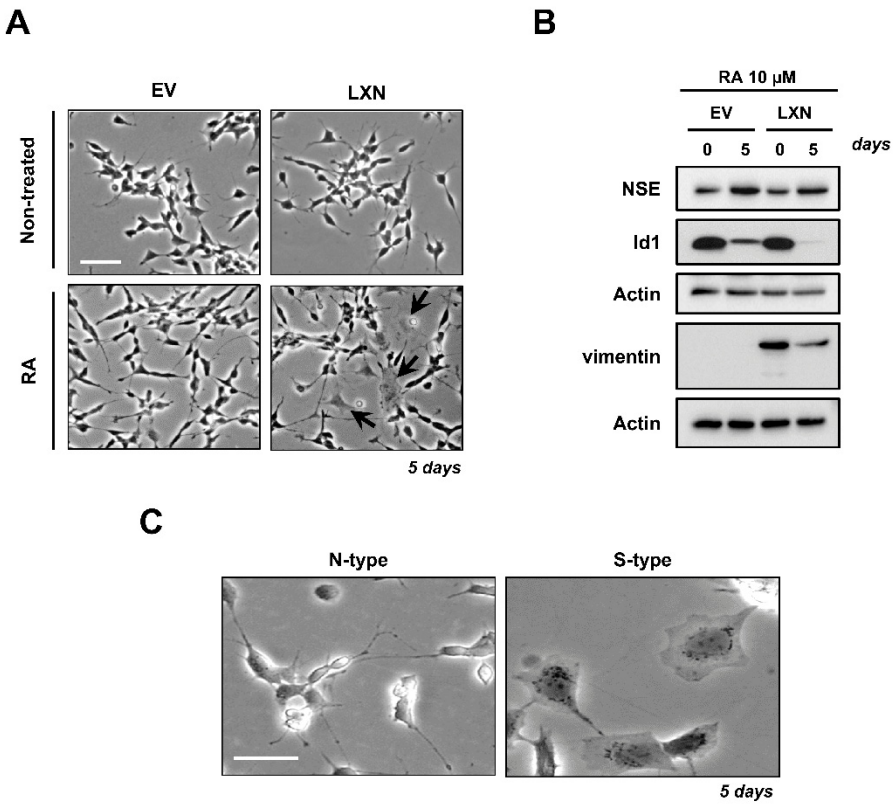

Figure 21. Latexin facilitates cell specification towards the S-type phenotype after RA treatment. *A,* Representative phase contrast microphotographs of empty vector-transfected (EV) or latexin-overexpressing (LXN) cells treated with 10 µM RA for 5 days. S-type cells are highlighted by arrows. *B*, EV or LXN cells were treated for 5 days with 10 µM RA and protein extracts were obtained before (0) or after 5 days of treatment. Protein levels of neuron specific enolase (NSE), inhibitor of DNA binding 1 (Id1), and vimentin were analyzed by *Western blotting*. β-actin was used as loading control. *C*, Representative bright field microphotographs showing the typical morphology of N-type and S-type cells. *Scale bars* = 70 µm.

To ascertain which biochemical mechanisms are governing the transdifferentiation effect in which latexin is implicated in, we focused on the PI3K/PDK1/Akt and p42/p44MAPK pathways since they are among the most intracellular pathways involved in RA-promoted differentiation (Datta et al. 1999; Encinas et al. 2002). To this end, we first analyzed the phosphorylation status of ERK (**Figure 22A**) and Akt (**Figure 22B**). As shown in **Figure 22A**, RA-induced ERK activation was enhanced in latexin-overexpressing cells. Remarkably, such activation was sustained over the course of the treatment (**Figure 22A**). On the other hand, the sole overexpression of latexin was sufficient to promote Akt phosphorylation in cells growing in complete media, in the absence of RA (**Figure 22B**). In the same line, latexin-overexpressing cells also showed an early, prominent and time-maintained phosphorylation of Akt when cultured in the presence of RA (**Figure 22B**).

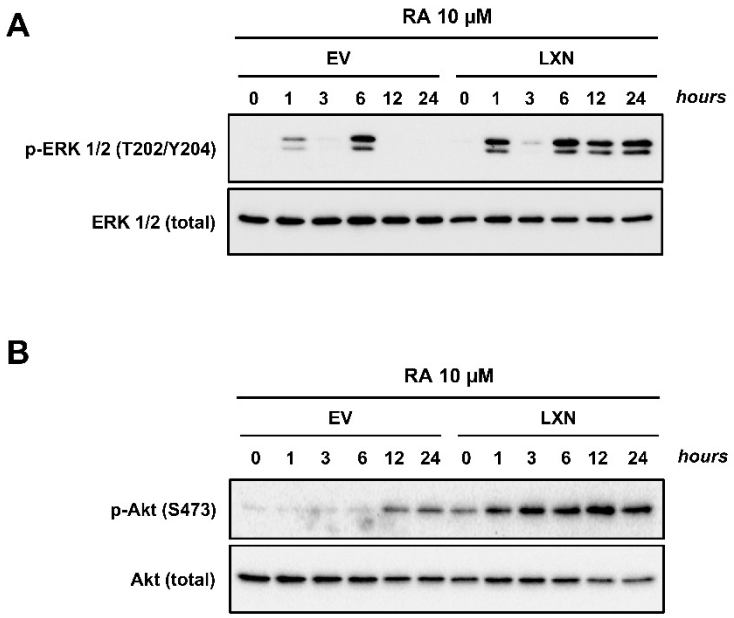

Figure 22. RA-induced phosphorylation of both Akt and ERK is enhanced in latexin-overexpressing cells. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were treated with 10 µM RA or left untreated, and protein extracts were obtained before treatment (0) or at the specified times of treatment. *A,* Levels of total ERK 1/2 and phospho-ERK 1/2 were analyzed by *Western Blotting. B,* Levels of total Akt and phospho-Akt S473 were analyzed by *Western blotting*.

These findings led us to analyze whether the inhibition of any of these pathways could influence the above-mentioned latexin-promoted effects. As a first approach, cells were cultured in the presence of 10 µM U0126, a selective inhibitor of MEK1/2 (Duncia et al. 1998). Thus, the addition of the inhibitor did not apparently influence either N-type differentiation or S-type appearance in latexin-overexpressing cells cultured in the presence of RA during 5 days (**Figure 23A**). Interestingly, when cells were cultured for longer times in the presence of RA, while empty vector-transfected cells gradually aborted neuronal differentiation, latexin-overexpressing cells exhibited extensive neuritic processes after 15 days in culture (**Figure 23B**). However, the presence of 10 µM U0126 did not prevent such differentiated phenotype (**Figure 23B**). Therefore, we analyzed the phosphorylation status of ERK upon treatment with U0126. As shown in **Figure 23C** the addition of 10 µM U0126 prevented RA-mediated activation of ERK in both empty vector-transfected and latexin-overexpressing cells (**Figure 12C**). We further corroborated the effect of inhibiting ERK on the emergence of S-type cells promoted by RA. Thus, N-type cells were removed with PBS (see *Materials and Methods*), while maintaining the strongly attached S-type cells. **Figure 23D** shows that the enrichment of S-type population observed in latexin-overexpressing cells was only slightly attenuated upon ERK inhibition (**Figure 23D**). Taken altogether, it seemed that the p42/p44MAPK pathway was not essential for latexin-mediated cellular effects.

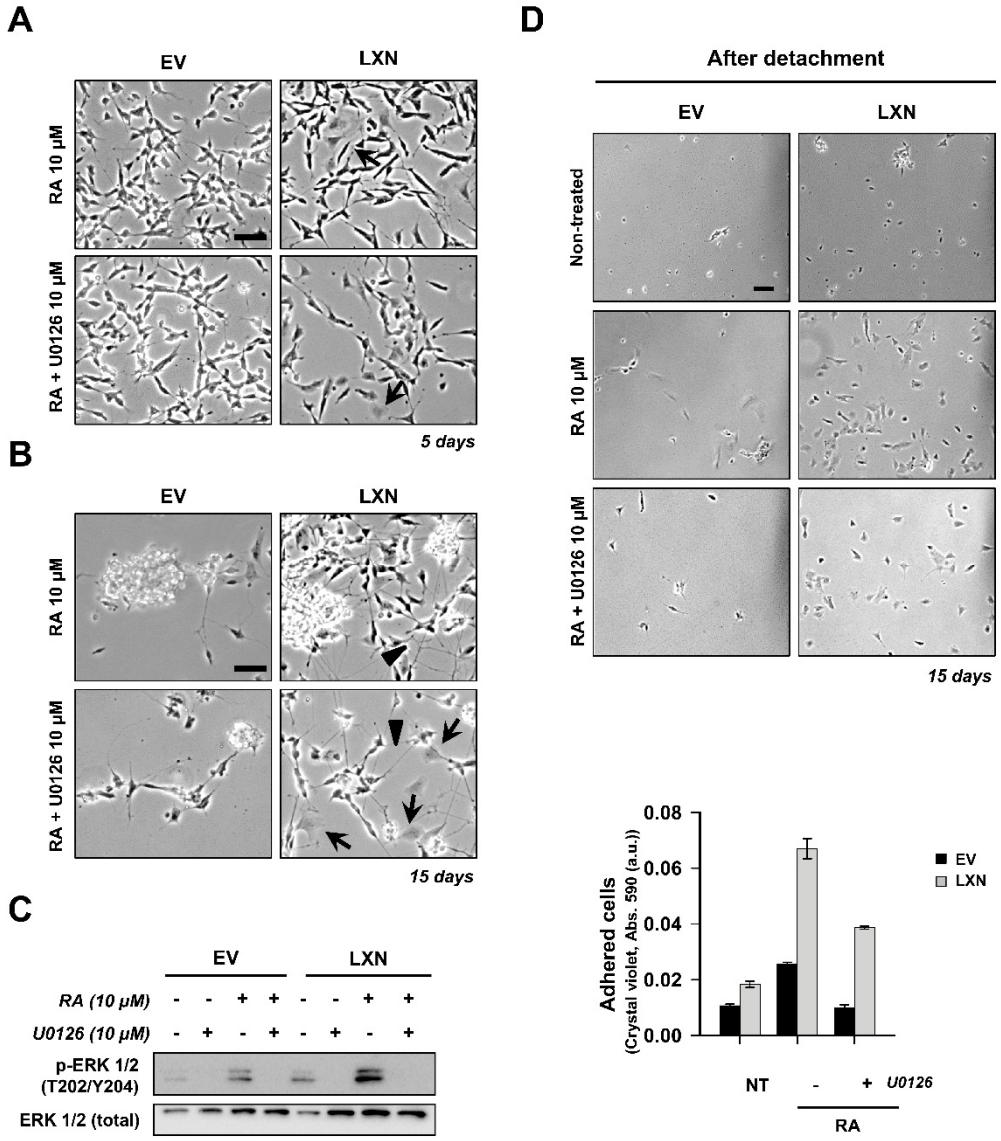

Figure 23. U0126 treatment does not significantly alter RA-promoted N-type differentiation or S-type emergence. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells cultured for 1 hour in the presence of 10 µM U0126, were treated with 10 µM RA. Phase contrast microphotographs representative of three independent experiments of cells treated during 5 (*A*) or 15 (*B*) days are shown. *C*, EV or LXN cells cultured for 1 hour in the presence or in the absence of 10 µM U0126 were treated with 10 µM RA for 1 hour or left untreated. Protein extracts were obtained after the indicated treatments and the levels of ERK 1/2 total, phospho-ERK 1/2 were analyzed by *Western blotting*. *D*, EV or LXN cells cultured for 1 hour in the presence or in the absence of 10 µM U0126 were treated with 10 µM RA for 15 days or left untreated. Representative phase contrast microphotographs of the remaining adhered cells after performing the "Detachment protocol" described in the Materials and Methods section are shown. Adhered cells from upper panels were quantified by staining cells with crystal violet as described in Materials and Methods. The graph shows arbitrary units (a.u.) of the absorbance measured at 590 nm (Abs. 590). Values are represented as mean ± S.D. (n=3). Paired Student's t tests analysis was used to determine the statistical significance. $p \leq 0.01$ was considered to be significant.*Scale bars* = 70 µm.

Moreover, we wanted to ascertain the impact of inhibiting the PI3K/PDK1/Akt pathway. Accordingly, we employed GSK2334470, a PDK1 specific inhibitor (Najafov et al. 2011). Cells cultured for up to 15 days with RA in the presence of 10 μM GSK2334470 gradually impaired both cell survival and cell differentiation (**Figure 24A**). We corroborated this effects by employing 1 μM PI-103 (PI3K inhibitor (Fan et al. 2006)) and 10 μM MK-2206 (Akt inhibitor (Hirai et al. 2010)) (**Figure 24B**).

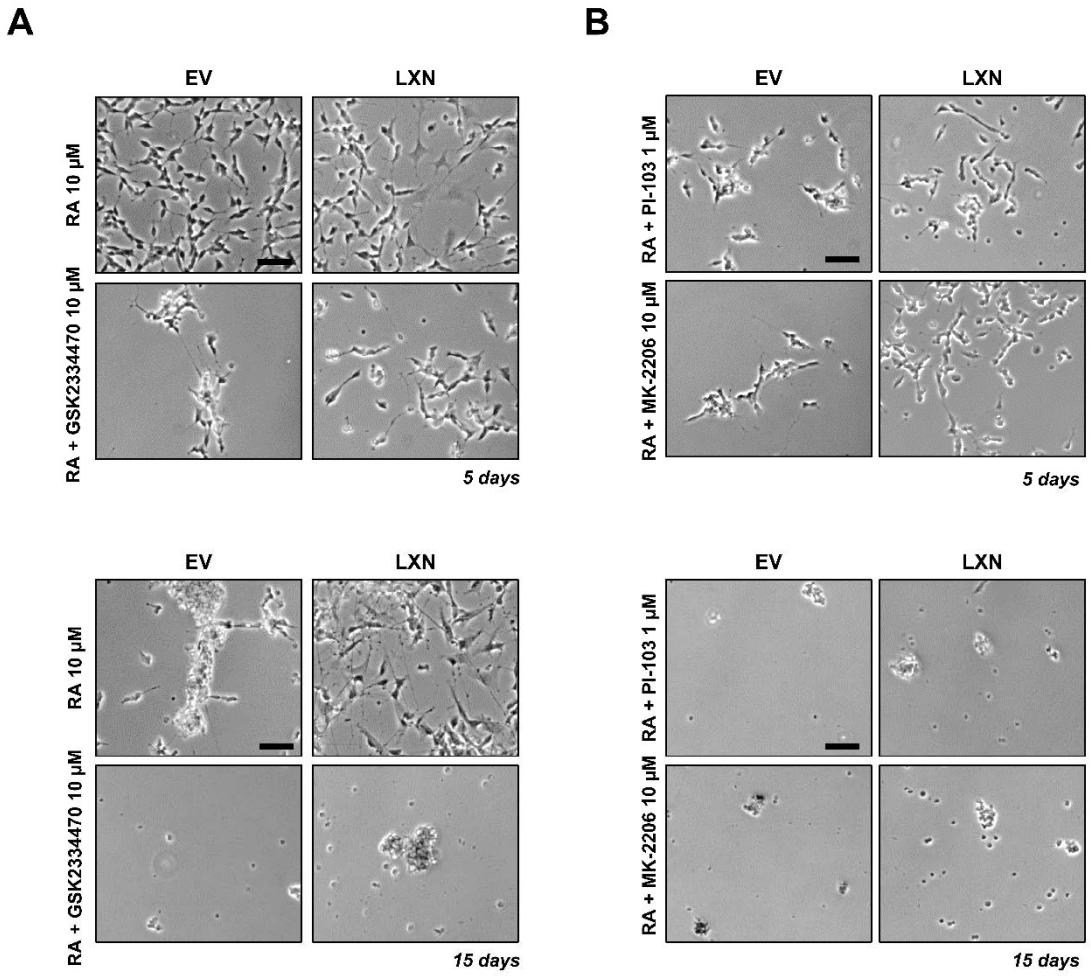

Figure 24. High concentrations of the PI3K, PDK1 or Akt inhibitor impair cell survival of SH-SY5Y cells. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells cultured for 1 hour in the presence of 10 μM GSK2334470 were treated with 10 μM RA. *A*, Phase contrast microphotographs representative of three independent experiments of cells treated during 5 (*upper panel*) or 15 (l*ower panel*) days are shown. *B*, EV or LXN cells cultured for 1 hour in the presence of 1μM PI-103 or 10 μM MK-2206 were treated with 10 μM RA during 5 (*upper panel*) or 15 (l*ower panel*) days and representative phase contrast microphotographs are shown. *Scale bars* = 70 μm

With the aim to modulate such cellular response, we lowered the concentration of GSK2334470 to 3 μM. As shown in **Figure 25A**, empty vector-transfected and latexin-overexpressing cells cultured for 5 days with RA in the presence of 3 μM GSK2334470 remained viable and RA-mediated neurite outgrowth was not impaired. After 15 days of treatment, however, while RA-treated empty vector-transfected cells died, RA-treated latexin-overexpressing cells retained extensive neuritic processes (**Figure 25A**). One of the most striking findings was

the apparent absence of RA-induced S-type cells when the PDK1 inhibitor was employed (**Figure 25A**). Thus, we analyzed the phosphorylation status of Akt and a downstream substrate upon GSK2334470 treatment. The addition of 3 μM GSK2334470 to the culture media avoided RA-mediated threonine (T308) and serine (S473) Akt phosphorylation in empty vector-transfected cells (**Figure 25B**). However, this PDK1 inhibitor did not fully prevent Akt phosphorylation in either untreated or RA-treated latexin-overexpressing cells (**Figure 25B**). This residual Akt phosphorylation could be likely mediating the differential cellular effects observed between empty vector-transfected and latexin-overexpressing cells, both cultured in the presence of RA and 3 μM GSK2334470 up to 15 days (**Figure 25A**). To corroborate that Akt was activated after RA treatment, we checked the phosphorylation status of Proline-Rich AKT1 Substrate 1 (PRAS40) at T246, a site specifically phosphorylated by Akt (Kovacina et al. 2003). As shown in **Figure 25B**, RA induced the phosphorylation of PRAS40 at T246 in both empty vector and latexin-overexpressing cells. In accordance with the previously observed phosphorylation status of Akt, untreated latexin-overexpressing cells already presented enhanced levels of PRAS40 phosphorylation (**Figure 25B**). Moreover, the increase in PRAS40 phosphorylation induced by RA was clearly accentuated in latexin-overexpressing cells (**Figure 25B**). Finally, PRAS40 phosphorylation was only partially inhibited in latexin-overexpressing cells treated with 3 μM GSK2334470, correlating with Akt phosphorylation status (**Figure 25B**). Then, we extended such results by employing lower concentrations of the PI3K and Akt inhibitors, namely 100 nM for PI-103 or 3 μM for MK-2206. As shown in **Figures 25A** and **25C**, RA-induced N-type differentiation was maintained in latexin-overexpressing cells whereas S-type phenotype was apparently abolished, regardless of the inhibitor employed.

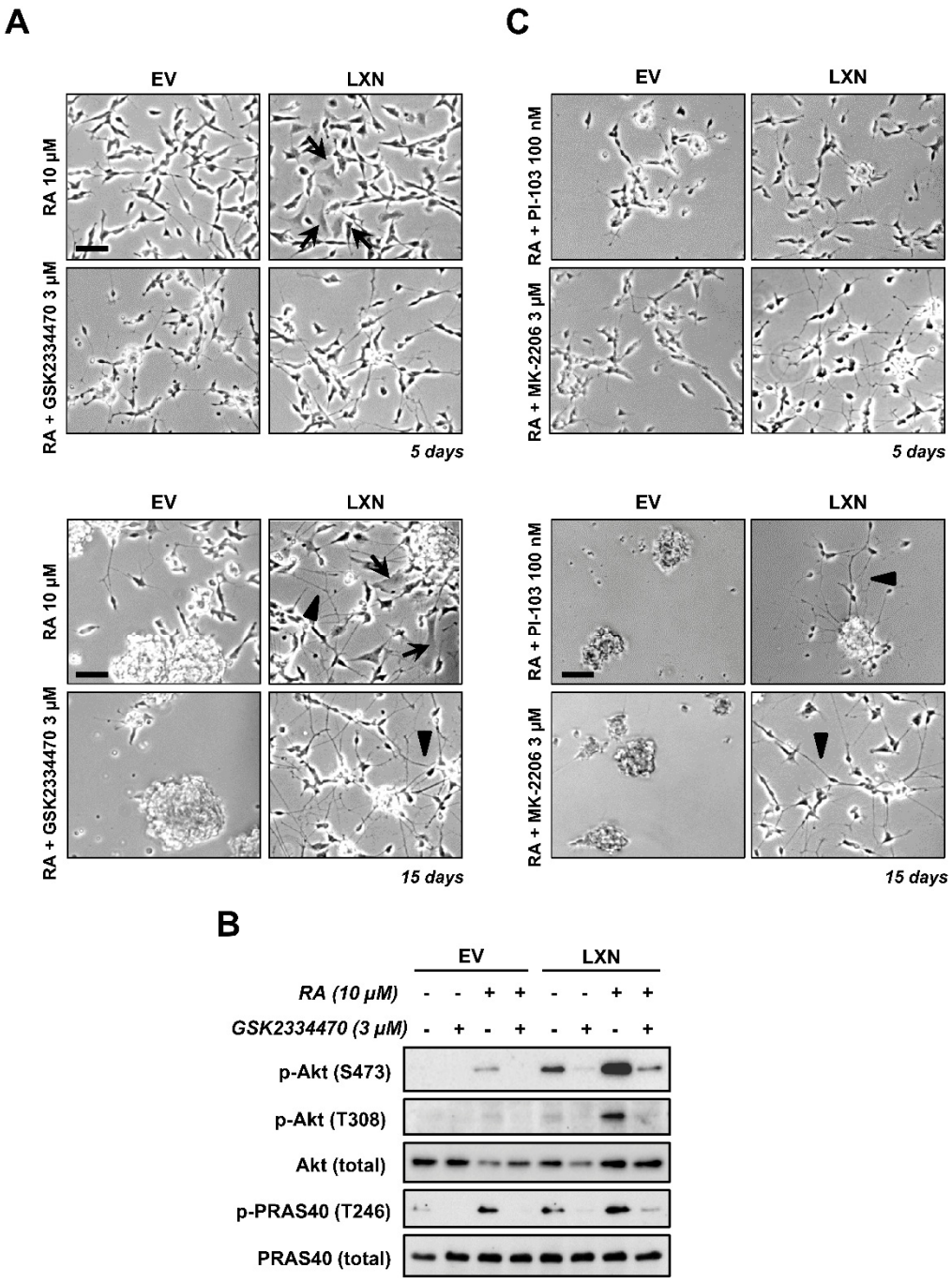

Figure 25. The partial inhibition of the PI3K/PDK1/Akt axis does not impair neuronal differentiation of RA-treated latexin-overexpressing cells. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells cultured for 1 hour in the presence of 3 µM GSK2334470, were treated with 10 µM RA. *A*, Phase contrast microphotographs representative of three independent experiments of cells treated during 5 (*upper panels*) or 15 (*lower panels*) days are shown. *B*, EV or LXN cells cultured for 1 hour in the presence or in the absence of 3 µM GSK2334470 were treated with 10 µM RA for 12 hours or left untreated. Protein extracts were obtained after the indicated treatments and the levels of Akt, phospho-Akt T308, phospho-Akt S473, PRAS40 and phospho-PRAS40 T246 were analyzed by *Western blotting*. *C*, EV or LXN cells cultured for 1 hour in the presence of 100 nM PI-103 or 3 µM MK-2206 were treated with 10 µM RA. Phase contrast microphotographs representative of three independent experiments of cells treated during 5 (*upper panels*) or 15 (*lower panels*) days are shown Note that latexin overexpression allows the extension of neuritic processes in the presence

of the PI3K, PDK1 or Akt inhibitors used. S-type cells are highlighted by arrows. Neuritic-like extensions from N-type cells are indicated by arrowheads. *Scale bars* = 70 µm.

To corroborate the apparent disappearance of S-type cells when the PI3K/PDK1/Akt axis was inhibited, N-type cells were removed from the cell culture and quantification of the remaining adherent cells was carried out by crystal violet staining (see *Materials and Methods*). As shown in **Figure 26**, GSK2334470 treatment significantly reduced the number of S-type cells facilitated by latexin upon RA treatment. Because PI-103 is not only a class IA PI3K but also mTOR inhibitor (Knight et al. 2006) and MK-2206 does not equally inhibit all three Akt isoforms (Hirai et al. 2010), we employed GSK2334470 for further experiments. Taking all together, the PI3K/PDK1/Akt axis appears to be central in latexin-promoted cellular specification towards the S-type phenotype.

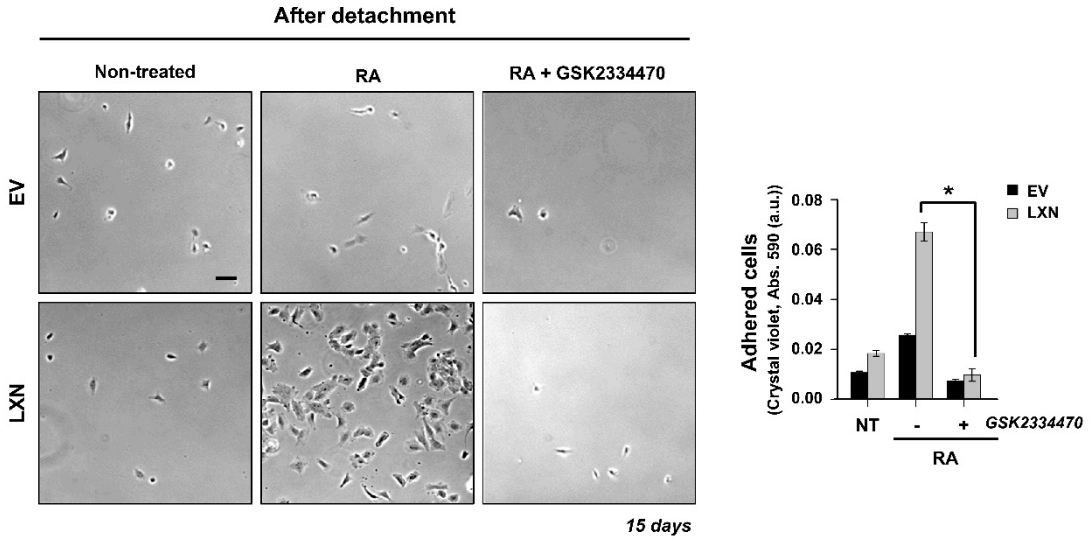

Figure 26. **Partial inhibition of PDK1 impedes the emergence of RA-promoted S-type phenotype in latexin-overexpressing cells.** Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells cultured for 1 hour in the presence or in the absence of 3 µM GSK2334470 were treated with 10 µM RA or left untreated (Non-treated, NT) for up to 15 days. Representative phase contrast microphotographs of the remaining adhered cells after performing the "*Detachment protocol*" described in the *Materials and Methods* section are shown. *Scale bar* = 70 µm. Adhered cells from upper panels were quantified by staining cells with crystal violet as described in *Materials and Methods*. The graph shows arbitrary units (a.u.) of the absorbance measured at 590 nm (Abs. 590). Values are represented as mean ± S.D. (n=3). Paired Student's t tests analysis was used to determine the statistical significance. $p \leq 0.01$ was considered to be significant.

2.2. LATEXIN OVEREXPRESSION ALLOWS LONG-TERM CELL SURVIVAL AND SENESCENCE UPON RETINOIC ACID TREATMENT

Since Akt was more efficiently activated in latexin-overexpressing cells upon RA treatment (**Figure 22**), we wondered if latexin-overexpressing cells could survive for longer periods in culture. As expected, both untreated empty vector-transfected and latexin-overexpressing cells showed a remarkable reduction of cell viability after 30 days in culture (**Figure 27**). In line with this, in cells transfected with empty vector, differentiation was gradually impaired over the course of the days, and cells tended to aggregate and form floating clumps, as expected in cells cultured in non-adherent surfaces (Acosta et al. 2009) (**Figure 27**). However, RA-treated latexin-overexpressing cells remained alive after maintaining cell cultures over 1 month without changing the culture media (**Figure 27**). In this case, N-type cells displayed a highly differentiated morphology with extensive neurites and S-type cells became larger and flatter (**Figure 27**).

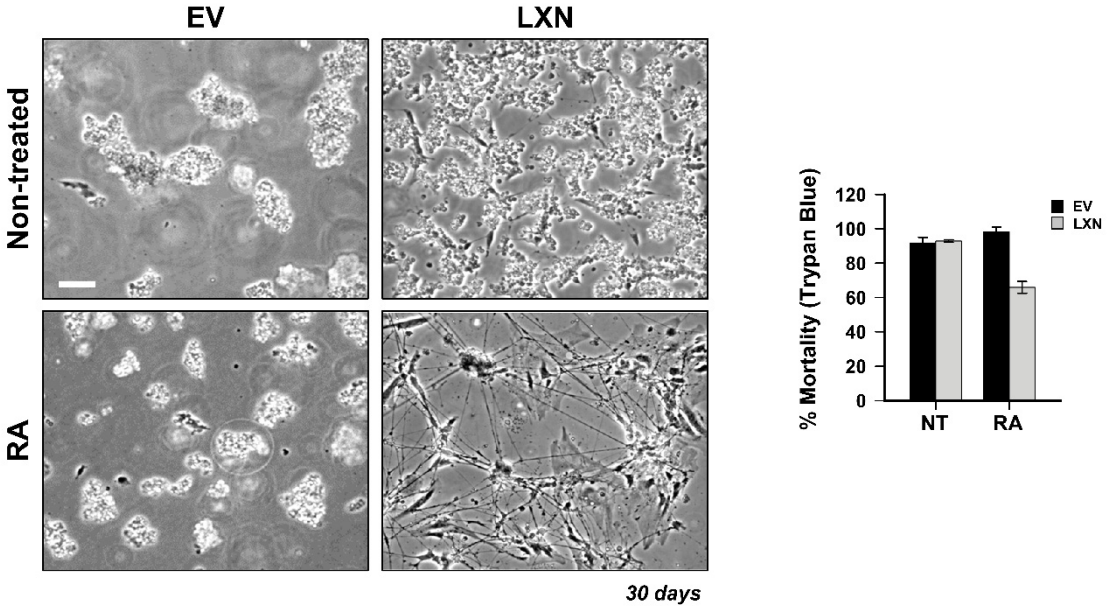

Figure 27. Latexin allows long-term cell survival in response to RA treatment. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were cultured during 30 days without changing cell culture media in the absence (Non-treated) or in the presence of 10 μM RA. Phase contrast microphotographs representative of three independent experiments are shown. Scale bar = 70 μm. Cell death was measured by trypan blue exclusion assay as described in "*Materials and Methods*". The values are represented as mean ± S.D. (n=3). Paired Student's t tests analysis was used to determine the statistical significance. $p \leq 0.01$ was considered to be significant.

Because such flattened and enlarged morphology and, as well, the high substrate adherence was suggestive of cellular senescence (Campisi 2005), we performed the senescence-associated β-galactosidase (SA-β-gal) assay, the most widely used method to detect senescent cells (Dimri et al. 1995). As shown in **Figure 28**, most of RA-induced S-type cells favored by latexin overexpression were senescent. However, a limited number of empty vector-transfected cells also showed SA-β-gal positivity after a 15-day culture (**Figure 28**). It must be

stressed the fact that confluent cells showing some degree of unspecific SA-β-gal activity is a recognized limitation of this assay (Dimri et al. 1995). In this sense, empty vector-transfected cells tended to grow in aggregates of rounded cells on top of one another, and SA-β-gal positive cells were detected within these clumps in RA-treated empty vector-transfected cells (**Figure 28**). Nevertheless, latexin-overexpressing cells still grew as a monolayer and SA-β-gal positive cells were spread along the culture plate. By detaching the N-type cells, we confirmed that the remaining adherent S-type cells were indeed SA-β-gal positive (**Figure 28**). Differences could not be quantified due to that empty vector-transfected cells, as mentioned before, were mainly organized as dense aggregates of cells after several days in culture.

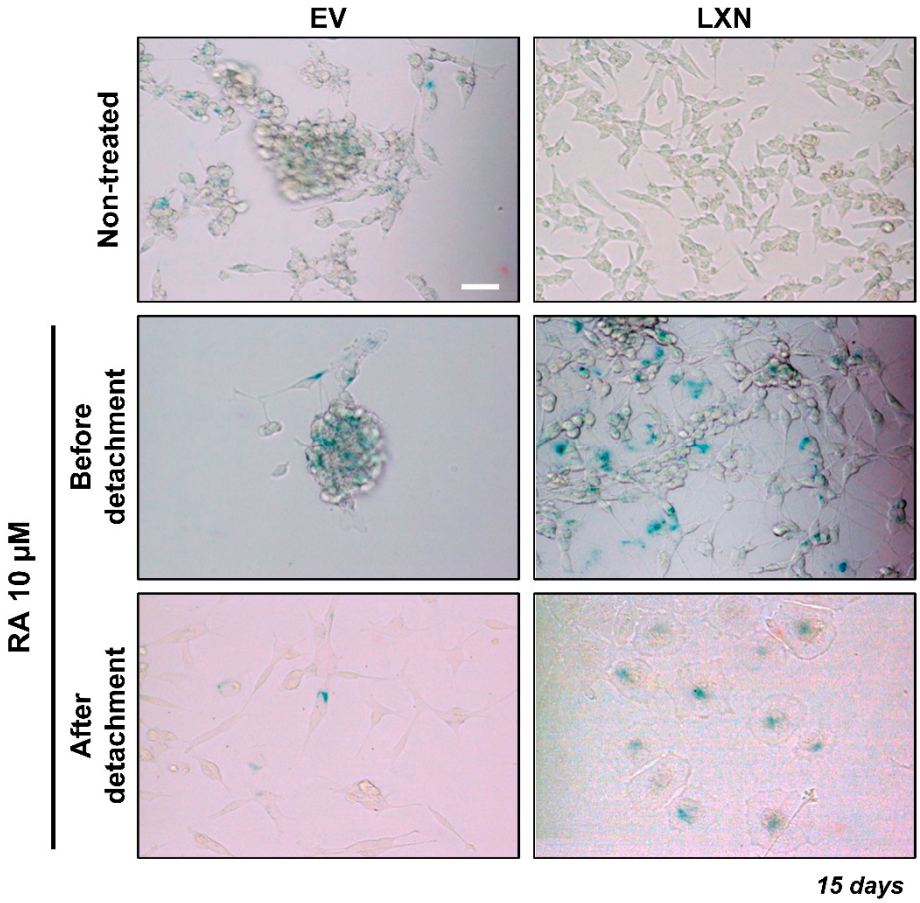

Figure 28. S-type cells promoted by RA treatment in latexin-overexpressing cells become senescent after long time in culture. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were treated with 10 µM RA, or left untreated (Non-treated) for the specified periods of time. Senescence-associated β-galactosidase (SA-β-gal) assay was performed as described in "*Materials and methods*" and visualized by bright field microscopy in cells treated during 15 days or left untreated (Non-treated). For cells treated with RA, SA-β-gal was carried out before or after performing the "*Detachment protocol*" detailed in the *Materials and Methods* section. Representative bright field microphotographs are shown. Scale bar = 50 µm

Additionally, we assess whether the above-mentioned effect was translated in differences in cell proliferation. To this end, the of 5-ethynyl-2'-deoxyuridine (EdU) assay was performed (Salic & Mitchison 2008). As shown in **Figure 29**, RA treatment during 5 days promoted a decrease in the proportion of EdU-incorporating cells in

both empty vector-transfected and latexin-overexpressing cells. Remarkably, and consistently with the results of SA-β-gal, at identical RA-treatment times, latexin-overexpressing cells showed a significant reduction in the number of EdU-positive cells compared with empty vector-transfected cells. Indeed, after 5 days of RA treatment, ~50% and ~25% of cells were EdU-positive in empty vector-transfected and latexin-overexpressing cells, respectively (**Figure 29**).

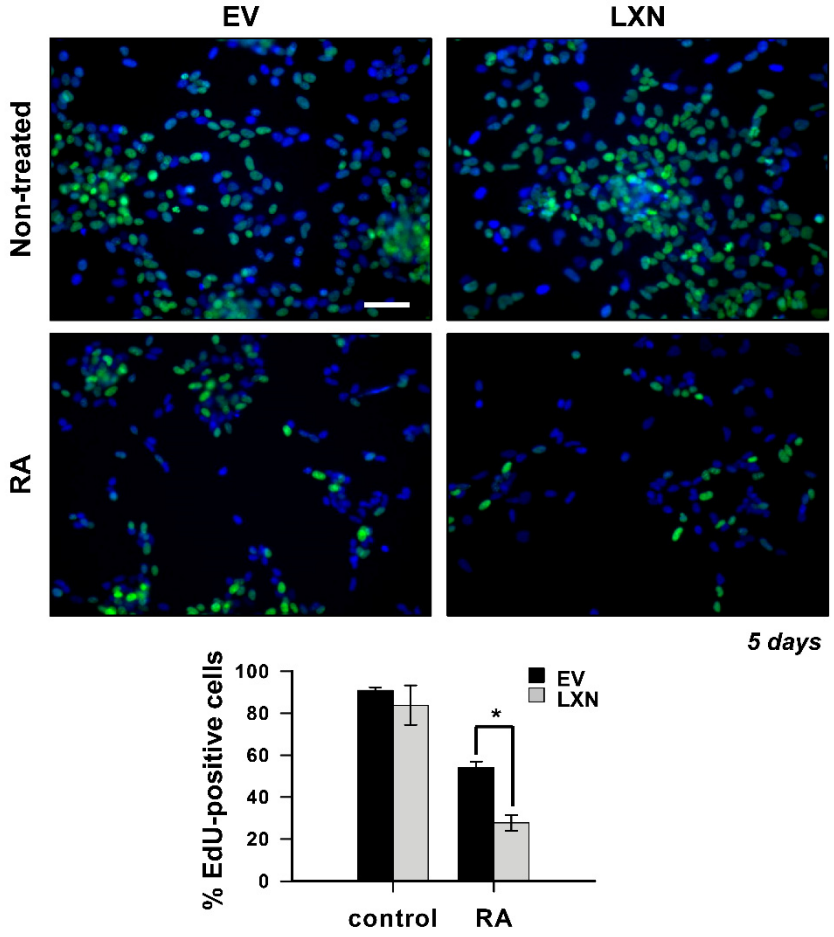

Figure 29. Latexin overexpression enhances the anti-proliferative effect of RA treatment. The 5-ethynyl-2'-deoxyuridine EdU incorporation assay was performed as described in "*Materials and Methods*" in empty vector-transfected (EV) or latexin-overexpressin (LXN) cells treated with 10 μM RA for 5 days or left untreated (Non-treated, NT). Representative fluorescence microphotographs showing EdU-positive cells (green) and Hoechst-stained nuclei (blue) are shown. Scale bar = 50 μm. The lower graph shows the percentage of EdU-positive cells over the total Hoechst-stained cells. The values are represented as mean ± S.D. (n=3). Paired Student's t tests analysis was used to determine the statistical significance. $p \leq 0.01$ was considered to be significant.

It has been previously reported the close association between mitochondrial elongation and senescence (Lee et al. 2007). Thus, cells were induced to differentiate with RA and the mitochondrial status was explored by using Mitotracker Red staining. Remarkably, in RA-treated latexin-overexpressing cells a proportion of cells, likely corresponding to the S-type phenotype, exhibited an enlarged and interconnected mitochondrial morphology. This phenotype was in sharp contrast with the punctuated pattern observed in either empty vector-

transfected cells or non-treated latexin-overexpressing cells (**Figure 30**). Taken together, these results indicated that latexin conferred long-term cell survival after RA treatment, allowing S-type cells to enter into a senescence program.

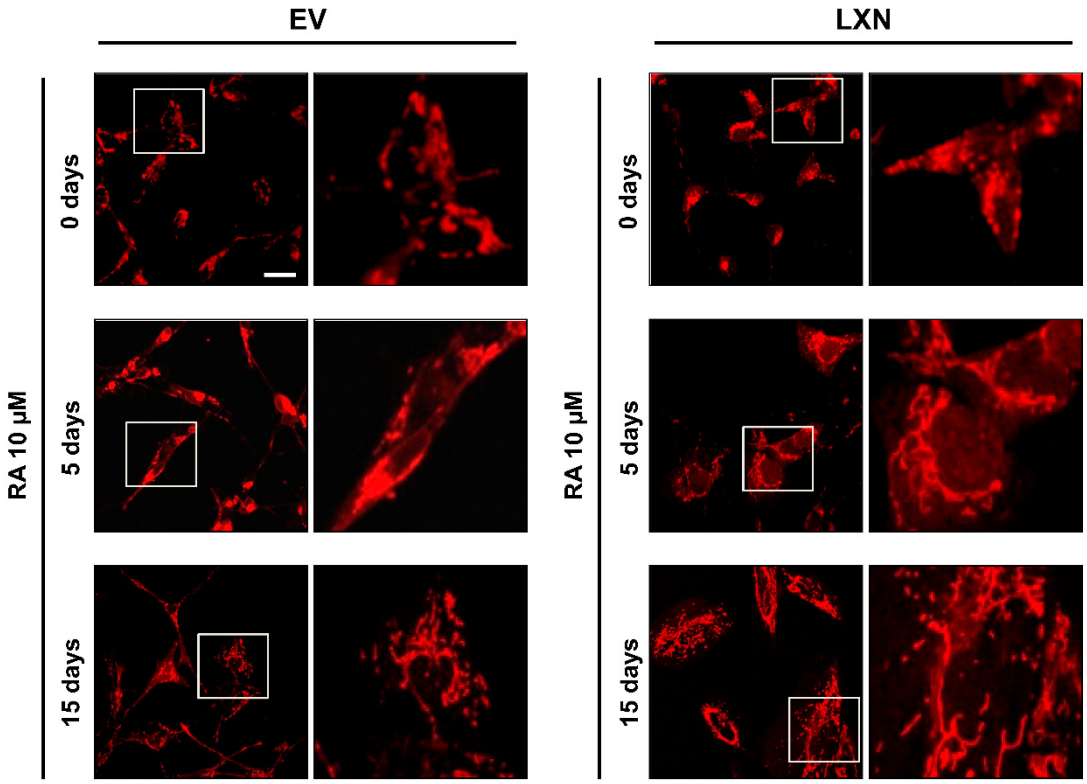

Figure 30. S-type cell facilitated by latexin overexpression in response to RA treatment exhibit mitochondrial elongation. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were treated with RA for 0, 5 or 15 days. Mitochondria were stained with 50 nM MitoTracker Red and visualized by confocal microscopy. *Scale bar* = 50 µm. The right panels in each condition are higher magnifications of the framed cells.

2.3. LATEXIN FACILITATES LONG-TERM CELL SURVIVAL AND SENESCENCE AFTER BRDU TREATMENT VIA PI3K/PDK1/AKT PATHWAY

On the basis of the previous results, we sought to examine whether the senescent phenotype promoted by latexin was specific of RA. To this end, we used the pro-senescence and S-type specific inducer 5-bromo-2'-deoxyuridine (BrdU) (Michishita et al. 1999; Sugimoto et al. 1988). Interestingly, SH-SY5Y cells upregulated latexin expression upon BrdU treatment, suggesting its potential involvement in the differentiation induced by this agent (**Figure 31B**). As shown in **Figure 31A**, BrdU-induced effects consisted in the progressive emergence of flattened cells, resembling the S-type phenotype, while N-type cells with the characteristic neuritic-like extensions were not predominantly promoted, in latexin-overexpressing cells. In line with these results, cells overexpressing latexin exhibited higher levels of the senescent marker vimentin (**Figure 31B**). When cells were exposed to BrdU for extended periods of time, S-type cells present in latexin-overexpressing

cells became flatter and larger, being reflective of cellular senescence. Conversely, empty vector-transfected cells did not exhibit S-type-like traits, but they gradually died over time of treatment (**Figure 31B**).

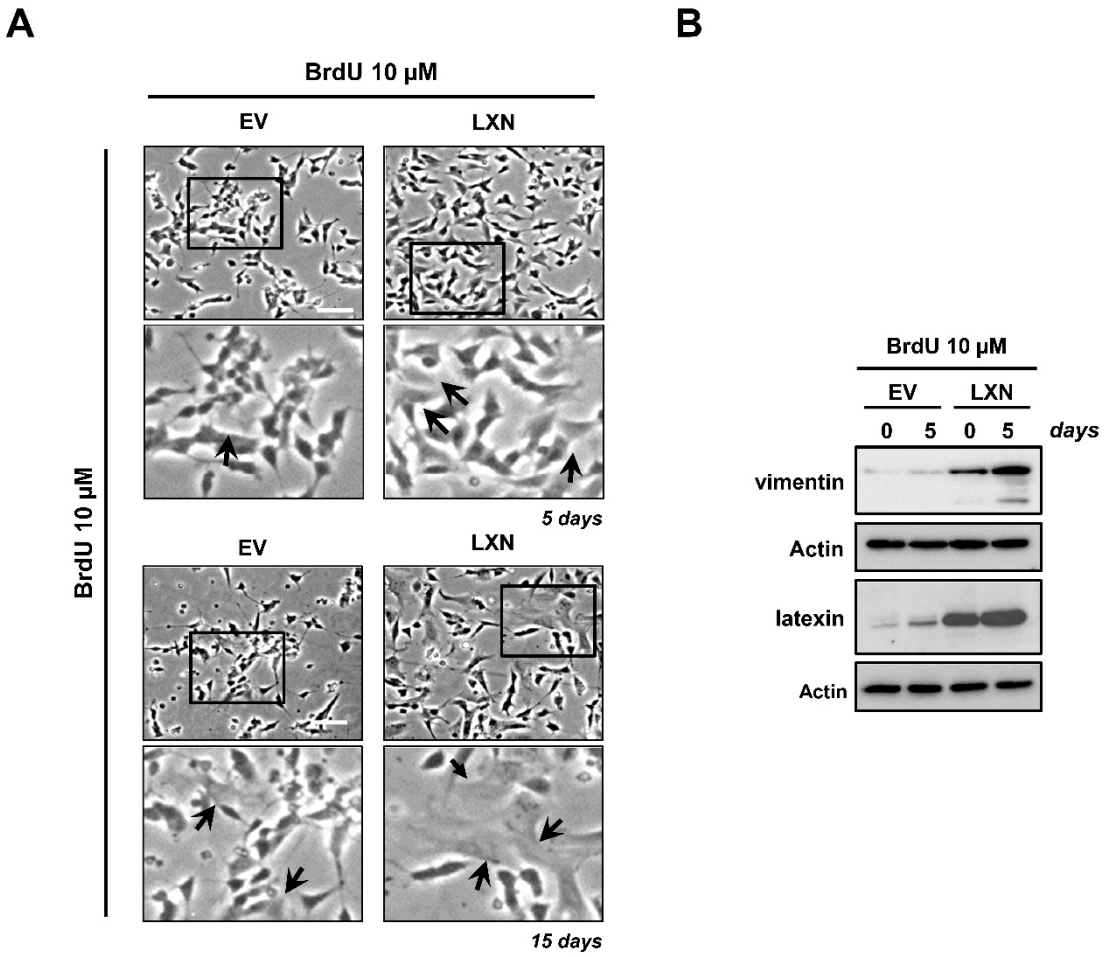

Figure 31. Latexin facilitates the emergence of S-type cells upon BrdU treatment. *A,* Phase contrast microphotographs representative of three independent experiments of empty vector-transfected (EV) or latexin-overexpressing (LXN) cells treated with 10 µM 5-bromo-2'-deoxyuridine (BrdU) for up to 15 days. The bottom panels in each condition are higher magnifications of the framed cells. S-type cells are indicated by arrows. *Scale bar* = 70 µm. *B,* EV or LXN cells were treated for 5 days with 10 µM BrdU and protein extracts were obtained before (0) or after 5 days of treatment. Protein levels of vimentin and latexin were analyzed by *Western blotting*. β-actin was used as loading control.

Thus, we evaluated SA-β-gal activity in cells treated with BrdU. As shown in **Figure 32A**, latexin significantly increased the number of BrdU-promoted S-type cells compared with empty vector-transfected cells. Nevertheless, since both BrdU and EdU are thymidine analogs, the EdU assay could not be performed in BrdU-treated cells. In addition, when exploring the mitochondrial morphology of cells cultured in the presence of BrdU during 5 days, we clearly observed a more elongated morphology in latexin-overexpressing cells compared to empty vector-transfected cells (**Figure 32B**). Altogether, these results indicated that latexin was also playing a pro-senescence role in BrdU-challenged cells by allowing long-term cell survival under unfavorable extracellular conditions.

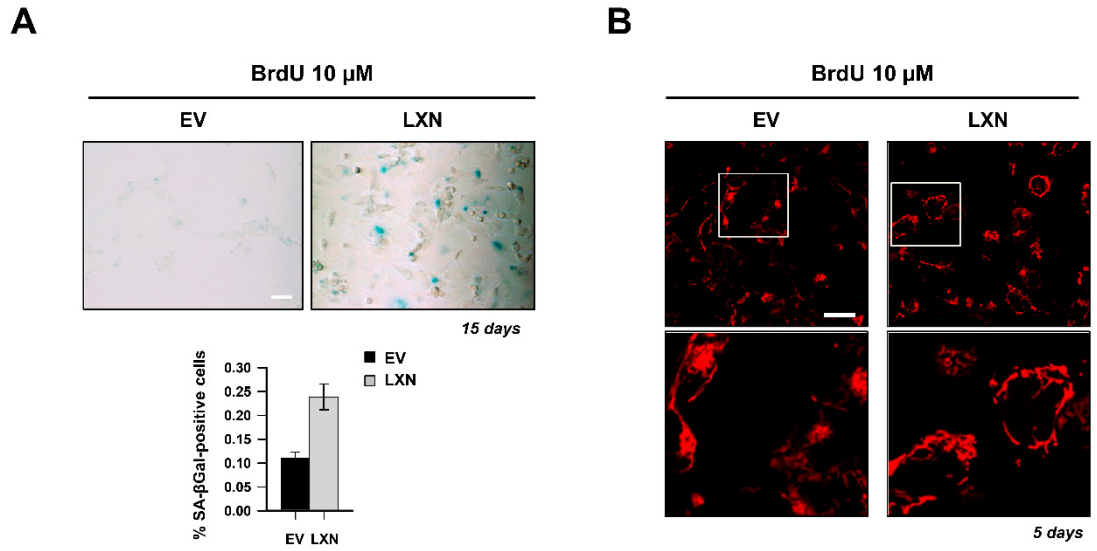

Figure 32. S-type cells facilitated by latexin upon BrdU treatment become senescent after long time in culture. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were cultured for up15 days in the presence of 10 µM BrdU or left untreated. *A,* Senescence-associated β-galactosidase (SA-β-gal) assay was performed and representative bright field microphotographs are shown. The lower graph shows the percentage of SA-β-gal-positive cells over the total cells. The values are represented as mean ± S.D. (n=3). *B*, EV or LXN cells treated for 5 days were stained with 50 nM MitoTracker Red and visualized by confocal microscopy. *Scale bars* = 50 µm.

Additionally, since latexin appears to favor cell survival and senescence upon BrdU exposure, we wanted to ascertain the potential involvement of the PI3K/PDK1/Akt pathway in such processes, as already observed after RA treatment. To address this issue, we first analyzed Akt phosphorylation levels in empty vector-transfected and latexin-overexpressing cells, cultured in the presence of 10 µM BrdU for up to 24 h. As shown in **Figure 33A**, both empty vector-transfected and latexin-overexpressing cells showed an increase in Akt phosphorylation levels at 1 h of BrdU treatment, followed by a decrease over time of treatment. However, the phosphorylation of Akt at both T308 and S473 sites was higher in latexin-overexpressing cells, compared to empty vector-transfected cells, over the course of BrdU treatment (**Figure 33A**). When exploring the impact of inhibiting PDK1 on the cellular phenotype promoted by BrdU, we observed that 3 µM GSK2334470 hampered the emergence of the S-type phenotype in latexin-overexpressing cells cultured in the presence of BrdU for 5 days (**Figure 33B**).

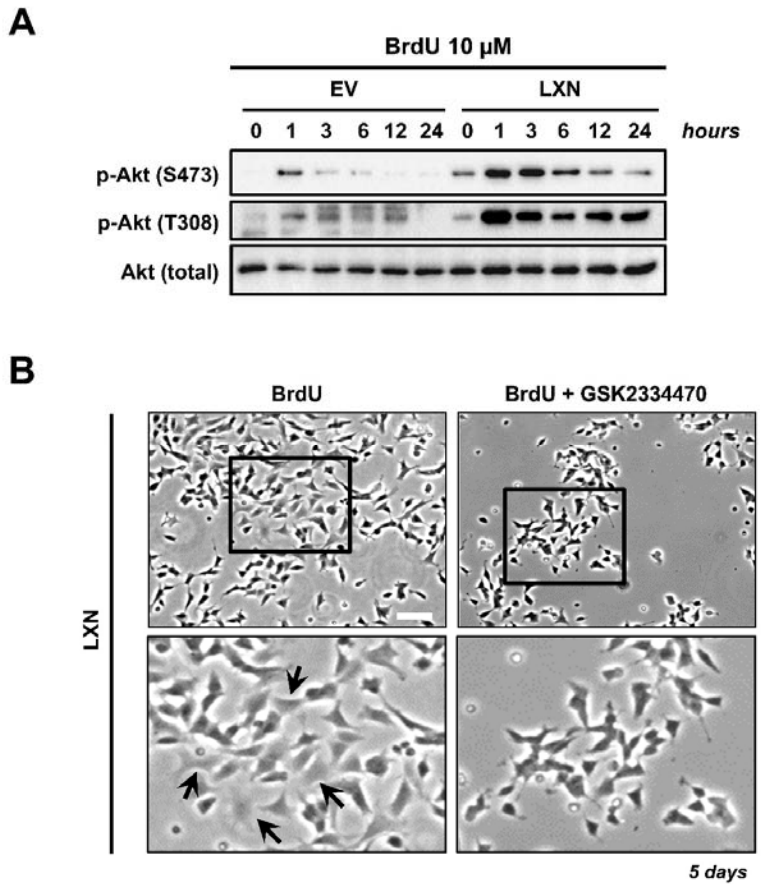

Figure 33. Akt activation is enhanced in latexin-overexpressing cells upon BrdU treatment. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were treated with 10 µM 5-bromo-2'-deoxyuridine (BrdU) or left untreated. *A,* Protein extracts were obtained before (0) or at the specified times of treatment. Protein levels of total Akt, phospho-Akt T308 and phospho-Akt S473 were analyzed by *Western blotting*. *B*, Representative phase contrast microphotographs of LXN cells treated with 10 µM BrdU for 5 days, in the absence or in the presence of 3 µM GSK2334470, are shown. The bottom panels in each condition are higher magnifications of the framed cells. S-type cells are indicated with arrows. *Scale bar* = 70 µm.

In line with these results, the senescent phenotype promoted by BrdU in cells overexpressing latexin was impaired after treatment with 3 µM GSK2334470, as evidenced by the drastic reduction in the number of SA-β-gal positive cells (**Figure 34A**). In accordance, the elongated morphology observed in BrdU-treated latexin-overexpressing cells was also disrupted when PDK1 was inhibited (**Figure 34B**). These findings indicated that PDK1/Akt pathway was also necessary for BrdU-prompted S-type lineage in latexin-overexpressing cells. Finally, the comparative analysis of the expression of vimentin, a described marker of S-type cells and senescence, in cells cultured during 15 days either in the presence or absence of 3 µM GSK2334470, treated with RA or BrdU or left untreated, allowed us to summarize the effects at long term of the PDK1/AKT pathway blockade on the S-type effect promoted by both differentiation agents. On one side, in empty vector-transfected cells, vimentin expression was only perceptible when BrdU was used as differentiation agent alone (**Figure 34C**). On the other hand, latexin-overexpressing cells exhibited a consistently higher expression of vimentin, compared to that of empty vector-transfected cells, being detected under most conditions surveyed. Expression

of this marker was already observed in the absence of any treatment, which could be suggestive of the latexin capability of allowing cells to undergo cellular senescence. Interestingly, although vimentin levels experienced a decrease at 5 days of RA (**Figure 21A**), in spite of S-type cells already became evident at this time, they were risen when differentiation was more advanced. Yet again, the highest expression of vimentin was found upon treatment with BrdU, in perfect agreement with the potent induction of S-type cells exerted by this agent coupled to the enhancing effect of latexin. Conversely, latexin-overexpressing cells cultured in the presence of GSK2334470 were invariably associated with a prominent decrease of vimentin protein levels, which is in accordance with the disappearance of S-type cells observed after GSK2334470 treatment.

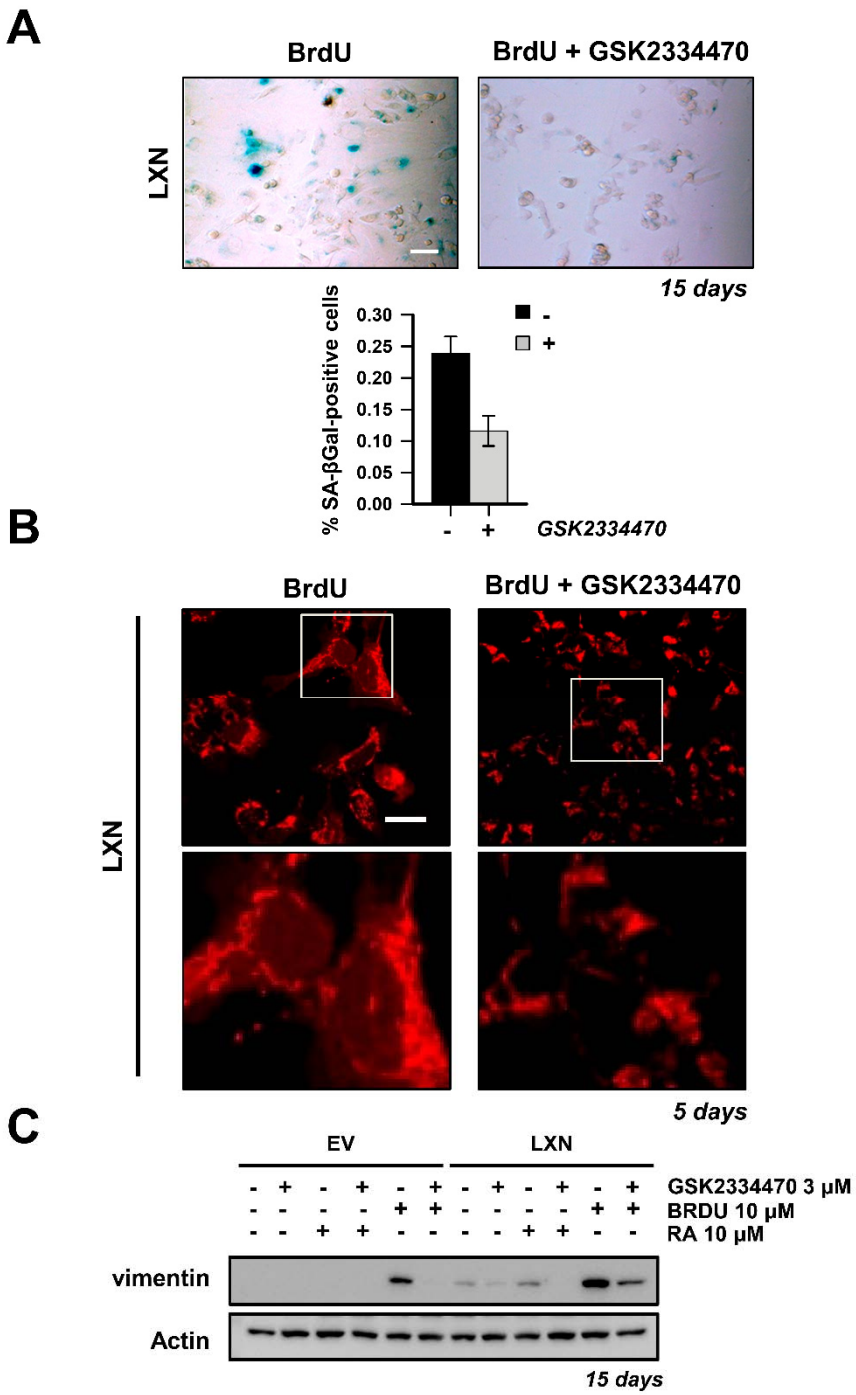

Figure 34. Cellular senescence promoted by latexin in response to BrdU treatment relies on PI3K/AKT pathway activation. *A*, Senescence-associated β-galactosidase (SA-β-gal) assay was performed in latexin-overexpressing (LXN) cells treated with 10 μM BrdU for 15 days, in the absence or in the presence of 3 μM GSK2334470, and analyzed by bright field microscopy. Representative images are shown. The lower graph shows the percentage of SA-β-gal-positive cells over the total cells. The values are represented as mean ± S.D. (n=3). *B*, LXN cells were treated with 10 μM BrdU for 5 days, in the absence or in the presence of 3 μM GSK2334470, and mitochondria were stained with 50 nM MitoTracker Red and visualized by confocal microscopy. *Scale bars* = 50 μm. *C,* EV or LXN cultured in the presence or in the absence of 3 μM GSK2334470 were either treated with 10 μM RA or 10 μM BrdU or left untreated. After 15 days, cell extracts were obtained and the protein levels of vimentin were analyzed by *Western blotting.* β-actin was used as loading control.

Taken together, we evidenced that PI3K/PDK1/Akt axis activation is essential for the emergence of RA-induced S-type emergence facilitated by latexin. The activation of this pathway also allowed latexin-overexpressing cells to survive for long periods under unfavorable extracellular conditions and to undergo cellular senescence. In agreement with these results, latexin also prompted the pro-senescent effect induced by the differentiating agent BrdU. To our best knowledge, this is the first time that a pro-differentiation and senescence role of latexin was unraveled.

3. CHAPTER 3. LATEXIN MODULATES RETINOIC ACID ACID-INDUCED GENE EXPRESSION IN SH-SY5Y CELLS.

There are several studies reporting the involvement of latexin relevant processes such as inflammation (Aagaard et al. 2005), control of hematopoietic stem cell numbers (Liang et al. 2007) or cancer (Yong Li et al. 2011; Muthusamy et al. 2013; Ni et al. 2014). Moreover, we have previously demonstrated the involvement of latexin in RA-induced differentiation in SH-SY5Y cells. However, the precise mechanism of action still remains largely unknown. There are no studies focused on the genetic regulation promoted by latexin. For this reason, we took advantage of genome-wide expression profile and bioinformatic tools to analyze the transcriptomic signature of SH-SY5Y cells overexpressing latexin and treated with RA. Unveiling the landscape of gene expression promoted by latexin will be critical for identifying new target genes and signaling pathways transcriptionally regulated by this protein.

3.1. OVERVIEW OF SEQUENCING DATA AND EVALUATION OF OVERALL GENE EXPRESSION

In order to gain better insights into the precise molecular mechanisms underlying the previously described phenotypic effects promoted by latexin, high-throughput profiling of gene expression by RNA sequencing (RNA-seq) was performed. Concretely, we characterized the transcriptional profile in samples corresponding to empty vector-transfected SH-SY5Y cells, empty vector-transfected SH-SY5Y cells treated with RA, latexin-overexpressing SH-SY5Y cells, and latexin-overexpressing SH-SY5Y cells treated with RA, after 5 days of incubation. Illumina sequencing produced high quality reads from all samples. Among the aligned reads, on average 85% were mapped to unique genome regions. On the basis of their detected RNA levels, differentially expressed genes (DEGs) between samples were identified through pairwise comparisons, as follows: RA-treated empty vector-transfected relative to empty vector-transfected cells (comparison 1), latexin-overexpressing cells relative to empty vector-transfected cells (comparison 2), RA-treated latexin-overexpressing cells relative to latexin-overexpressing cells (comparison 3) and RA-treated latexin-overexpressing cells relative to RA-treated empty vector-transfected cells (comparison 4). A schematic representation of the relationship between sample conditions and the comparative analysis performed is presented in **Figure 35A**. An initial overview of the transcriptional profiles of the different samples was provided by the volcano plots representations of the previously established comparisons (**Figure 35B**). Overall, these representations indicated a high quality of the transcriptional profiles, since a large number of the identified DEGs bore statistical significance values, in terms of false discovery rate (FDR), below the 0.01 threshold.

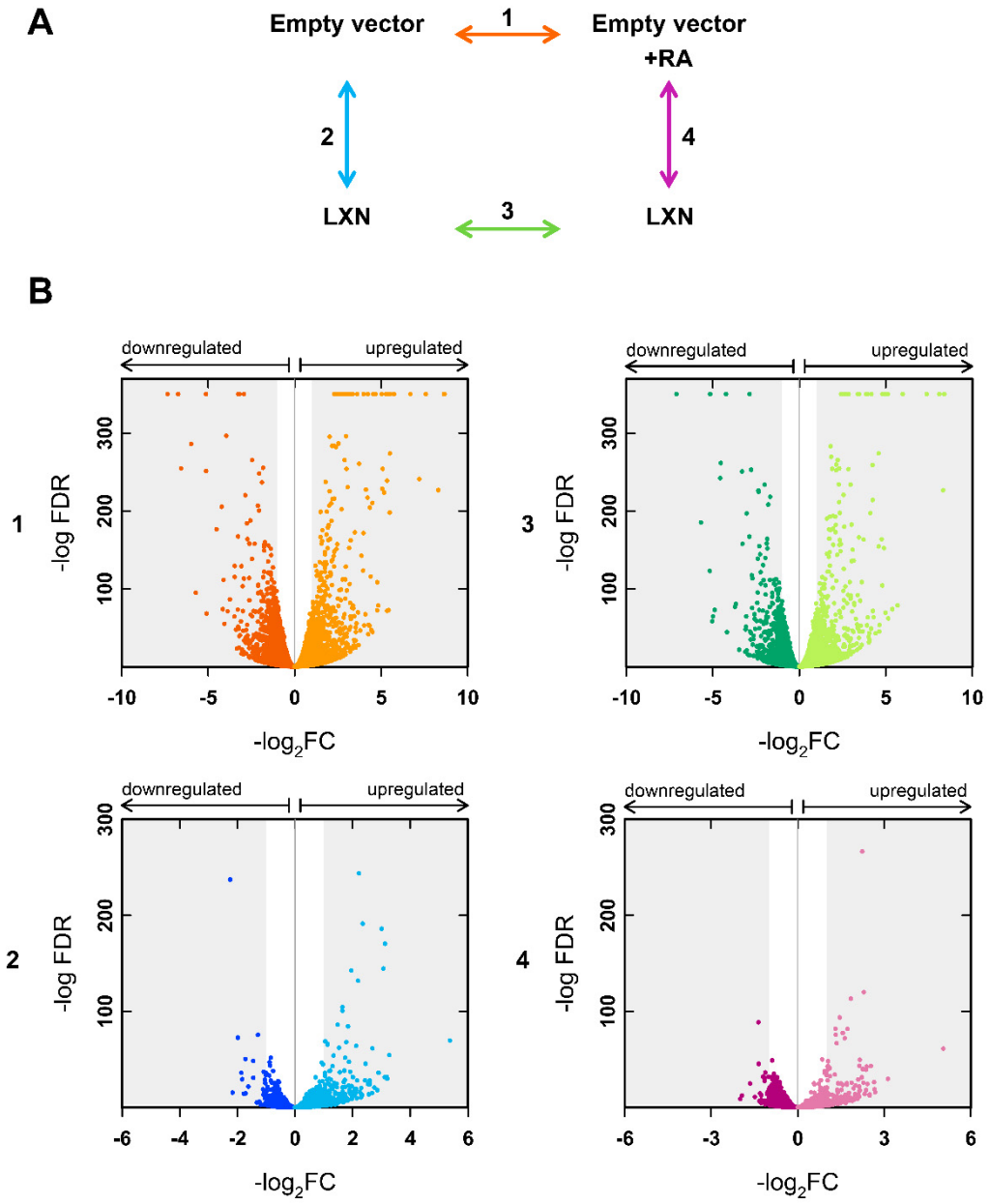

Figure 35A. Schematic representation of the comparative analysis performed and overview of the overall gene expression profiles. *A*, Schematic representation of the pairwise comparisons analyzed. Differential expressed genes (DEGs) were identified from the following pairwise comparisons: RA-treated empty vector-transfected relative to empty vector-transfected cells (comparison 1, orange), latexin-overexpressing cells relative to empty vector-transfected cells (comparison 2, blue), RA-treated latexin-overexpressing cells relative to latexin-overexpressing cells (comparison 3, green) and RA-treated latexin-overexpressing cells relative to RA-treated empty vector-transfected cells (comparison 4, purple). *B*, Distribution of DEGs in each pairwise comparison were displayed as volcano plot representations. These representations associate the magnitude of the change in expression (fold change, FC) and the statistical significance (false discovery rate, FDR).

More specifically, volcano plots of comparisons 1 and 3 showed that DEGs in empty vector-transfected and latexin-overexpressing cells in response to RA treatment followed a similar distribution (**Figure 36B**). Indeed, pairwise correlation of fold change (FC) values for genes with FDR < 0.01 between conditions 1 and 3 yielded

a high Pearson's coefficient, with very few genes showing opposed expression changes (**Figure 36A**). This indicated that the effects induced by RA at the transcriptional level, followed a similar distribution in empty vector-transfected and latexin-overexpressing cells. Volcano plots of conditions associated to latexin overexpression (comparison 2 and 4) presented a lower overall number of DEGs compared to conditions associated to RA treatment (comparisons 1 and 3) (**Figure 36B**). Likewise, pairwise correlation of FC values revealed a significant correlation between DEGs in comparisons 2 and 4, though significantly lower than in RA conditions with a higher proportion of genes with opposed expression changes (**Figure 36C**). This finding suggests that latexin promoted a similar distribution of changes in gene expression, regardless of RA treatment (**Figure 36C**). Finally, changes in gene expression promoted by RA in either empty vector-transfected cells (condition 1) or latexin-overexpressing cells (comparison 3) did not significantly correlate with those promoted by latexin overexpression solely (comparison 2) (**Figures 36B and 36D**).

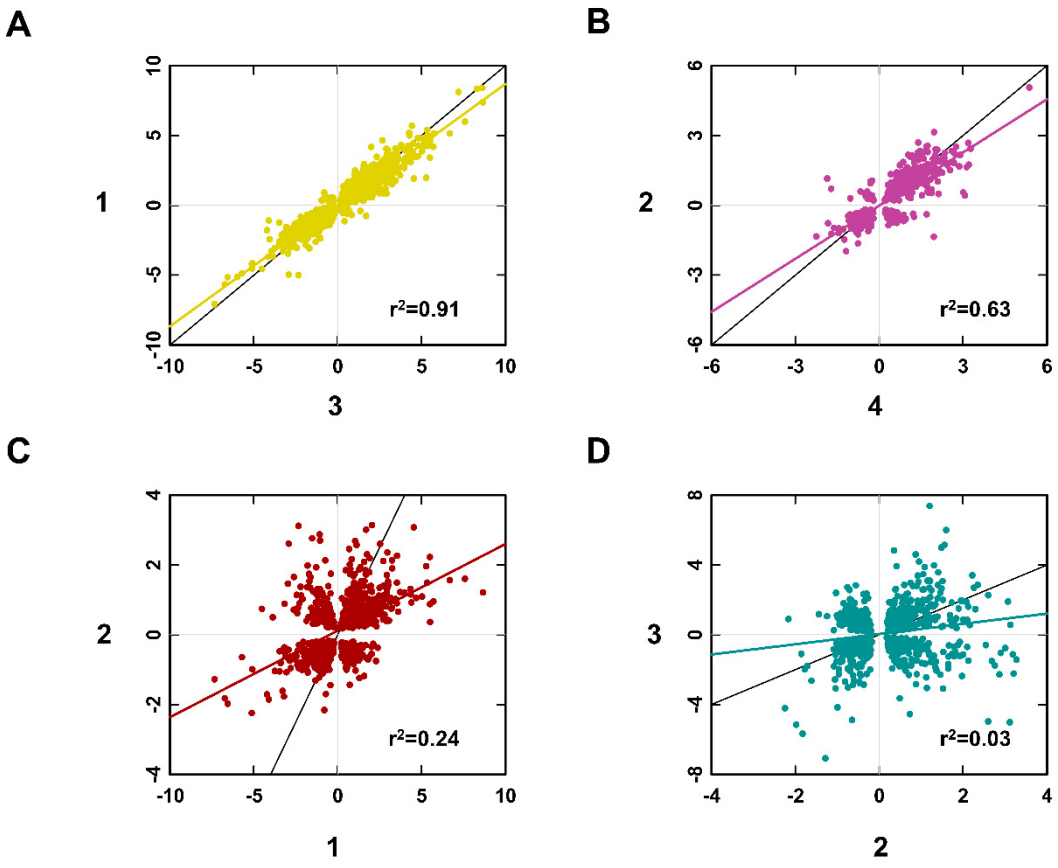

Figure 36. DEGs fold changes of RA treatment or latexin-overexpression in different cellular conditions correlate better than relative to each other. Fold change correlation (-$\log_2$) of DEGs with FDR < 0.01 between: *A,* Comparison 1 and comparison 3. *B*, Comparison 1 and comparison 2. *C*, Comparison 2 and comparison 4. *D*, Comparison 2 and comparison 3.

In order to retrieve relevant gene ontologies (GOs) associated to each comparison, stringent cutoffs were applied so as to define DEGs datasets of an appropriate size. This was achieved by selecting DEGs with associated FDR < 0.001 and an absolute FC > 1. By applying these filters, pairwise comparison of empty

vector-transfected vs RA-treated empty vector-transfected cells (comparison 1) identified a total of 1915 DEGs, with 1136 upregulated and 779 downregulated in RA-treated empty vector-transfected cells. The number of DEGs identified in latexin-overexpressing cells compared to empty vector transfected cells (comparison 2) was 207, with 180 upregulated and 27 downregulated in latexin-overexpressing cells. Pairwise comparison of latexin-overexpressing cells vs RA-treated latexin-overexpressing cells (comparison 3) identified a total of 1515 DEGs. Among them, 981 were upregulated and 534 were downregulated in RA-treated latexin-overexpressing cells. Finally, 202 DEGs were identified in RA-treated latexin-overexpressing cells relative to RA-treated empty vector-transfected (comparison 4), with 169 upregulated and 33 downregulated in latexin-overexpressing cells treated with RA (**Table 9**).

Table 9. Summary of DEGs in each pairwise comparison.

Comparison	DEGs FDR < 0.001	Upregulated	Downregulated
1	1915	1136	779
2	207	180	27
3	1525	981	534
4	202	169	33

3.2. CHANGES IN THE TRANSCRIPTIONAL PROFILE RESULTING FROM LATEXIN OVEREXPRESSION

We first aimed to define the transcriptional profile of latexin-overexpressing cells compared to empty vector-transfected cells (comparison 2). Interestingly, the sole overexpression of latexin resulted in large changes of gene expression. Concretely, a total of 207 genes were differentially expressed in latexin-overexpressing cells relative to empty vector-transfected cells; 180 were upregulated and only 27 were downregulated, indicating the tendency to upregulation exerted by this protein (FDR< 0.001 and FC > 1) (**Figure 37A**). The DAVID Functional Annotation Clustering Tool (Sherman et al. 2007; Huang et al. 2008; Huang et al. 2007) was employed to identify significantly enriched GO categories associated to those DEGs (FDR < 0.05). Upregulated DEGs were categorized into three different categories of GOs: Molecular Function, Cellular Component and Biological Process (Ashburner et al. 2000). Within Biological Process category, the most significant GOs belonged to ‘skeletal system development’ and ‘cell adhesion’ followed by ‘cell migration’ and ‘neuron differentiation’ processes (**Figure 37B**). Remarkably, the most prominent Cellular Component categories were ‘extracellular region’ and ‘extracellular matrix’ (**Figure 37B**). On the other hand, GOs categorized within Molecular Function were enriched in ‘extracellular matrix constituent’ (**Figure 37B**). Notably, downregulated DEGs did not result in any statistically enriched GO term. Moreover, to ascertain the potential pathways associated with those DEGs, we employed the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto 2000), which revealed that ‘ECM-receptor interaction’ and ‘Pathways in cancer’

were significantly enriched. Downregulated DEGs did not result in any statistically enriched pathway. Of note, among the upregulated genes we could identify important regulators of RA signaling such as the Cellular Retinoic Acid Binding Protein 1 (CRABP1), the Cellular Retinoic Acid Binding Protein 2 (CRABP2), and the Retinoic Acid Receptor Beta (RARβ), all of them important regulators of RA signaling (Noy 2010).

Overall, the overexpression of latexin resulted in large changes of gene expression, most of them related to cell adhesion processes. However, as previously described, the sole overexpression of latexin did not result in noticeably morphology changes in cells cultured for up to 5 days (Chapter 1). Nevertheless, such functions associated with cell adhesion processes were consistent with the differential behavior observed when maintaining cells for up to 10 days in culture. More concretely, we observed that the sole overexpression of latexin avoided the tendency of empty vector-transfected cells to grow in clumps of rounded cells on top of one another (**Figure 37C**). Taken altogether, analyzing the DEGs in this condition could shed light into the genes regulated by latexin but also into those likely responsible of such differential response observed after long-term in culture.

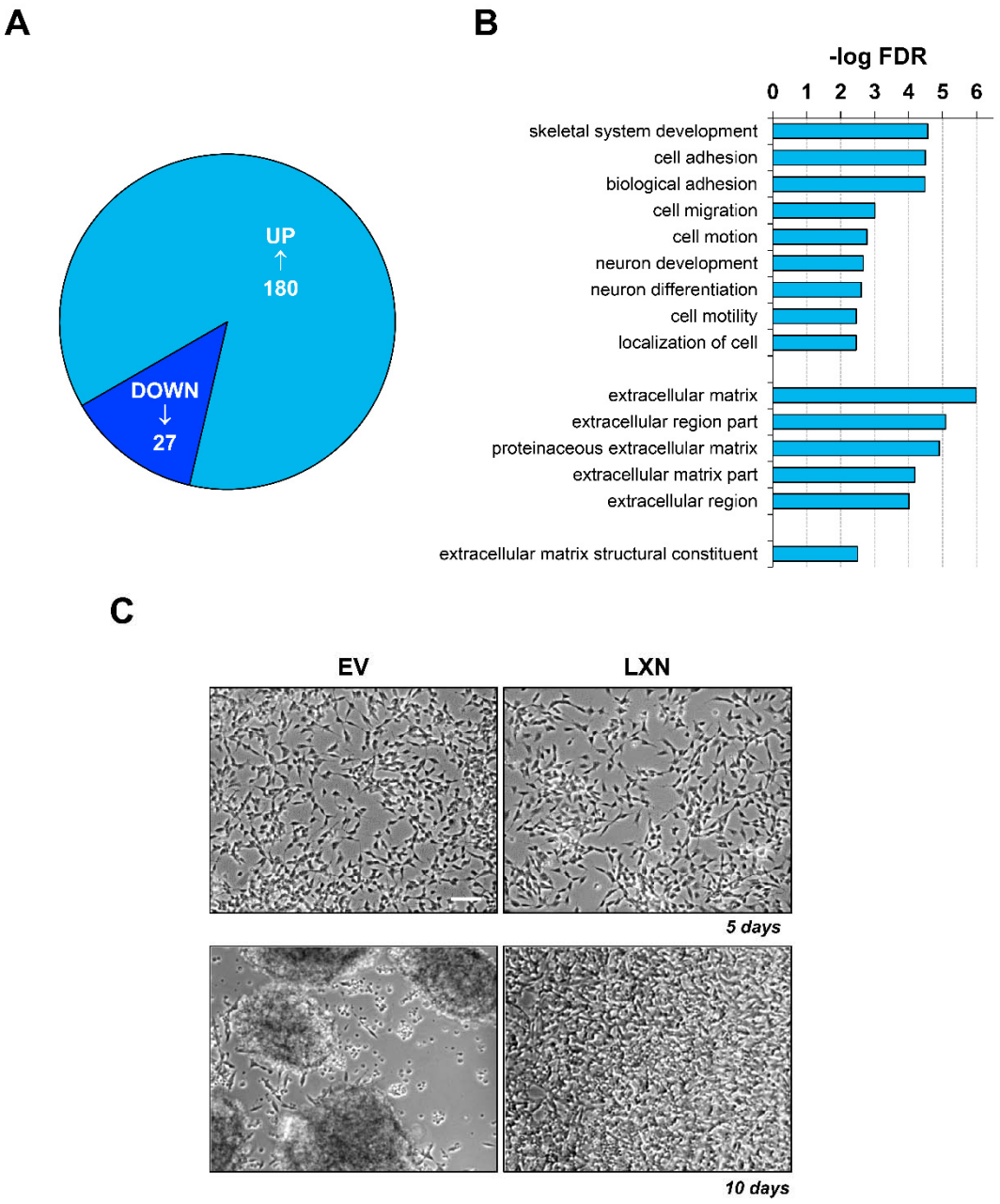

Figure 37. Changes in the transcriptional profile provided by latexin overexpression. *A,* By filtering differential expressed genes (DEGs) with associated FDR < 0.001 and an absolute FC > 1, latexin-overexpressing cells compared to empty vector transfected cells (comparison 2) regulated 207 genes, 180 were upregulated and 27 were downregulated. *B,* Gene Ontology (GO) enrichment analysis was performed by DAVID Functional Annotation Clustering Tool. Biological Process, Cellular Component and Molecular functions with a FDR < 0.05 are shown (log FDR). *C*, Empty vector transfected (EV) or latexin-overexpressing (LXN) cells were cultured for up to 10 days. Representative phase contrast microphotographs for each condition are shown. Scale bar = 70 μm.

3.3. CHANGES IN THE TRANSCRIPTIONAL PROFILE PROVIDED BY RA TREATMENT

Once we have established the GOs and biological pathways associated to the overexpression of latexin, we sought to determine the landscape of gene expression promoted by RA treatment alone. To this end, we first explored the transcriptional profile of empty vector-transfected cells induced to differentiate with RA for 5 days. As shown in **Figure 38A**, RA-treatment resulted in large changes of gene expression in empty vector-transfected cells, showing a greater tendency to the upregulation (FDR < 0.001 and FC >1). GO analysis performed by DAVID revealed that RA promoted substantial expression changes in genes related to several biological functions (FDR < 0.05). More concretely, the 1136 upregulated DEGS were predominantly associated to biological processes such as 'cell adhesion', 'neuron development', 'synaptogenesis', 'extracellular structure organization', 'morphogenesis' and 'transmission of nerve impulse' (**Figure 38B**). Downregulated DEGs were enriched in biological processes including 'M phase', 'cell cycle', 'cell division' and 'positive regulation of cell proliferation' (**Figure 38C**). Consistently, transcriptional changes induced by RA in empty vector-transfected cells are in good agreement with those reported in the literature for wild type SH-SY5Y cells treated with RA (Wang et al. 2014). Overall, these data indicated that RA mainly upregulates the expression of genes involved in cell differentiation, morphogenesis and cell adhesion whereas it downregulates the expression genes involved in cell cycle control, being all canonical functions previously associated to RA (Pratt et al. 2003; Maden 2007; Niederreither & Dollé 2008).

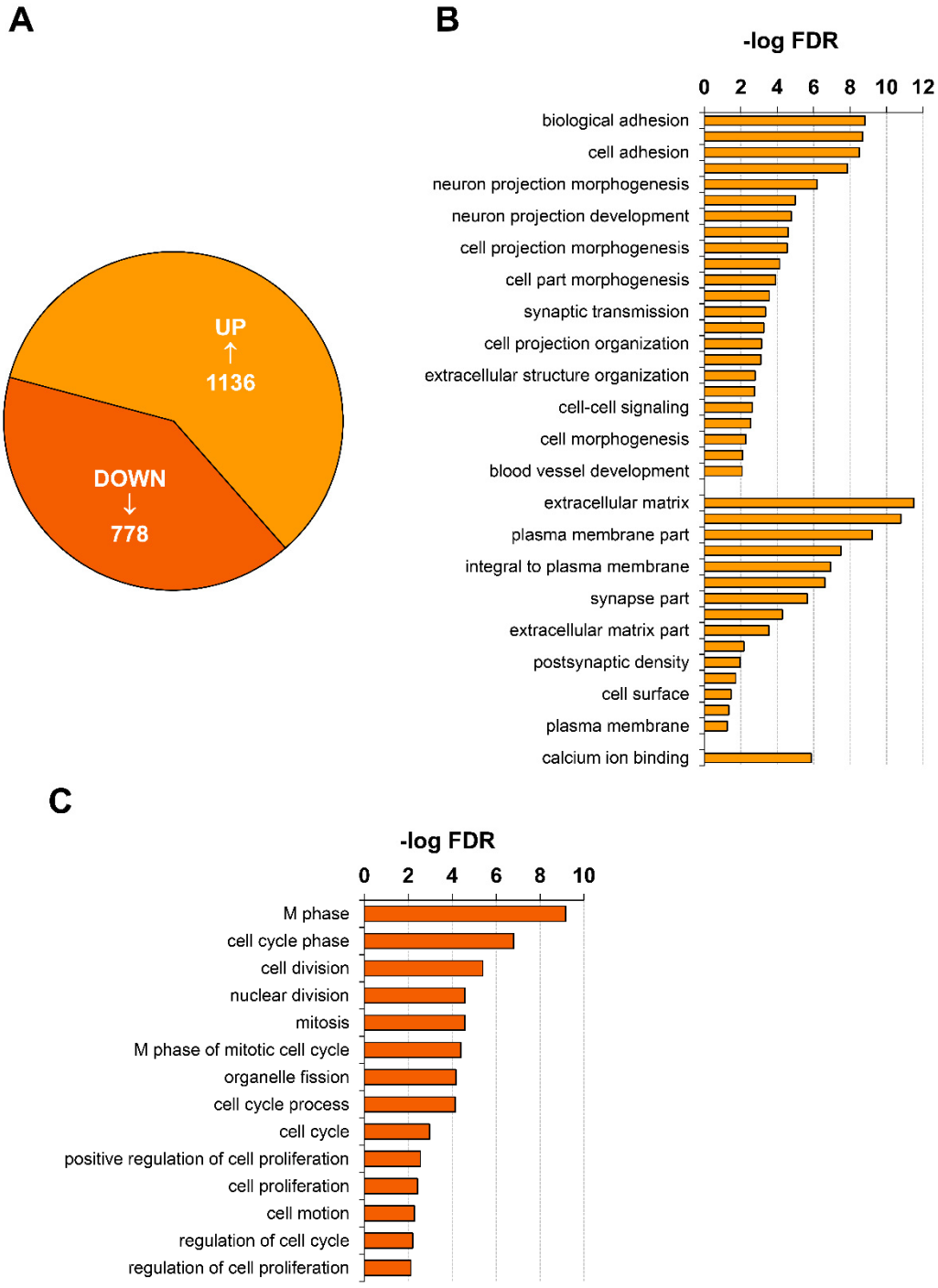

Figure 38. Changes in the transcriptional profile promoted by RA in empty vector-transfected cells. *A,* By filtering differential expressed genes (DEGs) with associated FDR < 0.001 and an absolute FC > 1, RA-treated empty vector-transfected cells compared to empty vector transfected cells (comparison 1) regulated 1914 genes, 1136 were upregulated (light orange) and 778 were downregulated (dark orange). Gene Ontology (GO) enrichment analysis was performed by DAVID Functional Annotation Clustering Tool. Biological Process, Cellular Component and Molecular functions with a FDR < 0.05 are shown (log FDR) for: *B*, upregulated DEGs. *C*, Downregulated DEGs.

Since we have previously established that latexin-mediated effects require the synergy with RA, we aimed to characterize whether DEGs promoted by either latexin overexpression or RA treatment were coincident. For this purpose, Venn diagrams were created to identify common or differential up or downregulated genes between comparison 1 and 2 (**Figure 39**). As shown in **Figure 39**, 65 genes were commonly upregulated in both 1 and 2 comparisons whereas 115 genes were only differentially upregulated by latexin overexpression. These results indicated that the transcriptional changes induced by latexin overexpression were not mainly comprised in the changes promoted by RA in empty vector-transfected cells. Remarkably, shared DEGs within the upregulated gene set predominantly represented 'extracellular matrix organization', 'cell adhesion 'and 'skeletal system development' (**Figure 39**). On the other hand, the 115 genes exclusively upregulated by latexin were only significantly associated with morphogenesis biological processes. Notably, DEGs that were only upregulated by RA predominantly represented 'cell adhesion' and 'synaptogenesis' processes (**Figure 39**). When considering the downregulated genes, 16 genes appeared downregulated in the two sets (comparison 1 and 2), whereas 11 were only downregulated in latexin overexpressing cells. However, downregulated genes were not significantly associated with any GO or pathway (FDR < 0.05). Taken all together, the sole overexpression of latexin is able to regulate the expression of a large number of genes, which are predominantly related to cell adhesion and neuron development functions. In addition, transcriptional changes promoted by the overexpression of latexin did not largely overlap with those induced by RA, suggesting putative divergent functions between both, latexin expression and RA treatment.

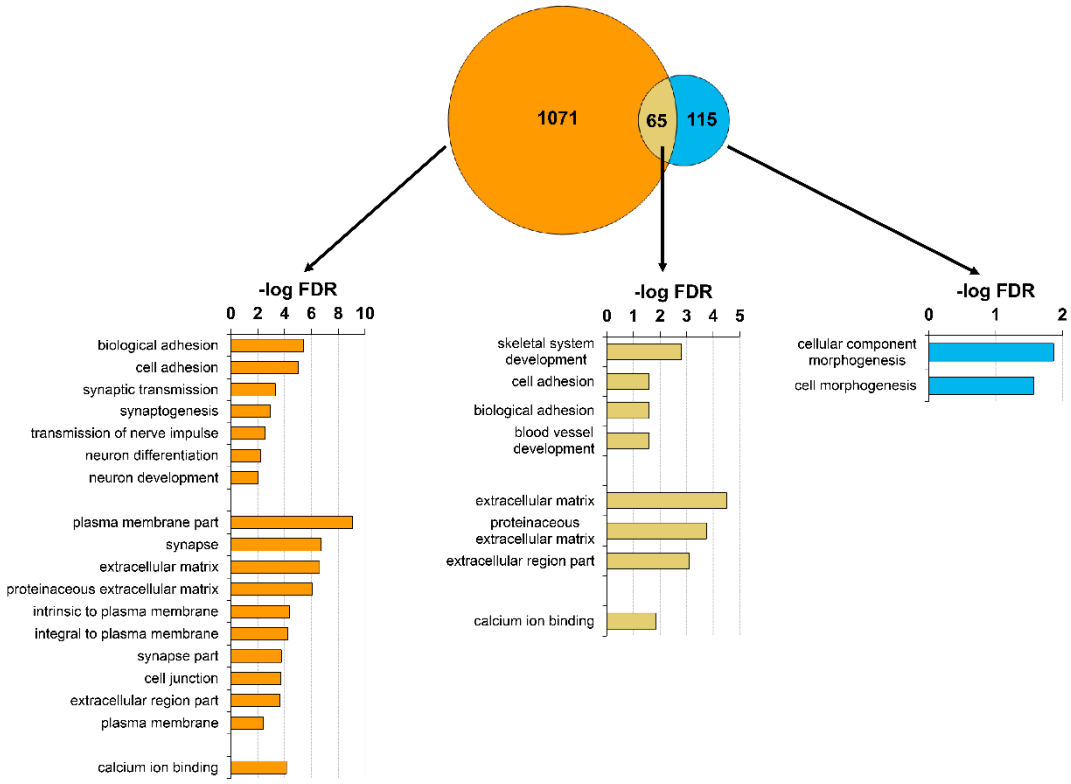

Figure 39. Latexin-induced changes in gene expression are not highly overlapped with those promoted by RA treatment. Venn diagram showing differential and common differential expressed genes (DEGs) with associated false discovery rate (FDR) < 0.001 and fold change (FC) > 1 between comparison 1 and 2. Of note, 65 genes were commonly upregulated by latexin overexpression and RA-treatment in empty vector-transfected cells while 115 were only upregulated by latexin overexpression.

Once we have ascertained the effects that latexin overexpression and RA treatment promote at the transcriptional level, we next analyzed how RA modified the gene expression profile when cells are overexpressing latexin (comparison 3). In cells overexpressing latexin, RA treatment significantly upregulated 981 genes and downregulated 534 genes (FDR < 0.001 and FC >1) (**Figure 40A**). Notably, RA-regulated DEGs were lower in cells overexpressing latexin compared with cells stably transfected with empty vector. This effect was also translated in a great reduction of GOs obtained by DAVID analysis. Moreover, when analyzing the 981 upregulated DEGs, the most significant Biological Processes belonged to the category 'cell adhesion' and 'extracellular structure organization'. Among the Cellular Component part, 'plasma membrane', 'extracellular matrix', 'synapse' and 'cell junction' were the most relevant terms (**Figure 40B**). The only statistically enriched GO within Molecular Function was 'calcium ion binding'. When analyzing downregulated DEGs, the most significant GOs belonged to the categories 'neuron differentiation', 'cell cycle phase', 'cellular component morphogenesis' and 'M phase' (**Figure 40C**). Interestingly, the most prominent term within the Cellular Component category of downregulated DEGs was 'chromosomal part' (**Figure 40C**). Regarding Molecular Function, the only statistically enriched term associated to downregulated DEGs was 'transmembrane receptor protein tyrosine kinase activity' (**Figure 40C**).

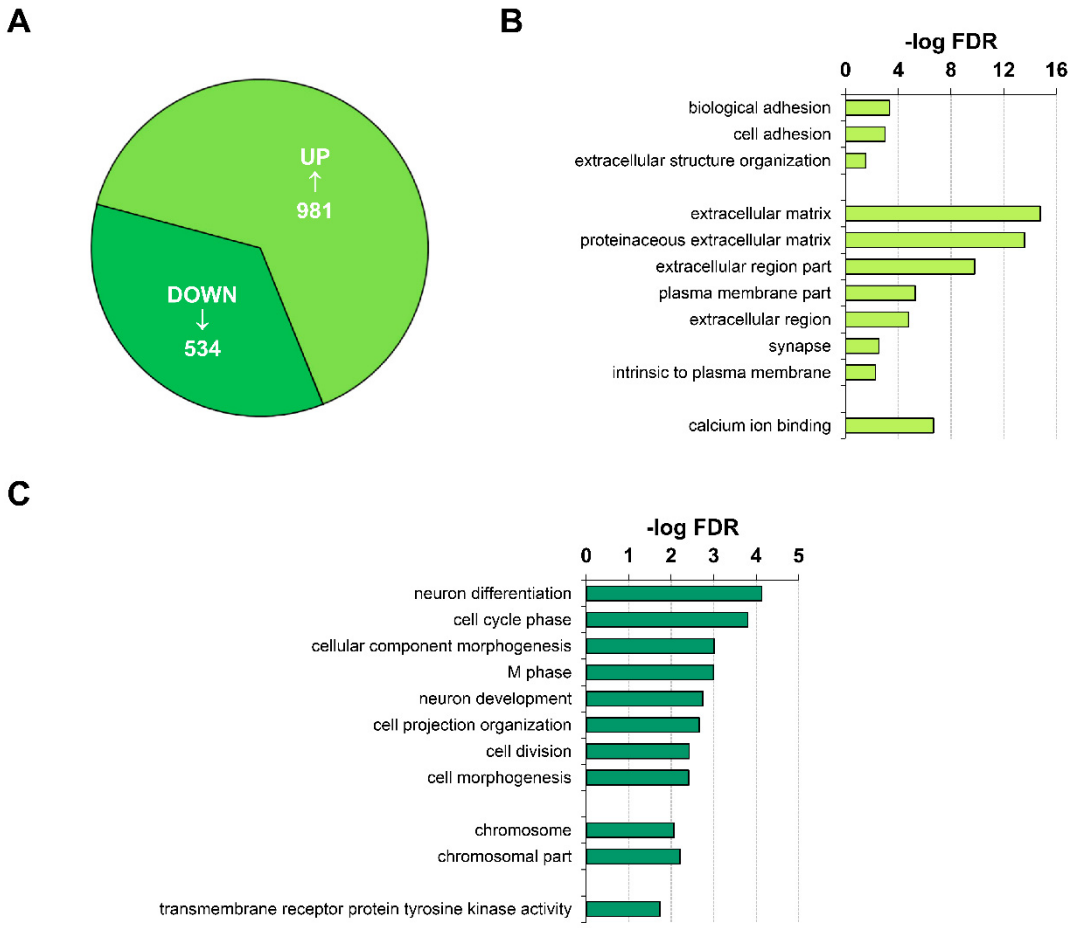

Figure 40. Changes in the transcriptional profile promoted by RA in cells overexpressing latexin. *A,* By filtering differential expressed genes (DEGs) with associated FDR < 0.001 and an absolute FC > 1, RA-treated empty vector-transfected cells compared to empty vector transfected cells (comparison 1) regulated 1914 genes, 1136 were upregulated (light green) and 778 were downregulated (dark green). Gene Ontology (GO) enrichment analysis was performed by DAVID Functional Annotation Clustering Tool. Biological Process, Cellular Component and Molecular functions with a FDR < 0.05 are shown (log FDR) for: *B*, upregulated DEGs. *C*, Downregulated DEGs.

We then compared the gene expression profiles of empty vector-transfected cells and latexin-overexpressing cells in response to RA treatment (comparison 1 and 3) (**Figure 41**). Among the upregulated DEGs, 690 were coincident between both comparisons, 291 were exclusively upregulated in latexin-overexpressing cells in response to RA treatment and 446 were only upregulated in empty vector-transfected cells in response to RA treatment (**Figure 41**). Common upregulated DEGs between comparison 1 and 3 were mainly associated to 'cell-cell signaling', 'cell adhesion' and 'extracellular matrix' processes (**Figure 41**). Moreover, specific upregulated genes in empty vector-transfected cells in response to RA treatment were predominantly related to 'synapse organization' and 'plasma membrane'. No statistically enriched GO was obtained for the 291 genes exclusively upregulated in latexin-overexpressing cells determined after a correction was applied to the data at a FDR < 0.05.

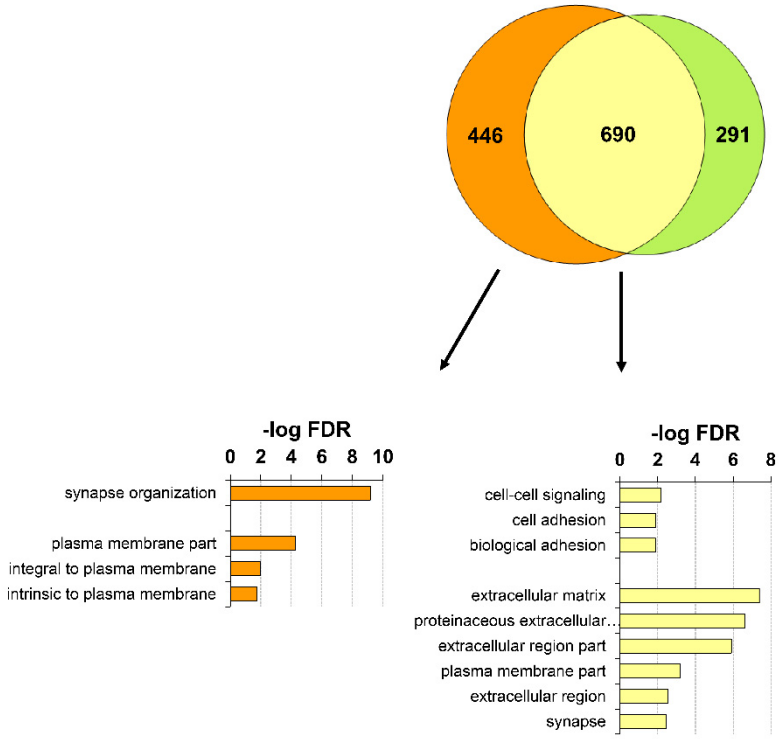

Figure 41. RA upregulation of gene expression is not completely coincident in empty vector-transfected cells and latexin-overexpressing cells. Venn diagram showing differential and common upregulated differential expressed genes (DEGs) with associated false discovery rate (FDR) < 0.001 and fold change (FC) > 1 between comparison 1 and 3. Of note, 690 genes were commonly upregulated by RA treatment in either cells transfected with empty vector or overexpressing latexin, 446 were exclusively upregulated by RA in empty vector-transfected cells and 291 were specifically upregulated by RA in latexin-overexpressing cells. Biological Process, Cellular Component and Molecular Function Gene ontologies (GOs) were obtained by DAVID Functional Annotation Clustering Tool in either common or differential DEGs.

Regarding downregulated DEGs, 418 genes were common between both comparisons, 361 specifically downregulated in empty vector-transfected cells in response to RA treatment and 116 exclusively downregulated in latexin-overexpressing cells in response to RA treatment (**Figure 42**). Remarkably, common downregulated DEGs were mainly associated to 'neuron differentiation', 'cell division' and 'neuron projection morphogenesis'. The 361 downregulated genes by RA treatment in empty vector transfected cells were enriched in 'M phase' and 'cell cycle phase' processes, whereas none significantly enriched term was obtained for the 116 downregulated genes in comparison 3 (**Figure 42**). Overall, the transcriptional effect of RA treatment was largely coincident in empty vector-transfected cells and latexin-overexpressing cells. However, RA-induced changes in gene expression were less prominent in cells overexpressing latexin. Moreover, cell adhesion and extracellular matrix (ECM) related processes were commonly promoted by RA treatment in either empty vector-transfected cells or latexin-overexpressing cells. In contrast, synapse organization process was only promoted by RA treatment in empty vector transfected cells.

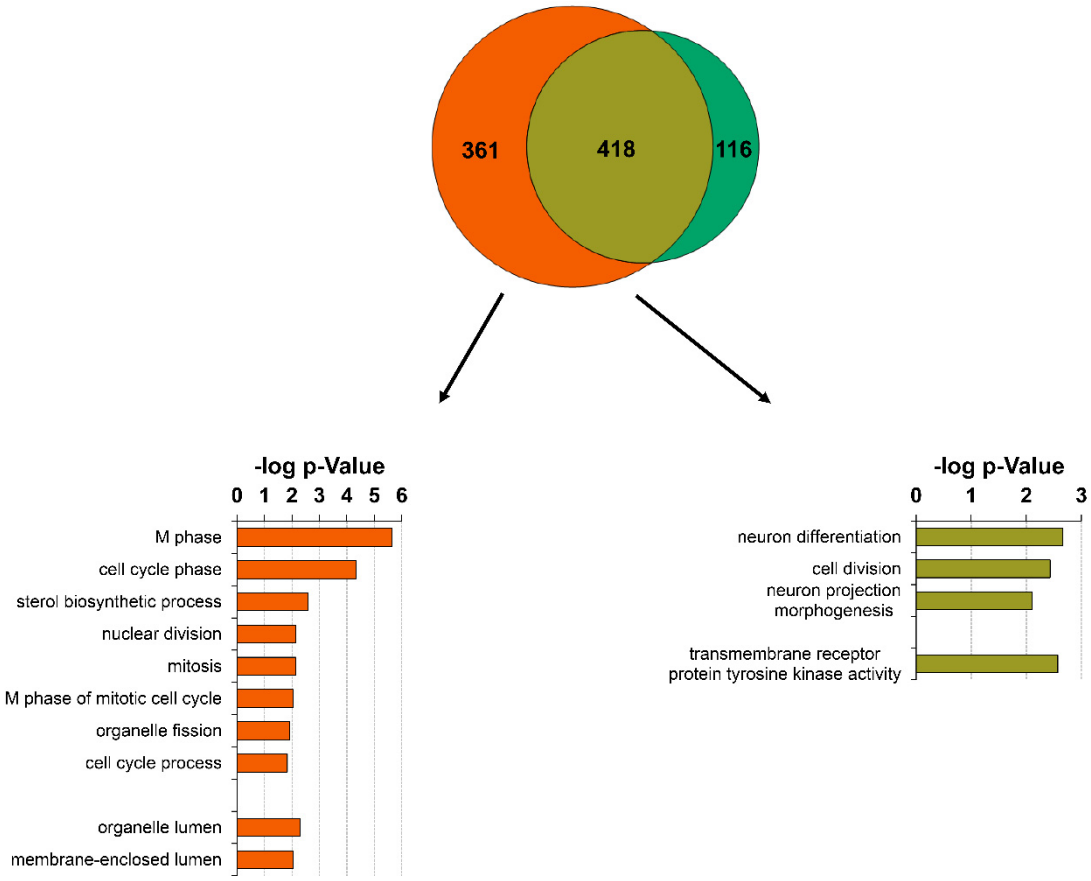

Figure 42. RA upregulation of gene expression is not completely coincident in empty vector-transfected cells and latexin-overexpressing cells. Venn diagram showing differential and common downregulated differential expressed genes (DEGs) with associated false discovery rate (FDR) < 0.001 and fold change (FC) > 1 between comparison 1 and 3. Of note, 418 genes were commonly downregulated by RA treatment in either cells transfected with empty vector or overexpressing latexin, 361 were exclusively downregulated by RA in empty vector-transfected cells and 116 were specifically downregulated by RA in latexin-overexpressing cells. Biological Process, Cellular Component and Molecular Function Gene ontologies (GOs) were obtained by DAVID Functional Annotation Clustering Tool in either common or differential DEGs.

3.4. DIFFERENTIAL TRANSCRIPTIONAL PROFILE OF RA-TREATED LATEXIN-OVEREXPRESSING CELLS COMPARED TO RA-TREATED EMPTY VECTOR-TRANSFECTED CELLS

Finally, we analyzed the transcriptional differences between RA-treated empty vector-transfected cells and RA-treated latexin-overexpressing cells (comparison 4). Notably, 169 genes were upregulated and 33 were downregulated in RA-treated latexin-overexpressing cells (FDR < 0.001 and FC >1) (**Figure 43A**). The most significant GOs belonged to the categories ‘cell motion’, ‘migration’, ’response to mechanical stimulus’ and ‘extracellular structure organization’ (**Figure 43B**). Remarkably, ‘extracellular region’ and ‘extracellular

matrix' were the most significantly enriched terms within the Cellular Component category (**Figure 43B**).

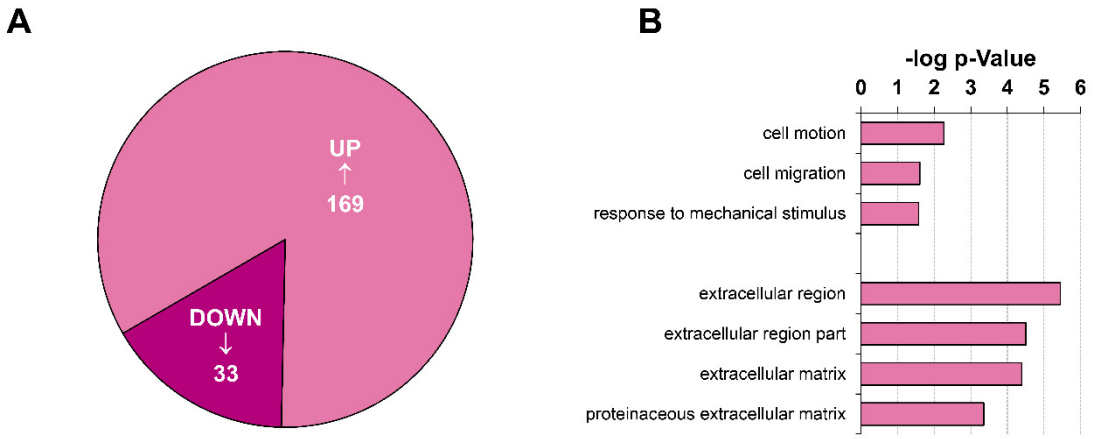

Figure 43. Transcriptional profile comparison between RA-treated latexin-overexpressing cells and empty vector-transfected cells. *A,* By filtering differential expressed genes (DEGs) with associated FDR < 0.001 and an absolute FC > 1, RA-treated latexin-overexpressing cells compared to RA-treated empty vector transfected cells (comparison 4) regulated 202 genes, 169 were upregulated (light purple) and 33 were downregulated (dark purple). *B,* Gene Ontology (GO) enrichment analysis was performed by DAVID Functional Annotation Clustering Tool. Biological Process, Cellular Component and Molecular functions with a FDR < 0.05 are shown (log FDR) for upregulated DEGs.

Moreover, the analysis of common and differential DEGs between conditions 2 and 4 would also shed light into the functions that are intrinsically regulated by latexin expression and the ones that latexin promotes synergistically with RA. As depicted in **Figure 44**, upregulated DEGs promoted by latexin overexpression either in the presence or in the absence of RA, were associated to 'collagen fibril organization' and 'extracellular matrix' terms. On the other hand, DEGs exclusive of non-treated latexin-overexpressing cells were enriched in 'cell adhesion' and 'skeletal system development' processes (**Figure 44**). DEGs only upregulated by RA-treated latexin-overexpressing cells did not result in any significant enriched GO.

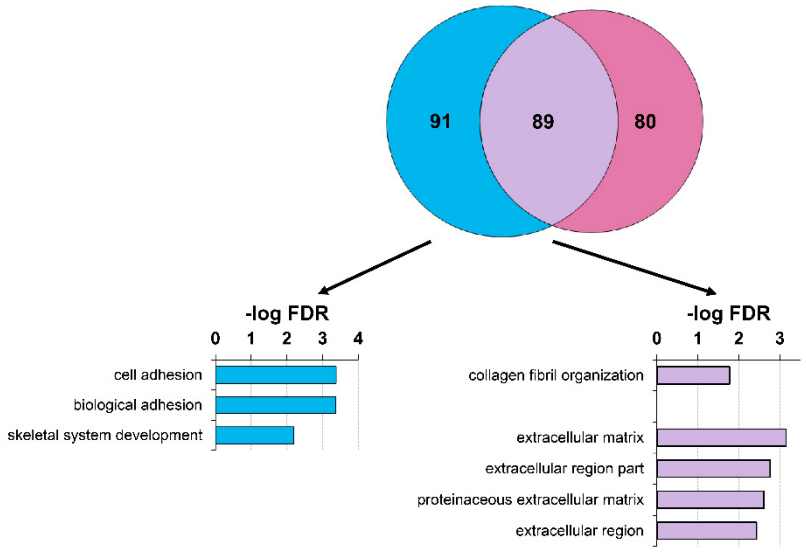

Figure 44. RA upregulation of gene expression is not completely coincident in empty vector-transfected cells and latexin-overexpressing cells. Venn diagram showing differential and common upregulated differential expressed genes (DEGs) with associated false discovery rate (FDR) < 0.001 and fold change (FC) > 1 between comparison 2 and 4. Of note, 89 genes were commonly upregulated by latexin expression in the presence or in the absence of RA treatment, 91 were exclusively upregulated by non-treated latexin-overexpressing cells and 80 were specifically upregulated by RA-treated latexin-overexpressing cells. Biological Process, Cellular Component and Molecular Function Gene ontologies (GOs) were obtained by DAVID Functional Annotation Clustering Tool in either common or differential DEGs.

Since we previously revealed that latexin facilitates the S-type phenotype in response to RA treatment in SH-SY5Y cells (Chapter 1 and 2), we next analyzed whether already reported markers of either N-type or S-type phenotypes were differential expressed in the overall conditions (**Figure 45**). As shown in **Figure 45**, latexin-overexpressing cells significantly promoted the upregulation of several markers of the S-type phenotype whereas N-type markers remained unaltered. Taken altogether, most of the genes differentially regulated in RA-treated latexin-overexpressing cells compared to RA-treated empty vector-transfected cells were associated to the ECM. In addition, the overexpression of latexin significantly promoted the upregulation, at the transcriptional level, of several markers characteristic of the S phenotype.

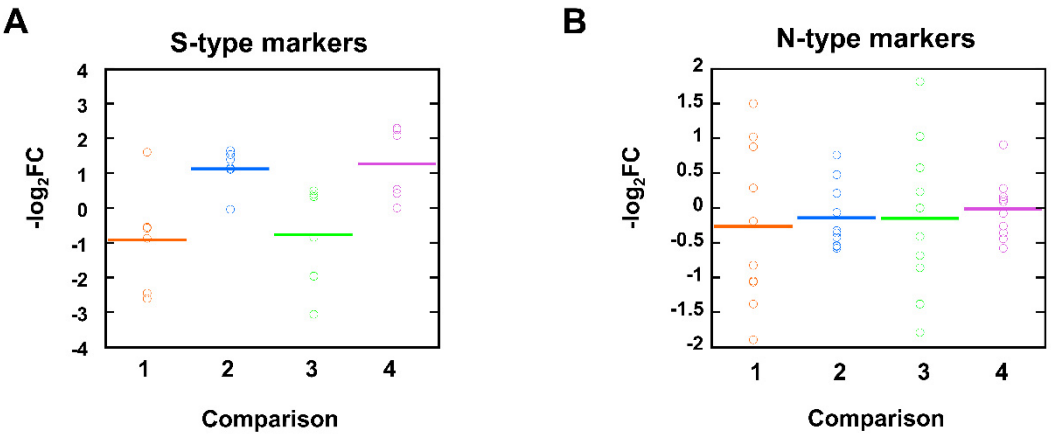

Figure 45. Latexin overexpression promotes gene expression upregulation of S-type markers. Scatter plots of the expression in log2 in all the conditions surveyed of several genes related to: *A*, S-type markers. *B*, N-type markers.

3.5. GENE VALIDATION BY REVERSE TRANSCRIPTION QUANTITATIVE PCR (QRT-PCR)

We selected nine genes (CD44, VIM, RARB, FOXC1, FGFR2, NTRK2, BDNF, CPA4 and LXN) to validate the expression profiles obtained from the RNA-seq analysis. We measured the RNA levels of each gene in the following conditions: empty vector-transfected cells, RA-treated empty vector-transfected cells, latexin-overexpressing cells and RA-treated latexin-overexpressing cells. Consistently, the selected genes tested by qRT-PCR exhibited the same range and direction of fold change than those obtained by the RNA-Seq analysis (**Figure 46**).

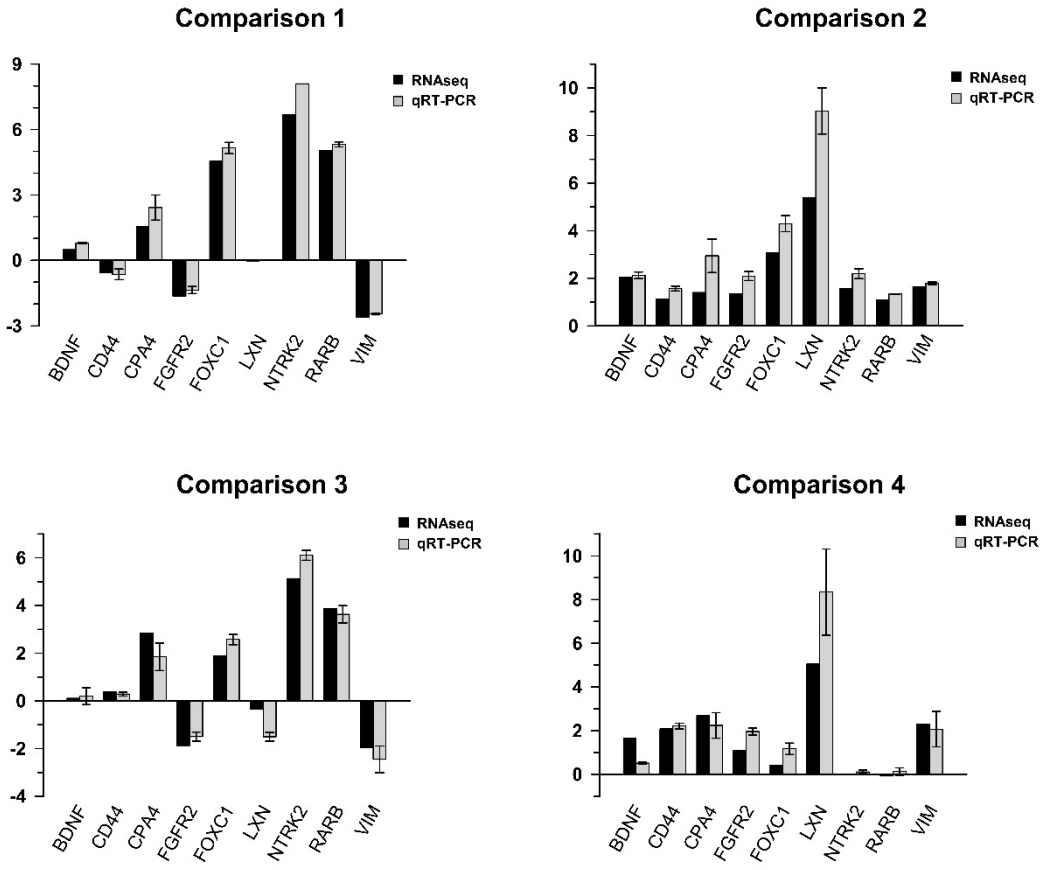

Figure 46. Gene validation by reverse transcription quantitative PCR. RNA levels of CD44, VIM, RARβ, FOXC1, FGFR2, NTRK2, BDNF, CPA4 and LXN was measured in the following conditions: empty vector-transfected cells, RA-treated empty vector-transfected cells, latexin-overexpressing cells and RA-treated latexin-overexpressing cells by qRT-PCR.

Taken altogether, the results presented in this Chapter indicate that latexin is able to regulate a large number of genes, most of them involved in cell adhesion, morphogenesis and development processes. Moreover, latexin also regulates the expression of genes associated with functions besides RA-differentiation, suggesting it could engage divergent functions upon the adequate stimuli. Surprisingly, the vast majority of genes upregulated by latexin either in the presence or in the absence of RA belong to the ECM, thus linking this structure to latexin-mediated effects in SH-SY5Y cells.

4. CHAPTER 4. LATEXIN-ASSOCIATED MORPHOLOGICAL EFFECTS IN OTHER CELLULAR MODELS

4.1. LATEXIN FACILITATES BRDU-INDUCED SENESCENCE IN NEUROBLASTOMA-DERIVED SK-N-LP CELLS

We have previously evidenced that latexin facilitates RA- and BrdU-induced transdifferentiation towards the S-type lineage. To extend our results to other cellular models, we explored the potential effects of latexin expression in different neuroblastoma cells. To this end, we first evaluated whether the expression of latexin could be modulated by RA or BrdU agents in a cell line characterized as S-type (SK-N-AS) or in another established as I-type (SK-N-LP). As shown in **Figure 47A,** both SK-N-LP and SK-N-AS cells upregulated latexin in response to BrdU treatment for 5 days. Interestingly, latexin upregulation upon BrdU treatment was remarkably more prominent in the I-type cell line SK-N-LP, suggesting the potential involvement of latexin in BrdU-mediated effects in this cell line (**Figure 47A**). Giving this result, and considering the bipotential ability of I-type cells to differentiate along both lineages, we focused on the SK-N-LP cell line for further experiments. Therefore, transiently and stable overexpression of latexin was performed in SK-N-LP cells (**Figure 47B**). SK-N-LP cell morphology was characterized by a round cell body, with few and short extensions and a slightly more proportion of cytoplasm than SH-SY5Y cells. As shown in **Figure 47C**, transiently overexpression of latexin resulted in an apparent rounding of cells. However, this differential effect was not further observed in cells stably overexpressing latexin (**Figure 47D**).

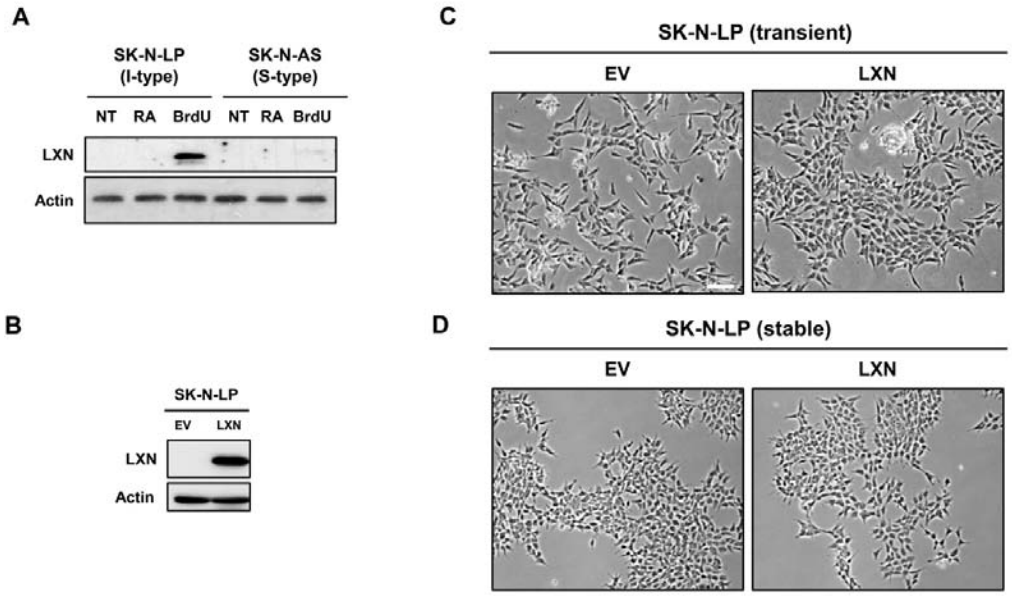

Figure 47. Latexin expression is upregulated upon BrdU treatment in SK-N-LP and SK-N-AS cells. *A,* Wild type SK-N-LP cells and SK-N-AS cells were treated with 10 μM RA or BrdU for 5 days or left untreated (non-treated, NT). Latexin expression was analyzed by *Western blotting*. β-actin was used as loading control. *B,* SK-N-LP cells were stably transfected with empty pcDNA3 or

pcDNA3-latexin plasmids. Protein extracts from empty vector transfected (EV) or latexin-overexpressing (LXN) cells were analyzed by *Western blotting* to corroborate the overexpression of latexin. The membranes were reprobed with β-actin antibody to check equal loading. *C,* Phase contrast microphotographs representative of transiently and stably transfected EV and LXN SK-N-LP cells. *Scale bars* = 70 μm.

We next sought to determine whether latexin expression resulted in phenotypic changes upon BrdU induction in SK-N-LP cells, as already observed in SH-SY5Y cells. Notably, BrdU exhibited an anti-proliferative in SK-N-LP cells, being this process apparently promoted in latexin-overexpressing cells (**Figures 48A and 48B**). In addition, SK-N-LP cells cultured in the presence of 10 μM BrdU adopted a more flattened morphology, resembling the S-type phenotype, in both empty vector-transfected and latexin-overexpressing cells (**Figure 48A**). After 15 days of treatment, BrdU-induced S-type cells became flatter, being the number of flattened cells apparently higher in latexin-overexpressing cells was (**Figure 48B**). Moreover, as time in culture increased, cells gradually died, and this effect was apparently attenuated in BrdU-trated latexin-overexpressing cells (**Figure 48B**). Since we have previously demonstrated the relevance of the PI3K/Akt axis on the transdifferentiation effect promoted by latexin (Chapter 2), we surveyed the impact of inhibiting PDK1 in SK-N-LP cells. As shown in **Figures 48A and 48B**, BrdU-treated cells cultured in the presence of 3 μM GSK2334470 exhibited a marked reduction in the overall cell numbers. Although Brdu-treated latexin-overexpressing cells cultured in the presence of 3 μM GSK2334470 exhibited a reduction of cell numbers compared with empty vector cells, cell death was not apparently promoted in latexin-overexpressing cells (**Figures 48A and 48B**).

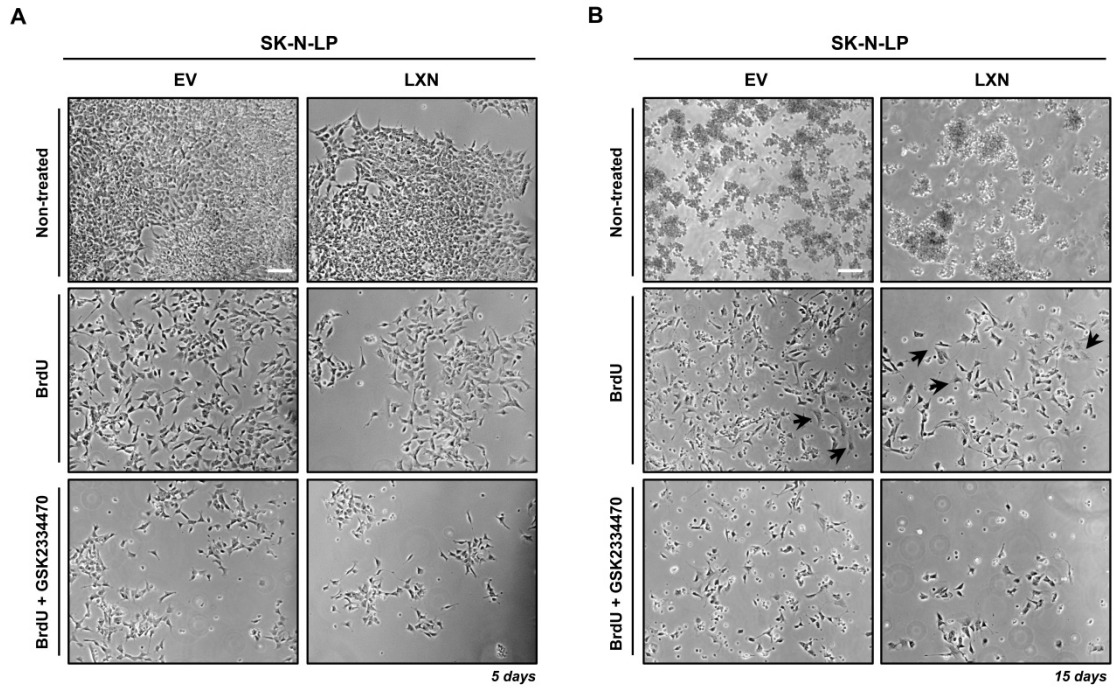

Figure 48. BrdU-induced S-type phenotype is facilitated by latexin overexpression in SK-N-LP cells. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells cultured for 1 hour in the presence or the absence of 3 μM GSK2334470 were treated with

10 µM BrdU or left untreated (non-treated). Phase contrast microphotographs representative of three independent experiments of cells treated during 5 days (*A*) and 15 days (*B*) are shown. S-type cells are highlighted by arrows. *Scale bar* = 70 µm.

A deeper analysis of cell morphology revealed cells displaying neuritic extensions were more abundant in empty vector-transfected cells (**Figure 49A**). Noticeably, cells with a flattened morphology, resembling the S-type phenotype, were more prominent in BrdU-treated latexin-overexpressing cells compared with empty vector-transfected cells. Since this flattened morphology was reminiscent of cellular senescence, we assessed whether this cellular response was activated upon BrdU treatment in SK-N-LP cells (**Figure 49B**). As shown in **Figure 497B**, latexin slightly increased the number of SA-β-Gal cells upon BrdU treatment. However, PDK1 inhibition did not significantly resulted in a great decrease in the number of senescent cells (**Figure 49B**). Taken altogether, latexin-overexpression facilitated BrdU-induced senescence in the I-type cell line SK-N-LP. In addition, the PI3K/Akt pathway did not play an essential role in this process as in the case of SH-SY5Y cells.

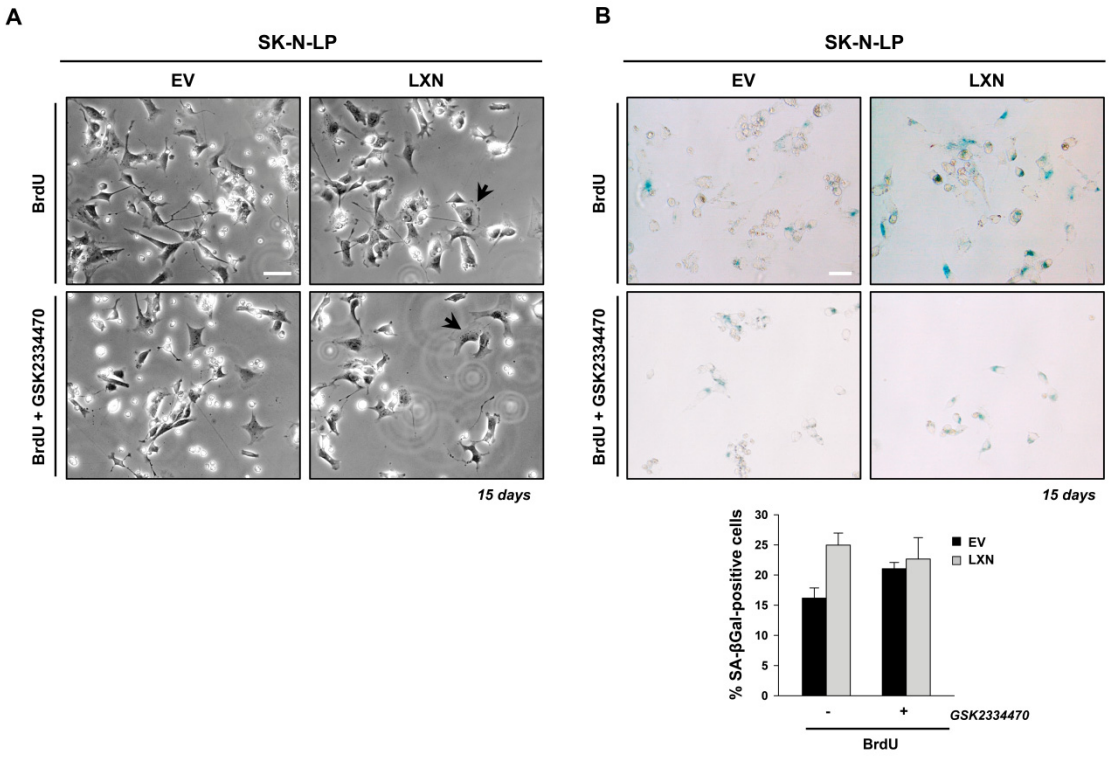

Figure 49. Latexin prompts BrdU-induced cellular senescence in SK-N-LP cells. Empty vector-transfected (EV) or latexin-overexpressing (LXN) cells cultured for 1 hour in the presence or the absence of 3 µM GSK2334470 were treated with 10 µM BrdU or left untreated (non-treated) for 15 days. *A*, Representative bright field microphotographs showing the cell morphology. S-type cells are highlighted by arrows. *B*, Senescence-associated β-galactosidase (SA-β-gal) assay was performed and analyzed by bright field microscopy in cells treated during 15 days or left untreated (Non-treated, -). The lower graph shows the percentage of SA-β-gal-positive cells over the total cells. The values are represented as mean ± S.D. (n=3). Scale bars = 50 µm.

4.2. LATEXIN PROMOTES CELL MORPHOLOGY CHANGES IN LN-18 CELLS

With the aim to extend our results to other cellular paradigms, we investigated the potential role of latexin in human glioblastoma-derived cells. To this end, we first screened for latexin protein expression in a panel containing different human glioblastoma-derived cells and a non-tumoral sample from primary astrocytes (**Figure 50A**). In contrast to the common lack of latexin expression observed in neuroblastoma cells, the U87-MG cell line exhibited endogenous latexin expression. Surprisingly, latexin was also highly expressed in the non-tumoral sample corresponding to primary astrocytes (**Figure 50A**). We next aimed to select a cell line a cell line bearing latexin endogenous expression (U87-MG cells) and another lacking latexin expression (LN-18 cells) to analyze the potential effects that exerted by this protein. For this reason, we corroborated if these cell lines were RA-responsive by evaluating the expression of latexin upon RA treatment. As shown in Figure **Figure 50B**, RA treatment upregulated latexin expression in both LN-18 cells and U87-MG cells. We first focused on LN-18 cells by performing a constitutive and stable transfection (**Figure 50C**).

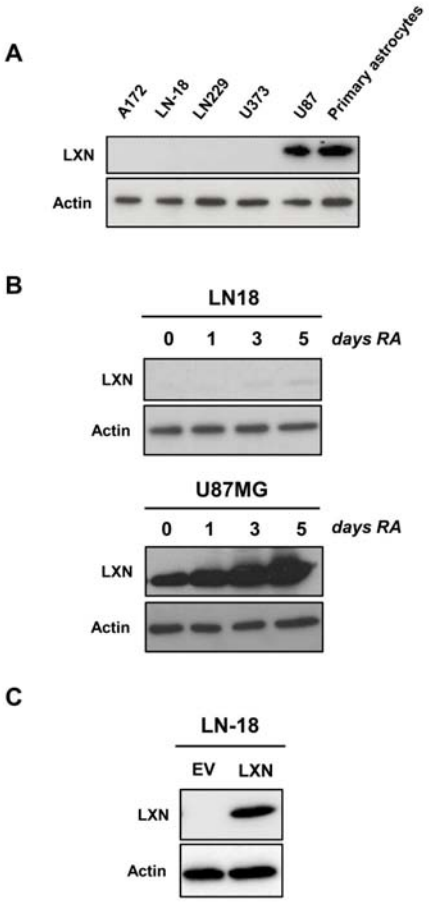

Figure 50. Latexin is heterogeneously expressed in glioblastoma-derived cells. *A,* Protein extracts from different glioblastoma cell lines and primary astrocytes cells were obtained and *Western blotting* against latexin was performed. β-actin was used as loading control. *B,* Wild type LN-18 cells and U87-MG cells were treated with 10 μM RA for up to 5 days and protein extracts were obtained

before (0) or after treatment at the indicated times (1, 3, and 5 days). Latexin expression was analyzed by *Western blotting*. β-actin served as loading control. *C,* LN-18 cells were stably transfected with empty pcDNA3 or pcDNA3-latexin plasmids. Protein extracts from empty vector-transfected (EV) or latexin-overexpressing (LXN) cells were analyzed by *Western blotting* to corroborate the overexpression of latexin. The membranes were reprobed with β-actin antibody to check equal loading.

Since we previously observed that this cell line was RA-responsive (**Figure 50B**), RA was first employed to assess the potential effects of latexin overexpression. As shown in **Figure 51A**, RA treatment did not provoke remarkable morphologic changes in LN-18 cells. In addition, the RA-promoted anti-proliferative effect observed in other cell lines was not evident in LN-18 cells (**Figure 39A**). However, when carefully examining cell morphology, we noticed that latexin-overexpressing cells exhibited dense shadings at the edge of the cells, features that are evocative of the process of membrane ruffling (O'Connor et al. 2000; Kitambi et al. 2014) (**Figures 51A and 51B**). Because RA did not promote noticeable morphological effects in LN-18 cells and neither ceased cell proliferation, cell morphology examination was hindered in these cells. For this reason, different differentiation and senescence inducers were employed.

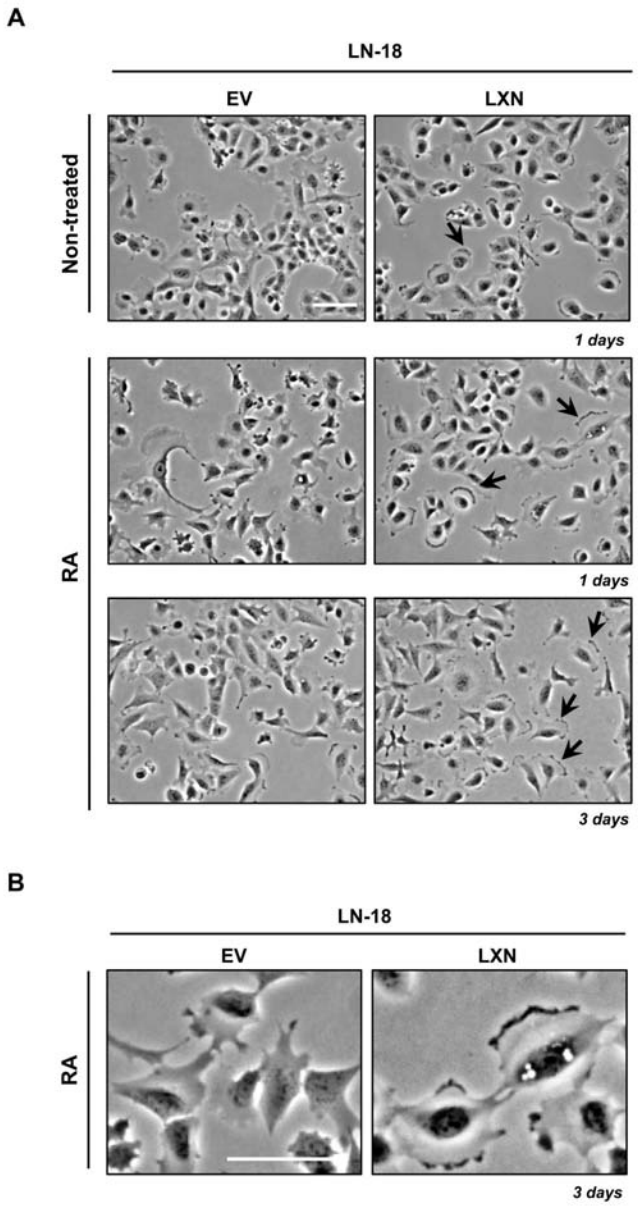

Figure 51. Latexin-overexpressing LN-18 cells exhibit membrane ruffling. *A,* Representative phase contrast microphotographs of empty vector-transfected (EV) or latexin-overexpressing (LXN) LN-18 cells treated with 10 μM RA for 1 and 3 days or left untreated (Non-treated). Membrane ruffling is indicated by arrows. *B*, High magnification of EV and LXN cells treated for 3 days with RA. *Scale bars* = 70 μm.

Considering that we have previously established that latexin facilitates cellular senescence (Chapter 2), we selected well-known inductors of this cellular response namely doxorubicin (Lee et al. 2011), berberine (Liu et al. 2015) and BrdU (Michishita et al. 1999). Doxorubicin induced cell morphology changes and cease of cell proliferation in LN-18 cells (**Figure 52A**). Particularly, 2.5 μM doxorubicin induced cytoplasm extension cell rounding (**Figures 52A and 52B**). Interestingly, doxorubicin-induced morphology was more rounded and the cell boundaries were markedly more homogeneous in latexin-overexpressing cells (**Figure 52B**). In contrast, in empty vector-transfected cells cellular edges were less well-defined, with protrusive regions

(**Figure 40B**). In addition, membrane ruffling was also observed associated to latexin expression in cells treated with doxorubicin (**Figures 52A and 52B**).

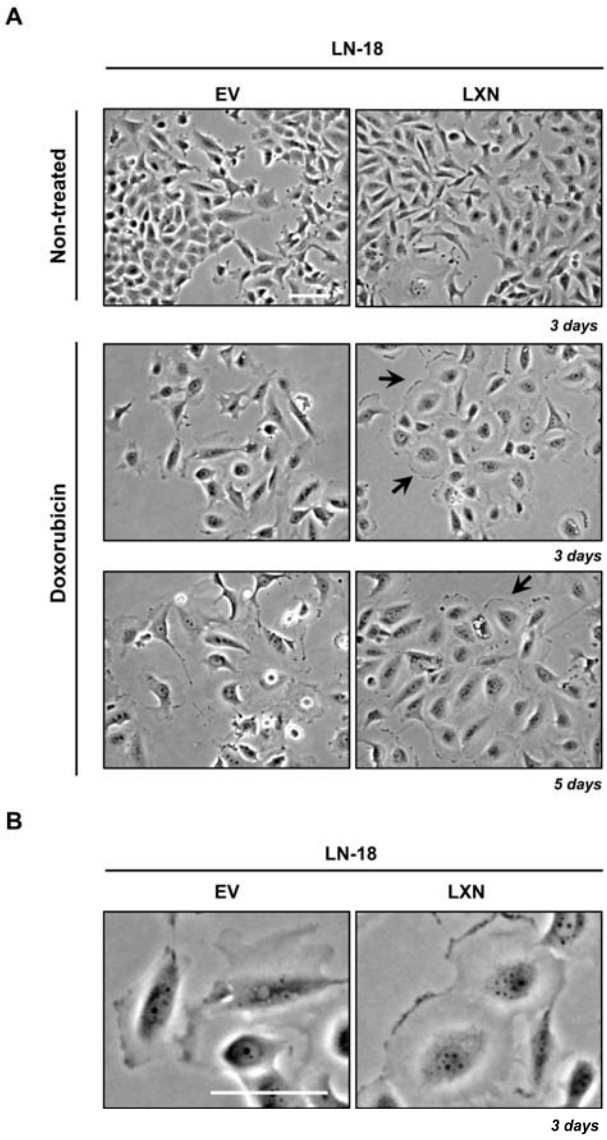

Figure 52. Latexin-overexpressing LN-18 cells exhibit a rounded cell morphology with extended cytoplasm after treatmen with doxorubicin. *A,* Representative phase contrast microphotographs of empty vector-transfected (EV) or latexin-overexpressing (LXN) LN-18 cells treated with 2.5 µM Doxorubicin for 3 and 5 days or left untreated (Non-treated). Rounded cell morphology is indicated by arrows. *B*, High magnification of EV and LXN cells treated for 3 days with 2.5 µM Doxorubicin. *Scale bars* = 70 µm.

On the other hand, LN-18 cells treated with 10 µM Berberine also exhibited cell shape alterations. More concretely, cells were larger with a higher proportion of cytoplasm than non-treated cells (**Figure 53A**). As observed in the previous treatments, latexin-overexpressing cells were characterized by a substantially increase of the cytoplasmic proportion (**Figures 53A and 53B**). However, the process of membrane ruffling was not as evident as in RA or doxorubicin treatments.

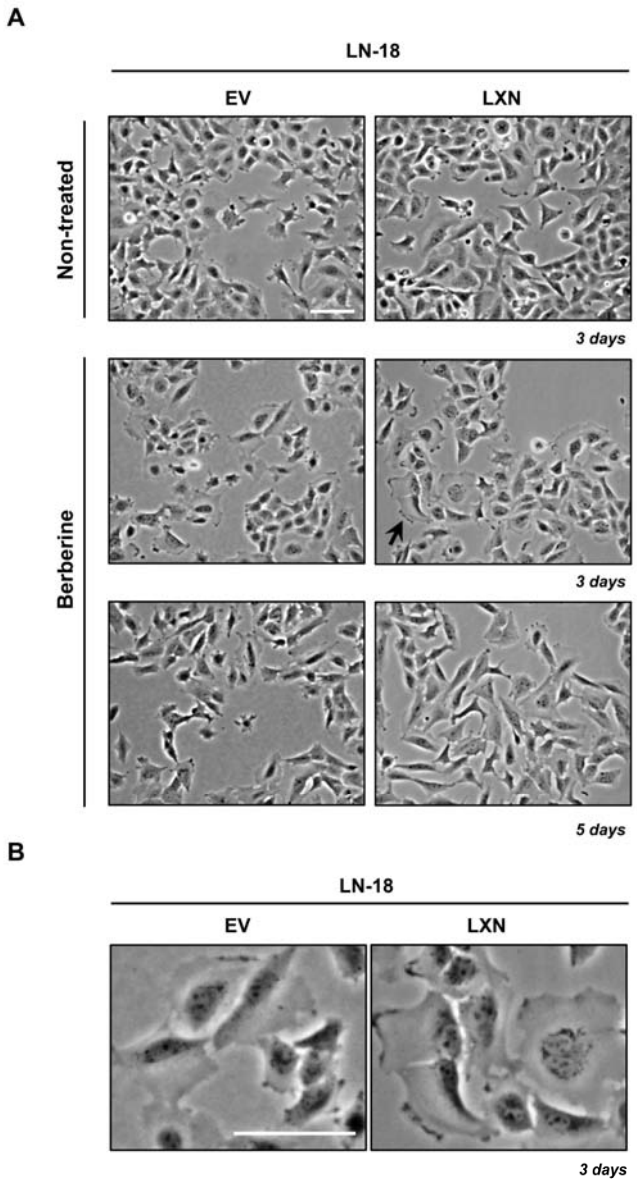

Figure 53. Differential morphology of Berberine-treated empty vector-transfected and latexin-overexpressing cells. *A,* Representative phase contrast microphotographs of empty vector-transfected (EV) or latexin-overexpressing (LXN) LN-18 cells treated with 10 μM Berberine for 3 and 5 days or left untreated (Non-treated). Membrane ruffling is indicated by arrows. *B*, High magnification of EV and LXN cells treated for 3 days with 10 μM Berberine. *Scale bars* = 70 μm.

Finally, BrdU-prompted cell shape changes mainly consisted in cell rounding, being this effect clearly more prominent in cells overexpressing latexin (**Figures 54A and 54B**). In addition, latexin-overexpressing cells also exhibited signs of membrane ruffling (**Figures 54A and 54B)**.

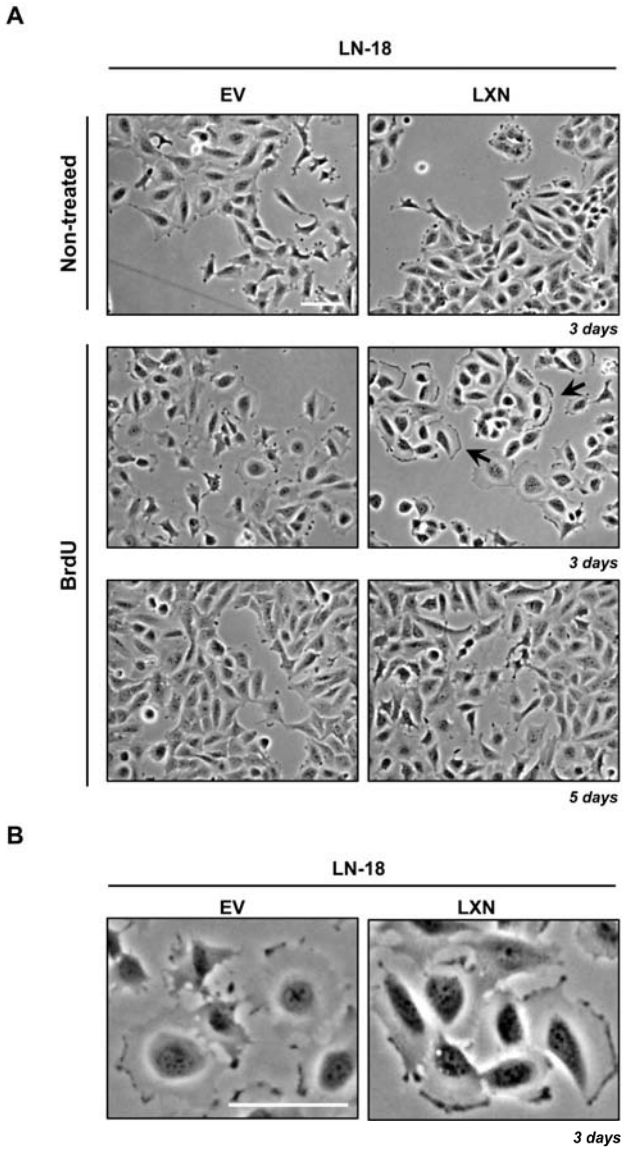

Figure 54. Brdu-treated latexin-overexpressing LN-18 cells exhibit membrane ruffling. *A,* Representative phase contrast microphotographs of empty vector-transfected (EV) or latexin-overexpressing (LXN) LN-18 cells treated with 10 μM BrdU for 3 and 5 days or left untreated (Non-treated). Membrane ruffling is indicated by arrows. *B*, High magnification of EV and LXN cells treated for 3 days with 10 μM BrdU. *Scale bars* = 70 μm.

4.3. LATEXIN FACILITATES DOXORUBICIN- AND BERBERIN-INDUCED SENESCENCE

Since the increase in the cytoplasmic area and also the cease of cell proliferation promoted by berberin or doxorubicin were evocative of cellular senescence, we analyzed whether this cellular response was engaged upon treatment with these agents. As shown in **Figure 55A** latexin facilitated doxorubicin- and berberin-induced cellular senescence. This effect led us to corroborate whether these agents could activate the expression of latexin. Surprisingly, latexin expression was only upregulated in response to RA or BrdU, as reported in SH-SY5Y cells (Chapter 2) (**Figure 55B**). Overall, in LN-18 cells latexin expression promoted cell

morphology changes mainly consisting in cytoplasmic extensions, membrane ruffling and, depending on the specific stimuli, cell rounding. In addition, latexin overexpression facilitated berberin- or doxorubicin-induced senescence.

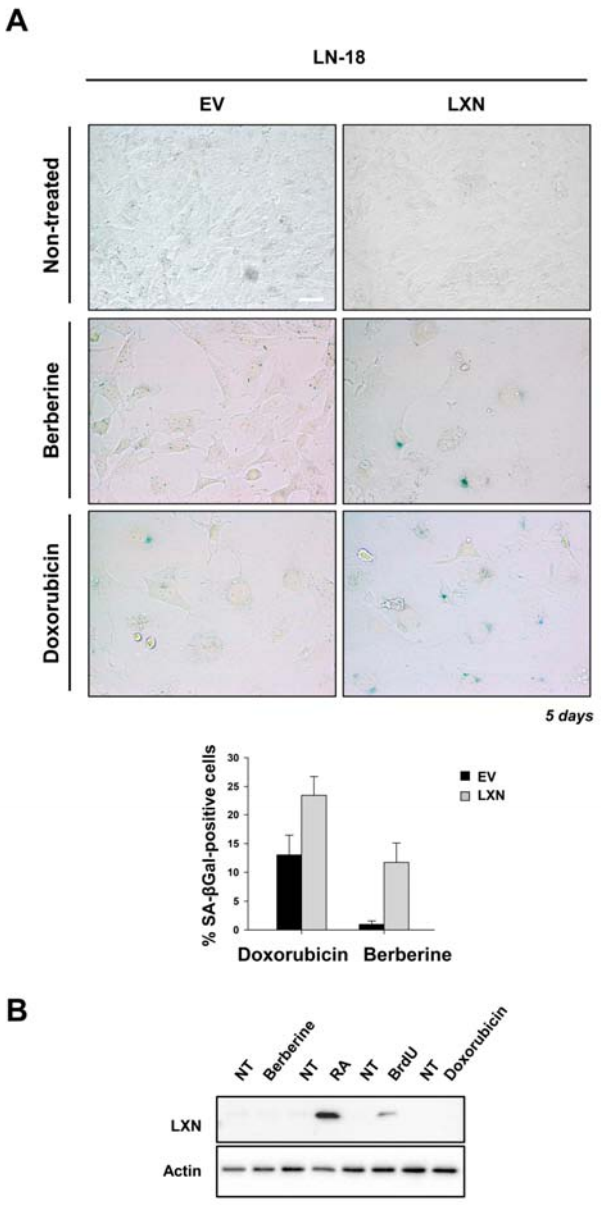

Figure 55. Latexin facilitates berberin- and doxorubicin-induced cellular senescence in LN-18 cells. *A*, Senescence-associated β-galactosidase (SA-β-gal) assay was performed in empty vector-transfected (EV) and latexin-overexpressing (LXN) cells treated with 10 μM Berberine or 2.5 μM Doxorubicin and analyzed by bright field microscopy. Representative images are shown. The lower graph shows the percentage of SA-β-gal-positive cells over the total cells. The values are represented as mean ± S.D. (n=3). *B*, Latexin expression levels in LN-18 cells treated for 5 days with 10 μM RA, 10 μM BrdU, 10 μM Berberine, 2.5 μM Doxorubicin or left untreated. The membranes were reprobed with β-actin antibody to check equal loading.

4.4. LATEXIN DOWNREGULATION INDUCES DIFFERENTIATION AND DECREASES CELLULAR SENESCENCE IN U87-MG CELLS

We next aimed to shed light into the potential effect of knocking down latexin in a cell line with high endogenous expression of this protein. We selected U87-MG cells since they endogenously express latexin and are well-established models of senescence (Rebbaa et al. 2003; Lee et al. 2011; Liu et al. 2015). As shown in **Figure 56A**, small interfering RNA (siRNAs) were effective at downregulating latexin expression, being LXN_1 siRNA the most efficient (**Figure 56A**). When examining cell morphology, we observed that the donwregulation of latexin did not result in remarkably changes (**Figure 56B**).

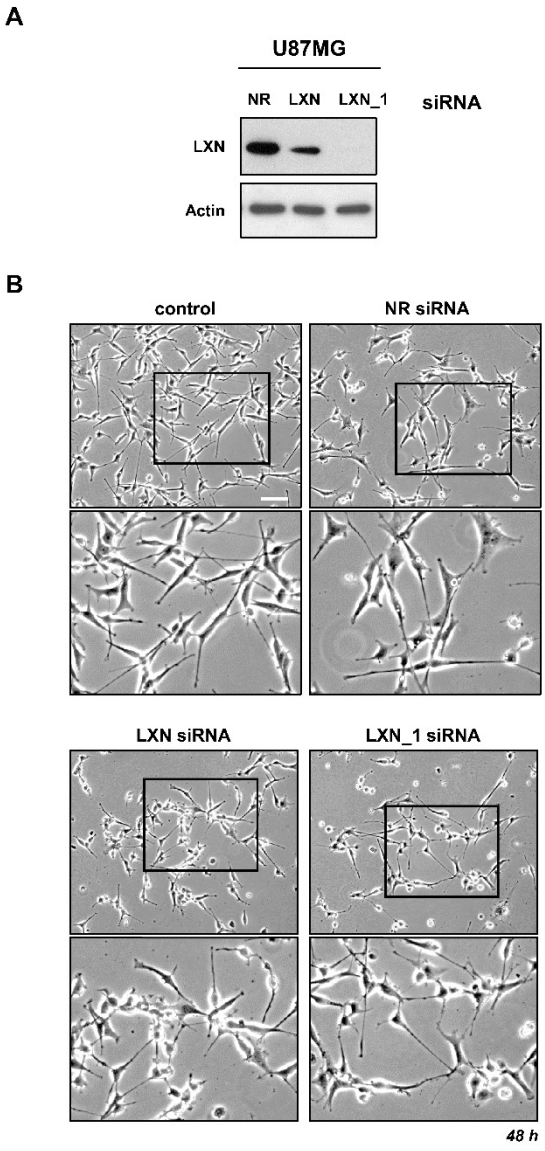

Figure 56. Effect of latexin silencing in U87-MG cell morphology. *A,* U87-MG cells were transfected with two different siRNA against LXN (LXN siRNA, LXN_1 siRNA), against a non-relevant sequence (NR) or non-transfected (control). After 48 hours, cells were detached and latexin knock down was corroborated by *Western blotting*. The membranes were reprobed with β-actin antibody to check equal loading. *B,* U87MG cells were transfected with two different siRNA against LXN (LXN siRNA, LXN_1 siRNA), against

a non-relevant sequence (NR) or non-transfected (control). Representative phase contrast microphotographs showing cells morphology of cells transfected for 48 hours. *Scale bar*= 70 μm.

Since the sole downregulation of latexin did not provoke remarkably cell morphology changes, we next analyzed whether its decreased expression levels could influence the effects promoted by different chemical agents. As shown in **Figure 57**, RA-induced latexin-downreguled cells exhibited a remarkable reduction of cell numbers with no apparent promotion of cell death (**Figure 57**). Moreover, LXN siRNAs resulted in cells displaying longer and thinner cytoplasmic extensions (**Figure 57**).

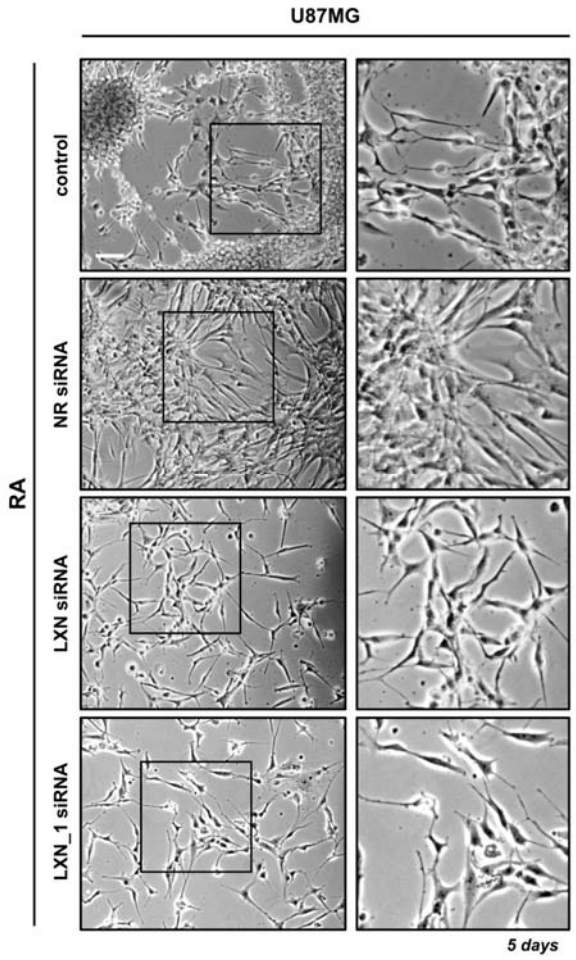

Figure 57. Effect of latexin silencing in the morphology of RA-treated U87-MG cells. U87-MG cells were transfected with two different siRNA against LXN (LXN siRNA, LXN_1 siRNA), against a non-relevant sequence (NR) or non-transfected (control). After 48 hours, cells were treated with 10 μM RA for up to 5 days. Representative phase contrast microphotographs of each condition are shown. *Scale bar*= 70 μm.

On the other hand, similar but more remarkable effects were observed when employing BrdU as differentiation agent. As shown in **Figure 58**, BrdU promoted more flattened morphologies in U87-MG cells. In contrast, knock down of latexin expression impeded this process since latexin-downregulated cells still exhibited long neuritic-like extensions, being this effect more evident in LXN_1 siRNA (**Figure 58**).

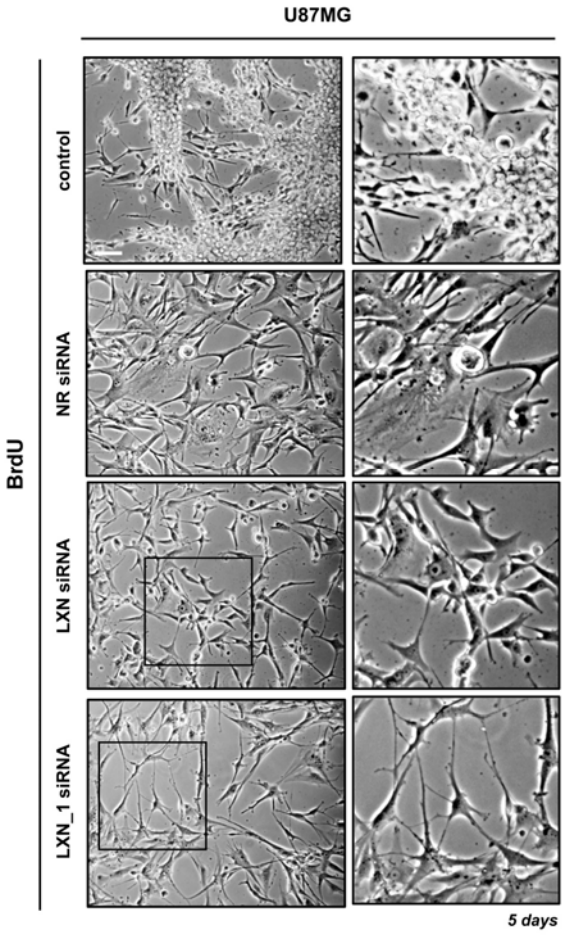

Figure 58. Effect of latexin silencing in the morphology of BrdU-treated U87-MG cells. U87-MG cells were transfected with two different siRNA against LXN (LXN siRNA, LXN_1 siRNA), against a non-relevant sequence (NR) or non-transfected (control). After 48 hours, cells were treated with 10 μM BrdU for up to 5 days. Representative phase contrast microphotographs of each condition are shown. *Scale bar* = 70 μm.

Finally, we analyzed the impact that the reducition of latexin expression could exert in doxorubicin-promoted effects. In accordance with its pro-senescence potential (Lee et al. 2011), doxorubicin-challenged cells exhibited a more flattened morphology and enlarged the cytoplasmic area (**Figure 59**). Strikingly, this effect was not evident in cells donwregulating latexin (**Figure 59**). More surprisingly, doxorubicin-treated latexin-silenced cells acquired an astrocytic-like morphology (**Figure 59**). We next assessed whether these different morphologies could be translated in differences in SA-β-Gal cell numbers. As shown in **Figure 59**, although LXN siRNA did not alter the number of SA-β-Gal positive cells, these numbers were significantly reduced in LXN_1-downregulated cells. Overall, silencing of latexin expression in U87-MG cells resulted in the acquisition of a cellular morphology reminiscent of astrocytes and in the impairment of cellular senescence.

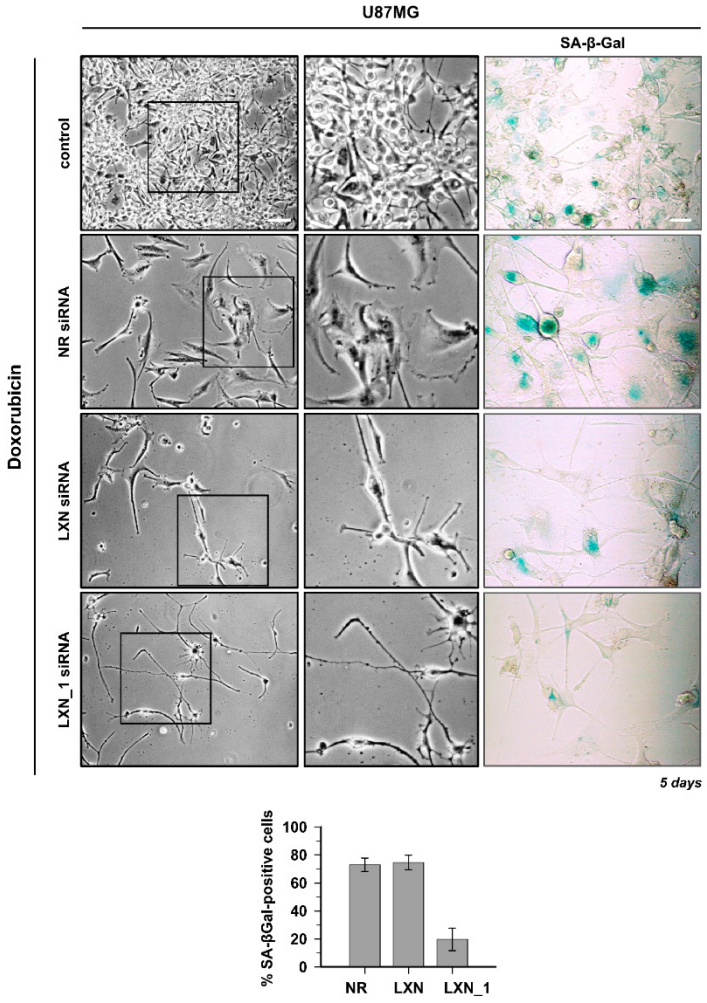

Figure 59. Latexin silencing diminishes doxorubicin-induced cellular senescence in U87-MG cells. U87-MG cells were transfected with two different siRNA against LXN (LXN siRNA, LXN_1 siRNA), against a non-relevant sequence (NR) or non-transfected (control). After 48 hours, cells were treated with 2.5 μM doxorubicin for up to 5 days. Representative phase contrast microphotographs of each condition are shown (*left and middle panel*). *Scale bars* = 70 μm. Senescence-associated β-galactosidase (SA-β-gal) assay was performed in each condition and analyzed by bright field microscopy. *Scale bars* = 50 μm. The lower graph shows the percentage of SA-β-gal-positive cells over the total cells. The values are represented as mean ± S.D. (n=3).

4.5. LATEXIN mRNA LEVELS ARE ASSOCIATED WITH PATIENT SURVIVAL IN NEUROBLASTOMA TUMORS WHILE THEY DO NOT SIGNIFICANTLY ALTER PATIENT OUTCOME IN GLIOBLASTOMA TUMORS.

Finally, we inquired if the expression of latexin could have a significant effect in patient outcome in neuroblastoma and glioblastoma tumors by using the R2 Mega-Sampler application in the bioinformatic program R2 (http:// r2.amc.nl). Kaplan-Meier survival analysis showed that low latexin mRNA expression was significantly associated with a worse overall survival when analyzing the Tumor Neuroblasoma SEQC dataset (498 samples, GEO ID: GSE49710) and the Tumor Neuroblastoma dataset Kocak (649 samples, GSE45547) (**Figure 60A**). Moreover, the association between latexin levels and patient survival was not significantly

altered in glioblastoma tumors. Likewise, when analyzing the Tumor Glioma French dataset (289 samples, GEO ID: gse16011) and the Tumor Glioblastoma TCGA dataset (540 samples), no significant differences were observed in terms of overall survival (**Figure 60B**). This results indicate that the mRNA levels of latexin are associated with a better clinical outcome in neuroblastoma tumors while they do not significantly change patient outcome in glioblastoma tumors.

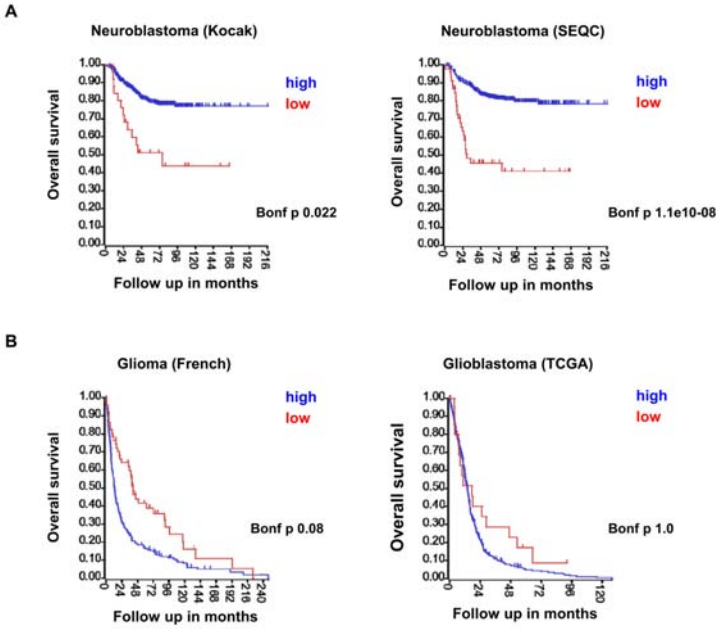

Figure 60. Latexin mRNA levels are associated with better overall survival in neuroblastoma tumors. The influence of latexin mRNA expression levels on the overall survival of neuroblastoma and glioblastoma patients was assessed by using the R2 Mega-Sampler application in the bioinformatic program R2 (http:// r2.amc.nl). A, Data relative to latexin mRNA expression levels in neuroblastoma was obtained from the public databases Kocak (n= 649) and SEQC (n = 498). Survival probabilities of two groups of neuroblastomas with low- or high-level latexin expression were estimated by the method of Kaplan-Meier (bonf = 0.022 and, bonf = 1.1e10-8respectively). B, Data relative to latexin mRNA expression levels in glioblastoma and gliomas was obtained from the public databases French (n = 289) and TCGA (n= 540). Survival probabilities of two groups of neuroblastomas with low- or high-level latexin expression were estimated by the method of Kaplan-Meier (bonf = 0.08 and bonf = 1.0, respectively)

Taken altogether, the results present in this Chapter unveil that latexin is able to facilitate cellular senescence in response to different chemical stimuli. In addition, latexin promotes cell morphology changes that are mainly associated to cytoplasmic enlargement. Moreover, latexin expression is presumably associated with better clinical outcome in neuroblastoma tumors.

V. DISCUSION

1. CHAPTER 1. THE ROLE OF LATEXIN IN RA-INDUCED DIFFERENTIATION IN SH-SY5Y CELLS

Latexin is a recently discovered and poorly known tumor suppressor gene whose mechanism of action is still unknown. The only known protein sharing sequence similarity with latexin is RARRES1, which is located adjacent to the latexin gene in chromosome 3 (Aagaard et al. 2005). Moreover, the expression of these paralogous genes is frequently downregulated by promoter hypermethylation in several types of cancers and the re-establishment of its expression can negatively affect tumor growth (Yong Li et al. 2011; Liu et al. 2012; Muthusamy et al. 2013; Chang et al. 2014; Ni et al. 2014; Youssef et al. 2004; Kloth et al. 2012). In this chapter, we first evidenced latexin is commonly downregulated in different neuroblastoma cell lines, contrasting with the heterogeneous pattern of expression of its familial related gene RARRES1. Remarkably, the lack of latexin expression could be reversed by treatment with either the differentiation agent RA or the demethylating agent 5-aza-dC. Moreover, to the best of our knowledge, no study has been published to date reporting mutations or any other genetic alterations able to deregulate latexin expression. Instead, many reports do actually indicate that epigenetic events are certainly involved in the regulation of its expression (Yong Li et al. 2011; Kloth et al. 2012; Muthusamy et al. 2013; Ni et al. 2014). This fact suggests that pharmacological re-activation of latexin expression is feasible. In order to unravel the potential tumor suppressor role of latexin, we first explored if its enhanced expression could have an impact on any of the well-established functions found deregulated in cancer namely cell proliferation, cell death or cell differentiation. Unexpectedly, either transiently or stable overexpression of latexin did not apparently have an anti-proliferative effect in SH-SY5Y cells. However, though cell growth analysis did not reveal significant differences between latexin-overexpressing and empty vector-transfected cells, the growth curve of the latter presented a much greater dispersion after 5 days in culture. This fact could indicate that, as time in culture progresses, latexin-overexpressing and empty vector-transfected cells can actually behave differentially, possibly as a result of a differential response to long-term culturing-induced stresses. Nevertheless, the potential effect of this source of cellular stress will be further addressed in Chapter II. Interestingly, overexpression of latexin neither resulted in activation of apoptosis. These results contrast with previous reports showing that latexin decreases cell growth and increases apoptosis in hematopoietic cells (Liu et al. 2012) and also that latexin sensitizes leukemogenic cells to gamma-irradiation (You et al. 2014). More precisely, in this latter study, You and colleagues reported that cells overexpressing latexin inefficiently activated caspase-3 and subsequently underwent necrosis upon gamma-irradiation (You et al. 2014). These findings are in accordance with non-published results from our group showing that cells overexpressing latexin displayed lower caspase-3 activation upon treatment with the chemotherapeutic drug camptothecin. Therefore, it is feasible to believe that latexin, through impairing the activation of caspase-3, could be engaging different cellular responses distinct from apoptosis. Despite studies reporting that latexin can activate cell death could seem inconsistent

with the results presented here, it is necessary to stress that proteins can have pleiotropic effects depending on the cellular context. In fact, there are many examples of proteins modulating different cellular responses depending on the cell type and cellular stress. One of the canonical examples is p53 which can activate apoptosis or senescence depending on its levels, kinetics, localization, transcriptional regulation, type of stress or cell type (Murray-Zmijewski et al. 2008).

We next focused on the potential involvement of latexin in cell differentiation processes. We first evidenced latexin is upregulated in response to RA treatment in neuroblastoma cells. Consistently, latexin expression in neurons has been reported to increase along the developmental stages (Arimatsu et al. 2003), which points out a potential role in differentiation for this protein. Therefore, we sought to determine whether the expression of latexin could influence the differentiation status of neuroblastoma cells. The most striking effect promoted by latexin was the early appearance of RA-induced S-type cells. Conversely, under our experimental settings, the well-established N-type differentiation promoted by RA was not apparently altered by latexin expression. It is important to highlight that neuronal differentiation is a complex mechanism tightly influenced by several conditions including, among others, growth factors, cell adhesion or the presence of differentiating agents (Dwane et al. 2013; Hämmerle et al. 2013; Hynds & Snow 2001). Accordingly, we screened different culturing conditions to distinguish the specific cellular effects mediated by latexin. Following this approach, we expected to clarify the cellular context and the precise cellular processes this protein is primarily involved in. Hence, we observed that the S-type emergence favored by latexin in the presence of RA is context-dependent since it requires the presence of serum in the culture medium. This fact is in perfect agreement with previous studies reporting that S-type cells emergence needs the presence of serum (Encinas et al. 2000) and that neuroblastoma cells are able to differentiate into a neuronal-like phenotype by the removal of serum from the culture medium (Cheung et al. 2009). In this sense, serum supplies growth factors, hormones, antioxidants, and proteins to the culture medium (Evangelopoulos et al. 2005). This implies that S-type cells harbor a greater dependence on such components to proliferate and survive than N-type cells. In this sense, latexin-overexpressing cells would presumably sensitize cells to the serum components that are required for S-type emergence.

Cell differentiation is also tightly regulated by extrinsic factors such as extracellular matrix (ECM) contacts. In this sense, ECM can sense environmental cues thus regulating cell proliferation, cell differentiation and cell survival (Hynes & Naba 2012). In particular, the ECM provides an extracellular environment that promotes neurite outgrowth in neuroblastoma (Brizzi et al. 2012). With the aim to mimic such extracellular conditions, cells were cultured on plates coated with laminin, fibronectin and collagen. Consistently, all three matrices supported neurite outgrowth, as previously reported (Dwane et al. 2013; Hynds & Snow 2001), although such process was not noticeably promoted by latexin overexpression. However, we cannot completely rule out a potential effect of latexin on neuronal differentiation since neither neurite length nor neurite branching were quantified. Remarkably, such culture coating conditions promoted the earlier appearance of S-type cells in both empty vector-transfected and latexin-overexpressing cells than when cells were cultured on non-coated plates. Given that finding, it is reasonable to think that latexin could be influencing the ECM composition or

structure to exert its S-type-promoting effect. In this sense, latexin is the only known endogenous inhibitor of zinc-dependent metallocarboxypeptidases (MCPs) in mammalians (Pallarès et al. 2005). Such enzymes are secreted to the ECM where they potentially cleave their target substrates (Tanco et al. 2010). In this sense, it can be speculated that latexin could modulate the ECM through the inhibition of MCPs. However, this speculation will be further addressed in the Chapter III.

In seeking conditions to maximize the differentiation effect observed in cells overexpressing latexin, different concentrations of RA were employed. Interestingly, the anti-growth effect of RA was evident at lower concentrations in latexin-overexpressing cells than in empty vector-transfected cells. This fact may indicate that the differentiation machinery is more efficiently activated in response to RA treatment in latexin-overexpressing cells, although it does not directly result in a noticeably promotion of neuronal differentiation. Consistently, it is conceivable to believe that such an increased efficiency is being funneled towards the S-type lineage differentiation. This idea would support the notion of latexin being a tumor suppressor since it has been reported that the S-type phenotype is less tumorigenic than the N-type (Walton et al. 2004). Accordingly, it would be of outstanding interest to determine which modulators are responsible of shifting RA activity towards one or another specific lineage. In addition, such increased RA sensitivity harbored by latexin-overexpressing cells would also explain why the epigenetic restoration of tumor suppressor genes expression, such as the RA receptor RARβ, returns RA sensitivity in some cell types (Xu 2007).

On the other hand, latexin-overexpressing cells exhibited greater tolerance to high concentrations of RA. This finding is of great importance since, in embryos, Vitamin A/RA deficiency can cause severe malformations but also excessive concentrations of these compounds can disrupt the normal development of several organs (Niederreither & Dollé 2008; Maden 2007). Accordingly, this agent is regarded as teratogen (i.e. an agent that can causes malformations in the embryo) (Lee et al. 2012; McCaffery et al. 2003). If these results could be extrapolated to normal tissues, these findings could implicate that the expression of latexin would attenuate the detrimental effects promoted by RA. Moreover, the cytotoxicity observed upon RA treatment can be rationalized considering that this agent, aside from its well-established ability to promote cell survival, can also prompt cell death (Noy 2010). Concretely, its pro-apoptotic effects have been associated to the downregulation of Bcl-2 and survivin and also with the ability of RA to regulate the expression of caspases (Raffo et al. 2000; Pratt et al. 2003). On the other hand, the pro-survival effect mediated by RA depends in a great extent on the activation of the PI3K/Akt pathway (López-Carballo et al. 2002). Thus, RA-promoted survival mechanisms appear more activated in latexin-overexpressing cells compared with empty vector cells when cells are treated with RA. In line with this, the PI3K/Akt pathway plays a pivotal role in this process.

In conclusion, the most evident mechanism of action of latexin in neuroblastoma cells does not seem to be related with either promotion of cell death, cell proliferation or neuronal differentiation. However, marked differences are observed in the promotion of RA-induced S-type phenotype by latexin. In addition, the optimization of the appropriate differentiation conditions in which latexin-mediated effects are more evident,

sheds light into the putative cellular context in which latexin could be exerting its function. In general terms, in a tumor context, latexin could act as a cell specification protein thus directing differentiation towards a specific phenotype. In this sense, the final outcome of such specification will depend on whether the characteristics of the promoted phenotype would be favorable or unfavorable for the progression of the tumor. On the other hand, in a healthy cellular context, tissues expressing latexin could be more susceptible to the action of morphogens such as RA and could attenuate the detrimental effects promoted by RA.

Taken altogether, the findings reported in this chapter provide the basis for further experiments to study in depth the cellular outcome that S-type cells facilitated by latexin are promoting and the signaling pathways behind this cellular response.

2. CHAPTER 2. LATEXIN FACILITATES LONG-TERM CELL SURVIVAL AND SENESCENCE VIA PDK1/AKT PATHWAY

In this chapter, we unravel that latexin facilitates cell specification towards the S-type lineage in response to either RA or BrdU treatment. As already mentioned, cell lines derived from neuroblastoma tumors display a phenotypic variety and three morphologically and biochemically different sub-types, termed N-, S- and I-types, can co-exist. Interestingly, such phenotypes can transdifferentiate under the adequate stimuli (Ross et al. 1983; Ciccarone et al. 1989; Piacentini et al. 1996). In this sense, RA possesses the ability to differentiate towards both N- and S-type phenotypes (Påhlman et al. 1984) whereas BrdU is able to induce a more homogeneous population of S-type cells in some neuroblastoma models (Sugimoto et al. 1988). Our results first evidence that latexin expression is downregulated in SH-SY5Y cells and that RA or BrdU treatment upregulates its expression, suggesting its potential involvement in the differentiation effect mediated by these agents. We demonstrate that latexin is able to shift the RA-induced differentiation outcome from the N-type towards the S-type phenotype, as corroborated by both the observed morphological changes and the increase in expression of the S-type marker vimentin. However, it is important to stress that changes in vimentin expression levels along RA treatment could seem contradictory with the emergence of the S-type phenotype observed. Thus, since vimentin is taken as *bona fide* molecular marker of S-type cells (Walton et al. 2004; Bell et al. 2013; Ross et al. 2003), the markedly elevated levels of vimentin expression already present in untreated latexin-overexpressing cells could seem inconsistent with the absence of S-type phenotypic traits in these cells. In addition, the levels of this protein tend to decrease upon RA treatment after 5 days. Such apparently paradoxical decline can be understood taking into account that RA has more propensity to induce neuronal-like differentiation than S-type emergence. Although a negative regulation of vimentin levels has not been reported to date associated to the N-type differentiation promoted by RA in SH-SY5Y cells, vimentin is known to be expressed during early development of neural crests, being essential for axonal initiation, but is subsequently replaced by other neurofilaments along the development (Boyne et al. 1996). In this sense, one possible scenario is that the initial reduction of vimentin levels observed in latexin-overexpressing cells at 5

days of RA treatment could be more related to the N-type differentiation than to the emergence of the S-phenotype. On the other hand, considering that both N and S subpopulations co-exist within the culture, the overall vimentin levels observed might rather constitute a reflection of the balance between these subtypes. Therefore, the reduction in the levels of this protein marker at 5 days of RA treatment would come as a result of culture subpopulations balance displaced towards the N-type cells undergoing neuronal-like differentiation. Accordingly, the levels of vimentin should be determined in separated populations of N and S cells to clarify this apparent discrepancy. However, vimentin levels turn to increase after 15 days of RA treatment, suggesting an equilibration in the balance between N- and S-type cells populations. Such a scheme for the variations of vimentin levels is further supported by the effect observed in latexin-overexpressing cells when BrdU is employed as differentiation agent, being able to induce a homogeneous population of S-type cells which is characterized by presenting vimentin levels already increased at 5 days of treatment. These observations raise the question as to whether vimentin actually represents an appropriate marker of S-type cells, as a final cellular differentiation marker, or this protein may rather be required over the process of cellular specification towards S-type phenotype. Aside from these assumptions, it is worth mentioning that this intermediate filament can adopt different structural forms ranging from small dots, short fibrils to long mature filamentous networks (Prahlad et al. 1998; Clarke & Allan 2002; Chou et al. 2007). These forms can interconvert depending on the specific requirements of the cell to perform different functions. In this sense, it is feasible to speculate that S-type formation could be characterized by a restructuring of vimentin assembly rather than by increased protein levels. In spite of these assumptions, latexin invariably facilitates the differentiation towards the S-type phenotype upon exposure to RA or BrdU. These results suggest that latexin could be a regulator of cell fate specification in this cellular model. This potential role is in agreement with the findings of Liang and colleagues, showing a negative correlation between latexin expression and the number of stem cells in the hematopoietic system (Liang et al. 2007) and also with latexin expression decreasing the mRNA levels of the stem cell transcription factors OCT4, NANOG, SOX2, KLF4 and MYCN (Muthusamy et al. 2013). Taken together, and considering our results, it is feasible to speculate that latexin could narrow cell fate choices, leading cellular differentiation towards a specific cell lineage.

Remarkably, the degree of cell differentiation is a relevant feature that, usually inversely correlates with the malignant progression of cancer (Sherr 2004), acquiring a special significance in the development of neuroblastoma (Garrett M Brodeur 2003). Indeed, neuroblastic differentiation and Schwann cell content correlate with better clinical outcome in neuroblastoma patients (Shimada et al. 1985). Accordingly, the S-type lineage harbors less malignant potential than the other two sub-types (Walton et al. 2004; Ross et al. 2003). In this sense, it can be hypothesized that latexin, if expressed, could exert a negative influence in the progression of neuroblastoma by promoting the S-type phenotype. However, as explained below, latexin also promotes long-term cell survival which could contradict the notion of latexin functioning as a tumor suppressor gene.

The pro-survival effect of latexin was evident in long-term cultured cells in the presence of RA without renewing cell culture media. Under these unfavorable conditions, latexin-overexpressing cells undergo two parallel processes: N-type cells display a highly differentiated morphology with extensive neurites, whereas S-type cells acquire a more flattened morphology. These effects can be explained by, at least, three different mechanisms. First, cell survival and differentiation are two closely coordinated processes, since differentiated cells, by extending long neurites, can establish cell-cell and/or cell-ECM contacts, providing the survival signals necessary to maintain cell viability. When these connections fail, cells aggregate in order to supply the required signals for preventing cell death (Santini et al. 2000), as observed in empty vector-transfected cells. Therefore, latexin could be promoting long-term cell survival by facilitating neuronal differentiation. However, this appears unlikely because neuronal differentiation towards N-type lineage does not seem to be enhanced by latexin after 5 days of RA treatment as evidenced by both cell morphology and N-type expression markers. Second, it has been reported that Schwann cells can drive cell differentiation and survival in neuroblastoma cells by secreting neurotrophins and other factors (Kwiatkowski et al. 1998; Ambros et al. 1996). Thus, in our cellular model, S-type cells could be responsible for N-type survival and cell differentiation. However, as discussed below, this assumption is unlikely because N-type phenotype can survive even in the absence of S-type cells. Finally, latexin could facilitate appropriate extra or intracellular conditions for the maintenance of long-term cell viability, rather than promoting cell differentiation. In such cellular context, cells would extend their lifespan, therefore enabling a sustained RA-triggered cell differentiation. In line with this idea, it has been reported that the overexpression of Bcl-X_L protects SH-SY5Y cells from staurosporine-induced caspase-dependent cell death allowing long-term cell survival and, therefore, the neuronal-like differentiation triggered by the alkaloid (Yuste et al. 2002). Taking into account these findings, it is feasible to think that latexin could function in a similar way than Bcl-X_L. Thus, cell resistance prompted by latexin towards the cytotoxicity of a non-renewed culture media could be related to an impairment in the activation of caspases, in agreement with You and colleagues, which reported that latexin overexpression impairs caspase-3 activation upon irradiation (You et al. 2014). However, it cannot be ruled out that latexin-mediated cell survival can arise from caspase-independent mechanisms.

The above-mentioned survival and differentiation effects promoted by latexin led us to focus on the PI3K/PDK1/Akt and p42/44 MAPK axis as potential mediators of these cellular processes. In this sense, our results employing the specific inhibitor of MEK1/2 are in agreement with previous studies showing that this inhibitor does not impair neurite outgrowth in neuroblastoma cells (Miloso et al. 2004; Qiao et al. 2012). On the other hand, high concentrations of specific inhibitors of the PI3K/PDK1/Akt pathway result cytotoxic in empty vector and latexin-overexpressing cells, regardless of RA treatment. Interestingly, the sole overexpression of latexin increases Akt phosphorylation levels already in basal growth conditions. This fact implies that cells overexpressing latexin are able to deploy a much efficient activation of the Akt pathway after certain pro-differentiation stimuli, such as RA or BrdU. Accordingly, the concentrations of PI3K/PDK1/Akt inhibitors required to completely abolish the activation of this pathway are greater in cells overexpressing

latexin, irrespectively of the pro-differentiation agent employed. In this sense, the partial inhibition of the PI3K/PDK1/Akt pathway impedes the emergence of the S-type phenotype while allowing the survival and differentiation of the N-type lineage in RA-treated latexin-overexpressing cells. Then, S-type cells harbor a higher dependence on the PI3K/PDK1/Akt pathway than N-type cells. This suggests that the S-type lineage could require the activation of this pathway to survive or, alternatively, to emerge after RA treatment. In order to discern between both possibilities, we employed BrdU because of its ability to induce a homogeneous population of S-type cells with no funneling towards the N-type phenotype (Sugimoto et al. 1988). The partial inhibition of the PI3K/PDK1/Akt pathway in BrdU-challenged latexin-overexpresing cells hinders S-type cells progression, rather than affecting the overall cell population viability, therefore suggesting that this pathway is necessary for S-type emergence. These evidences, taken altogether, indicate Akt can promote different cellular outcomes depending on its degree of activation. In this regard, low levels of Akt activity are sufficient to maintain cell survival while higher levels are required for certain cellular specification processes. More specifically, S-type cells need a robust Akt activation to emerge, while N-type cells survive and differentiate at lower levels. These observations could seem contradictory with the findings reported by Yan and colleagues in which S-type cells generated after inhibiting ALK display low levels of Akt activation (Yan et al. 2011). However, it must be stressed that the authors explore this pathway in stable cell lines with already formed S-type cells. In contrast, we focus on the process of emergence of the S-type lineage induced by differentiating agents, such as RA or BrdU. In this context, we speculate that Akt activation is required for the initial steps of cell specification leading the process towards the S-type lineage, but not for the survival of the already emerged S-type cells. This is in accordance with the Akt phosphorylation pattern detected over time of BrdU treatment, characterized by an initial increase followed by a decrease, which does not compromise the number of S-type cells observed after longer periods in culture. Thus, it can be speculated that this initial raise in Akt phosphorylation is required for the formation of S-type cells, whereas the following decrease may indicate that S-type cell maintenance do not require such levels.

Finally, S-type cells arising from RA- or BrdU-treated latexin-overexpressing cells undergo cellular senescence under unfavorable extracellular conditions as corroborated by SA-β-gal and EdU assays. In addition to these senescence canonical markers, the association between mitochondrial morphology and senescence has recently emerged. Indeed, elongated mitochondria has been detected in senescent cells (Mai et al. 2010). Strikingly, cells with elongated mitochondria have been proposed to be more resistant to apoptotic stimuli whereas fragmented mitochondria are more sensitive towards apoptotic triggers (Park et al. 2010). Mitochondrial morphology is a dynamic process which is orchestrated by different fusion and fission proteins such as Fis1, Opa1, Mfn1, Mfn2, SLP2 and MTP18. Interestingly, knockdown of the mitochondrial fission protein Fis1 is associated with mitochondrial elongation and senescent phenotype (Lee et al. 2007). Taken altogether, this evidences highlight the need to assess whether latexin could be regulating the expression of such proteins.

Regarding the putative effectors of cellular senescence in our experimental model, cells need the convergence of, at least, two players to undergo such cellular process: a cell specification agent (such as RA or BrdU) and

latexin. In this sense, it is known that RA or BrdU can induce cellular senescence in neuroblastoma cells (Acosta et al. 2009; Wainwright et al. 2001). In addition, latexin is a negative regulator of stem cells numbers and, for this reason, it has been proposed as a potential player in cellular aging (Liang & Van Zant 2008). However, the unfavorable extracellular conditions given, for example, by a non-renewed cell culture media, should be taken into account as a third potential key player. In fact, it has been already reported that cell culture conditions such as cell culture media and culture substrata are also effectors leading to the engagement of this cellular response (Ramirez et al. 2001). In this regard, RA- or BrdU-supplemented worn-out cell culture media drives cellular senescence in cells overexpressing latexin, but cell death in those transfected with empty vector. This observation suggests that latexin could be a key molecular player directing stressed tumor cells to enter into a cellular senescence program rather than undergoing apoptosis. This fact raises the question about what influences the choice between apoptosis and senescence of tumor cells facing a cellular stress. In this sense, damage intensity is one of the main mechanisms directing cell fates since apoptosis is normally engaged after intense damage, whereas senescence is triggered upon less severe injury (Childs et al. 2014). This observation suggests that latexin could be a molecular player directing stressed tumor cells towards senescence response, likely by modulating the cellular perception of damage intensity. It is conceivable to think that Akt phosphorylation prompted by latexin could be central to influence the cellular sensitivity towards an extracellular stress. In this sense, a strong activation of Akt could provide the adequate intracellular milieu allowing cells to raise their threshold to enter into apoptosis upon cellular stress. This idea is in accordance with the fact that latexin requires the synergy of RA or BrdU-triggered Akt activation to facilitate cellular senescence and with the subsequent decline of senescent cells when this pathway is inhibited. Accordingly, it seems that the level of Akt activation is essential for the induction of this cellular response. These findings are consistent with other studies reporting that low levels of Akt activity observed in PDK1 mutant mice are sufficient to support cell survival but not neuronal differentiation, which requires higher Akt activation (Zurashvili et al. 2013). Therefore, the fine-tuning of Akt activation seems to be essential for cells to survive or to enter into a particular differentiation program. In this sense, a high-order of cellular specification, such as senescence, requires a robust activation of the PI3K/PDK1/Akt pathway. Therefore, latexin acts as a molecular shifter directing stressed cancer cells towards cellular senescence, likely through the hyper-activation of Akt.

Collectively, aside from its pro-senescence effect, latexin favors long-term cell survival, which could seem contradictory with the tumor suppressor role traditionally assigned. In this sense, although cellular senescence is considered one of the major tumor-suppressive mechanisms in cancer (Campisi 2001), recent findings also indicate that that this cellular response can also contribute to the malignant transformation of neighboring cells (Young & Narita 2009; Campisi et al. 2011). Thus, senescence has a dual pro- and anti-tumor role depending on the cellular context (Campisi 2005). This duality depends on whether they are acute (beneficial) or chronic (harmful) senescent cells, the extracellular environment and the composition of senescence-associated secretory phenotype (SASP) (van Deursen 2014). Therefore, it should be explored whether cellular senescence promoted by latexin induces the production of SASP and, if such is the case, whether these secreted molecules

are able to influence either positively or negatively the survival of neighboring cells. In any case, it must be taken into account whether an anticancer drug is able or not to promote the expression of molecular shifters, such as latexin. For this reason, it should be of outstanding interest to determine whether the senescence response facilitated by latexin is beneficial or detrimental for cancer progression. This could help to envisage new potential antitumor approaches towards the induction or the repression of cellular senescence drivers, such as latexin.

3. CHAPTER 3. LATEXIN MODULATES RETINOIC ACID ACID-INDUCED GENE EXPRESSION IN SH-SY5Y CELLS

In the previous chapters, we have established that latexin is influencing the RA-promoted differentiation outcome in SH-SY5Y cells. Therefore, unravelling the molecular mechanisms behind this effect would shed light into the potential biological functions of latexin.

As explained before, the sole overexpression of latexin does not bring about any apparent sign of differentiation when cells are cultured under adequate extracellular conditions (Chapter I and II). However, when these cells are maintained for longer periods in culture, the growth pattern is drastically altered; latexin overexpressing cells are able to maintain a monolayer growth whereas empty vector-transfected cells gradually aggregate and form floating clumps. Accordingly, the sole overexpression of latexin is influencing cell behavior in such a way that only becomes evident when cells are facing a stress such as long-term cell culture in a non-renewed media. In line with this, it has been reported that the formation of cell clusters is a stress-response mechanism that cells develop to prevent cell death (Santini et al. 2000), therefore suggesting that latexin-overexpressing cells are able to better tolerate such kind of cellular stresses. Since non-treated samples subjected to the RNA-seq analysis have been maintained in culture for the same period than RA-treated cells, we are evaluating not only genes directly regulated by latexin expression but likely also those that would mediate this differential response exerted by latexin. For this reason, exploring the landscape of gene expression promoted by latexin would help clarifying not only genes directly promoted by latexin expression but also those that render cells enhanced growth capabilities. In addition, the transcriptional profile of cells stably overexpressing this protein could shed light into other potential molecular functions promoted by latexin in response to other stimuli distinct from RA. We first evidence that latexin promotes a large number of gene expression changes, although relatively subtle compared with those promoted by RA treatment, being the proportion of upregulated genes (86%) much higher than the downregulated (24%). Moreover, upregulated differential expressed genes (DEGs) are mainly associated to cell adhesion, skeletal development and neuron differentiation processes. Consistently, cell adhesion is probably one of the main features that distinguish the growth pattern observed in long-term cultured non-treated cells, since cell clusters observed in empty vector-transfected cells are a characteristic of neuroblastoma cells cultured under non-adherent conditions (Acosta et al. 2009). In this sense, it would be of great interest to determine if latexin expression *per se* promotes cell adhesion or if this cellular

response is rather a consequence of facing a cellular stress. More interestingly, DEGs upregulated by latexin overexpression are mainly associated to the extracellular matrix (ECM). This finding raises the possibility that latexin, via modifying the ECM composition, could be altering cell adhesion properties which ultimately leads to the differential growth pattern observed. However, such an interesting finding will be addressed below. Yet another relevant effect observed is that the overexpression of latexin induces expression changes in genes associated to RA signaling. Likewise, latexin upregulates the retinoic acid receptor beta 2 (RARβ2) or the cytosolic retinoic acid-binding proteins CRABP1 and CRABP2 whereas it donwregulates the fatty acid binding protein 5 (FABP5). Remarkably, the FABP5/CRABP2 ratio highly influences cellular outcome; higher levels of FABP5 than CRABP2 result in an anti-apoptotic and proliferative effect whereas the opposite ratio favors the anti-proliferative function of RA (Schug et al. 2007). These results are in accordance with the fact that RA exerts an anti-proliferative effect at lower concentrations in latexin-overexpressing cells compared with the higher concentrations required in empty vector-transfected cells (Chapter I).

Since distinguishable latexin-mediated effects require the synergy with RA, the evaluation of transcriptional changes mediated by RA when latexin is overexpressed or not, would shed light into the molecular basis of the above-mentioned transdifferentiation process. We first evidence that latexin and RARRES1 are not among the most upregulated genes upon RA treatment, which could implicate that these genes could represent early RA-induced genes. As expected, RA possesses the ability to regulate the expression of a vast number of genes, as previously described (Tanaka et al. 2007; Nakanishi et al. 2008), bearing a much greater capability to upregulate than to downregulate gene expression. Accordingly, RA treatment is associated to the regulation of genes involved in neuron differentiation and developmental processes. Interestingly, aside from these canonical functions, RA also upregulates the expression of a large number of genes involved in cell adhesion, a biological function also reported as essential during neurite outgrowth (Dwane et al. 2013). Unsurprisingly, downregulated genes are associated with cell division and M phase processes. These processes are consistent with those reported previously for RA treatment in SH-SY5Y cells (Wang et al. 2014) and are also in perfect agreement with canonical functions associated to RA at inducing cell differentiation and ceasing cell proliferation (Niederreither & Dollé 2008; Noy 2010; Tang & Gudas 2011). At this point, since latexin acts synergistically with RA, the comparison between common DEGs promoted by either RA in empty vector-transfected cells or by the sole overexpression of latexin would indicate common molecular mechanisms shared by both, latexin overexpression and RA treatment. More specifically, since latexin facilitates RA-induced S-type emergence and that RA harbors the potential to differentiate towards both N and S phenotypes, common changes in gene expression induced by either latexin overexpression or RA treatment would likely indicate which DEGs are responsible for the S-type appearance. On the contrary, DEGs exclusive of latexin overexpression could be suggestive of other cellular effects latexin could promote depending on different other cellular stimuli. Strikingly, despite latexin being regarded as a RA-responsive gene, not all the genes regulated by this protein are also involved in RA-differentiation. More concretely, among the 180 genes upregulated by latexin, only 65 are also upregulated by RA treatment. Consistently with the high adherence to the substrate

that exhibit S-type cells (Ross et al. 1983), those 65 common DEGs are associated to cell adhesion and ECM organization processes. These results raise the possibility that latexin-overexpressing cells could be more prepared to acquire the S-type phenotype likely by increasing the adhesion potential and by remodeling the ECM. Once again, it should be of remarkable interest to ascertain whether these genes are regulated because of the maintenance in culture or, alternatively, are intrinsically regulated by latexin expression. The 115 genes specifically upregulated by latexin overexpression are associated with cell morphogenesis processes. In this sense, cell morphogenesis is a developmental process transforming cell shape or size to acquire more mature characteristics, being fundamental in diverse cellular contexts namely differentiation, response to physiological stimuli, motility or embryonic development (Lecuit & Pilot 2003). Therefore, it is reasonable to believe that latexin, by modifying cell shape, could participate in processes such as cell specification or stress response, upon the adequate stimuli.

The analysis of DEGs promoted by RA when cells are overexpressing latexin would help to understand which alternative routes are taken the cells to promote the S-type phenotype. DEGs induced by RA in cells overexpressing latexin are, in general terms, lower than in cells transfected with empty vector. This fact can be explained, at least in part, considering that the overexpression of latexin already enhances the expression of several DEGs. Thus, if some of the genes that are upregulated by the sole overexpression of latexin coincide with those canonically activated by RA treatment, it is conceivable that in cells overexpressing latexin, RA would not need to promote the same magnitude of change to achieve similar final outcomes. However, this assumption does not explain the whole amount of upregulated DEGs and neither the downregulated ones. Thus, in cells overexpressing latexin, RA does not require to alter the expression of the same amount of genes to perform its action. Likewise, regulated genes by latexin overexpression could act coordinately to promote some cellular effects that, although not perceptible, ultimately lead to RA altering substantially less amount of genes to promote a specific function. In spite of that, enriched biological processes obtained by DAVID analysis associated to RA treatment in either empty vector-transfected or latexin-overexpressing cells are highly coincident. However, several remarkable differences can be appreciated. More concretely, the process neuron development is markedly less relevant during RA differentiation in latexin-overexpressing cells. Since the sole overexpression of latexin already induces gene expression changes related to neuron development and, considering that we have previously established that neuronal differentiation is not morphologically promoted in latexin-overexpressing cells (Chapter I and II), one scenario could be that the effects of RA inducing neuronal differentiation are attenuated in latexin overexpressing cells to reach a comparable neuronal differentiation status. This fact could implicate that cells overexpressing latexin are more prepared to receive a neuronal differentiation stimuli, but in the presence of an agent such as RA with the ability to differentiate along both lineages, they are more prone to facilitate the Schwannian differentiation. On the other hand, in latexin-overexpressing cells, RA also downregulates several genes involved in cell cycle processes but, strikingly, functions such as neuron differentiation and morphogenesis appear also downregulated.

Finally, analyzing the differences in the transcriptional profile of RA-treated empty vector-transfected cells and RA-treated latexin-overexpressing cells would shed light into the molecular basis that characterize the S-type phenotype and also could help to understand the long-term survival effect observed in latexin-overexpressing cells (Chapter II). We first observe that the main biological functions associated to upregulated genes in RA-treated latexin-overexpressing cells compared with RA-treated empty vector-transfected cells are cell migration, cell motion, and response to mechanical stimuli. These functions are consistent with S-type cells having different migration potential compared with N-type cells (Meyer et al. 2004) and also with the apoptosis resistance exhibited by S-type cells (Hopkins-Donaldson et al. 2002; Bian et al. 2004). Notably, although the sole overexpression of latexin upregulates genes involved in neuron differentiation, this process is not among the functions associated to upregulated genes in RA-treated latexin-overexpressing cells. Therefore, these results strengthen the assumption that the differentiated phenotype with extensive neurites of RA-treated latexin-overexpressing cells observed after 30 days in culture (Chapter II) is associated to a survival effect that allows a sustained neuronal differentiation rather than a promotion of neuronal differentiation. Yet another relevant feature observed after a close inspection of this comparison is that RA-treated latexin-overexpressing cells compared with RA-treated empty vector-transfected cells upregulate the expression of a cluster of genes belonging to mitochondrial ATP synthesis coupled to electron transport category.

Overall, one of the major findings of this study is that latexin, either in the presence or the absence of RA, upregulates the expression of a vast number of genes related to the ECM. The ECM is a dynamic network that regulate fundamental processes such as organ morphogenesis, differentiation, proliferation, adhesion, migration and tissue maintenance (Chen et al. 1997). Particularly, it also regulates several cancer properties such as angiogenesis, metastasis and proliferation (Pickup et al. 2014). For this reason, the potential involvement of the ECM in latexin-mediated effects is of special relevance to discern the role of latexin in SH-SY5Y cells. On one hand, changes in the ECM composition could be responsible of the PI3K/Akt pathway activation previously observed (Chapter II). In this sense, the transduction of ECM signals into the intracellular space is mainly mediated by the ECM receptors integrins (Legate et al. 2006; Brizzi et al. 2012). Such receptors can be intracellularly regulated by Integrin-linked kinases (ILK) which can, in turn, lead to cytoskeleton reorganizations and to the activation of several signaling cascades including the PI3K/Akt (Qian et al. 2005; Kimura et al. 2010). Alternatively, integrins can phosphorylate focal adhesion kinases (FAK), which can also activate the PI3K/Akt pathway (Velling et al. 2004). In addition, ECM proteins can interact with growth factors and cytokines thus serving as reservoirs for these molecules. Therefore, the ECM can mediate their local release in the adequate cellular context (Hynes & Naba 2012). In this sense, ECM proteins, via releasing growth factors or cytokines, can also activate such intracellular pathway. Notably, the notion of the ECM being cellular reservoirs of several factors is in the line with the long-term pro-survival effect mediated by latexin. On the other hand, the ECM has also a fundamental role in cell specification processes. As an example, it has been reported that soft matrices can direct stem cells towards specification into neuronal lineage (Engler et al. 2006; Georges et al. 2006). Thus, changes in the composition of the ECM along RA treatment would help to

understand how the ECM could mediate latexin-promoted effects. In line with this idea, ECM composition has been reported to dynamically change along the embryonic development. Indeed, embryonic ECM contain more in hyaluronan, fibronectin and proteoglycans such as versican whereas adult tissues tend to be enriched collagen, elastin and other ECM components (Nandadasa et al. 2014). Under our experimental settings, we observe several changes in the expression of genes associated to the ECM upon RA treatment and depending on latexin overexpression. For example, we observe that versican (VCAN), one of the major extracellular proteoglycans and a prototypical ECM protein of immature tissues (Hynes & Naba 2012), is markedly upregulated in latexin-overexpressing cells. However, this gene is significantly downregulated upon RA treatment in latexin-overexpressing cells. This finding could implicate that ECM in latexin-overexpressing cells could emulate an immature situation whereas the ECM composition in RA-treated latexin-overexpressing cells could be suggestive of a more differentiated event. However, this assumption would require experimental support. In spite of that, these results turn to indicate that variations in the ECM composition could play a key role in cell specification processes mediated by latexin. In line with this, it is important to mention that some of the S-type markers analyzed correspond to ECM proteins. Strikingly, upregulation of S-type markers is already observed in untreated latexin-overexpressing cells, suggesting that the overexpression of this protein could already condition the cells to experience such transdifferentiation process. Of note, the S-type marker vimentin (Acosta et al. 2009) shows the same pattern of expression than the one observed at the protein level (Chapter II), being downregulated by RA treatment but substantially enhanced by latexin overexpression. Similarly, the S-type marker CD44 (Walton et al. 2004) is also downregulated along RA treatment but latexin remarkably upregulates its gene expression. Surprisingly, CD44 displays opposed fold change direction when RA is added either in cells overexpressing latexin or transfected with the empty vector.

We have asserted that the ECM could play a pivotal role in latexin-mediated effects but the potential link between latexin and this dynamic structure has not been postulated yet. Interestingly, the regulation of the ECM involves several proteases including matrix metalloproteases (MMPs), mammalian disintregrin metalloproteinases (ADAMs) and other proteolytic enzymes (elastases, cathepsins, various serine esterase proteases, etc.). Latexin is the only known endogenous inhibitor of zinc-dependent metallocarboxypeptidases (MCPs) in mammalians (Pallarès et al. 2005). Strikingly, RNA-seq analysis revealed that carboxypeptidase A4 (CPA4) was significantly upregulated in latexin-overexpressing cells (1.4 fold higher). More interestingly, RA-treated latexin-overexpressing cells compared to RA-treated empty vector-transfected cells exhibited an increase in fold change of 2.69, thus representing the third most upregulated gene in latexin-overexpressing cells treated with RA. This fact implies that the synergy of latexin expression and RA treatment is determinant for CPA4 gene expression activation. Besides, under our experimental settings, CPA4 is one of the RA-responsive genes, suggesting a putative association between this enzyme and differentiation processes. In this sense, it has been known that MCPs can regulate the processing of neuropeptides and growth factors (Sapio & Fricker 2014; Sapio et al. 2015). Among MCPs, CPA4 and CPA6 have been identified in humans as secreted proteins from the A/B subfamily; CPA6 is bound to the ECM after secretion whereas CPA4 remains soluble

(Lyons et al. 2008; Tanco et al. 2010). Relevant CPA4 and CPA6 substrates are neurotensin, enkephalin, granins and angiotensin, which have been previously shown to play important roles in cell proliferation and differentiation (Lyons et al. 2010; Tanco et al. 2010). Therefore, it is conceivable that in our cellular model CPA4, via C-terminal cleavage of ECM components, could modulate the bioavailability and specific release of factors that could intimately regulate processes such as cell specification. However, whether this enzyme is biologically active or not when latexin is overexpressed must be elucidated. Taken all together, one scenario could be that latexin-mediated effects in SH-SY5Y cells require gene expression activation of CPA4.

Overall, the results presented in this Chapter provide extensive information about the transcriptional effect promoted by latexin expression in SH-SY5Y cells. One of the major findings is that the vast majority of genes regulated by latexin belong to the ECM. Given the reported intracellular location of latexin, the precise relationship between latexin and the potential remodeling of the ECM remains veiled.

4. CHAPTER 4. LATEXIN-ASSOCIATED MORPHOLOGICAL EFFECTS IN OTHER CELLULAR MODELS

We have previously characterized that latexin facilitates S-type differentiation in response to RA or Brdu treatment. In addition, we also shed light on the ability of this protein to modify the gene expression profile, particularly regulating genes associated to the ECM (Chapters I, II and III). When extending our results to other neuroblastoma cells, we first evidence that, compared to the S-type cell line, the I-type SK-N-LP cell line activates latexin expression more efficiently. In addition, BrdU harbors more capacity than RA to upregulate the expression of latexin, suggesting that latexin-mediated effects could be more pronounced when employing this differentiation agent. These findings are consistent with SK-N-AS cells already having a senescent-like phenotype. In this regard, it is feasible that these cells do not need a strong activation of cell specification programs to acquire a senescent phenotype. In contrast, I-type cells are considered either cancer stem cells (Ross et al. 1995) or intermediate cells between the N and the S phenotype (Ross et al. 2003). Therefore, it is likely that SK-N-LP cells require robust changes to activate cellular senescence and, presumably, that latexin plays a fundamental role in the acquisition of such phenotype. In line with this, latexin facilitates BrdU-induced senescence in SK-N-LP cells, although such effect is not as differentially promoted as in the N-type cell line SH-SY5Y (Chapter I and II). This could be due to empty vector-transfected SK-N-LP cells having a higher propensity to transdifferentiate towards the S-type phenotype than SH-SY5Y cells. This assumption is in accordance with the hypothesis that I-type cells are an intermediate phenotype between both N and S morphologies (Ross et al. 2003). In this regard, less pronounced latexin effects in this cell line could be understood in the context that the transdifferentiation from the N to the S phenotype probably needs a more robust activation of cell specification programs in which latexin could be participating compared with the transformation from an intermediate phenotype towards the Schwannian lineage. In any case, the importance of these findings resides in the fact that I-type cells are the most aggressive sub-type among

neuroblastoma phenotypes (Walton et al. 2004) and that latexin expression is favoring the appearance the least aggressive form as is the S-type phenotype (Ross et al. 2003). Yet another striking effect is that SK-N-LP cells gradually die along the course of BrdU treatment, and that this effect is apparently attenuated in latexin-overexpressing cells. Accordingly, latexin could be a determinant of the cell fate decision between apoptosis and senescence in this cell line. Importantly, in SK-N-LP cells the PDK1/Akt axis does not appear to play such a fundamental role in the emergence of S-type cells. Therefore, it can be inferred from these results that the development of the S-type phenotype in SK-N-LP cells does not harbor the same dependence on the PDK1/Akt pathway than in SH-SY5Y cells. This assumption might imply that different mechanism besides Akt activation could be required for the emergence of the S-type morphology in SK-N-LP cells or that basal levels of Akt phosphorylation in this cell line could be different than in SH-SY5Y cells, thus requiring higher concentrations of the inhibitor to promote similar effects.

We also aimed to unravel the potential role of latexin in a different cellular paradigm characterized by a distinct genetic background and cell specification programs. In addition, glioblastoma multiforme (GBM) are the most common lethal primary brain tumor in adults (Friedman et al. 2013), which adds further importance in unveiling the effect of latexin in such cellular model. One of the hallmarks of GBM is their extreme heterogeneity, mainly attributed to the presence of cancer stem cells (Yunqing Li et al. 2011). Therefore, the potential involvement in GBM of a protein that regulates cell specification processes cells is of outstanding interest. We reveal that in glioblastoma-derived LN-18 cells, latexin overexpression promotes the appearance of dense accumulations at the edge of the cell, which are reminiscent of the process of membrane ruffling. This process is characterized by flat sheets of membrane which are supported by the cytoskeleton and are indicative of polarization changes that cells acquire prior to cell migration (Choma et al. 2004; Gauthier et al. 2012). These phenotypic changes mainly involve focal adhesions and cytoskeleton re-organizations and both processes are connected to the ECM (O'Connor et al. 2000). These results, although preliminary, strengthen the previous finding that latexin mediates cell adhesion processes and points out to the ECM likely supporting this cellular function. However, it should be necessary to address whether latexin-overexpressing cells present migratory differences and also the potential involvement of the ECM in such process to completely understand the precise effect of latexin in GBM development. Moreover, in this cellular model is well-exemplified the differential capacity displayed by different senescence and differentiation inductors to alter cell morphology, and is also highlighted that latexin is able to modify the cellular morphology induced by these chemical agents. These results are in accordance with the previous assumption that latexin-overexpressing cells could also respond better to other stimuli besides RA and also with latexin promoting cell morphogenesis processes (Chapter III). Surprisingly, although latexin upregulation is only observed upon RA or BrdU treatment for up to 5 days, this protein only prompts subtle morphological changes upon induction with these agents. On the contrary, latexin-associated phenotypic changes are more evident when employing the isoquinoline alkaloid Berberin (Wang et al. 2012) and the genotoxic agent doxorubicin, despite these senescence inductors do not upregulate latexin expression after 5 days of treatment. This fact suggests that upon induction with these

agents, latexin could be regulated by different mechanisms or that the potential regulation of latexin exerted by these agents is more transient than the one promoted by RA or BrdU. Furthermore, latexin overexpression facilitates berberin- or doxorubicin-induced cellular senescence, although the presence of SA-β-Gal positive cells is markedly more reduced than in other cellular models. In this regard, LN-18 cells are reported to be more prone to undergo apoptosis rather than senescence upon induction with different stimuli, and this effect is mainly associated to the wild type expression of PTEN and the downregulation of Akt found in these cells (Lee et al. 2011). In this regard, it should be explored whether this axis is determinant in the pro-senescence latexin-induced effect in LN-18 cells. Therefore, U87MG cells are an appealing model since they express high endogenous levels of latexin and also because they express mutated PTEN, being a well-described model of senescence (Rebbaa et al. 2003; Lee et al. 2011). Interestingly, the downregulation of latexin in U87MG cells facilitates a cellular phenotype characterized by long and thin cytoplasmic extensions. More surprisingly, latexin-downregulated cells challenged with doxorubicin acquire an astrocytic-like phenotype accompanied by a reduction in the number of SA-β-Gal positive cells. Remarkably, the downregulation of latexin results in decreased cell numbers with no apparent induction of apoptosis. This fact, taken together with the reduction of senescence observed, points out that different non-cycling processes aside from cellular senescence must be undertaken in cells downregulating latexin. Since the cellular morphology observed in cells lacking latexin expression is evocative of astrocytic differentiation, one possible scenario is that latexin expression could be determinant in the cellular choice between senescence and terminal differentiation. Notably, the partial downregulation of latexin gives rise to intermediate phenotypes that are able to senesce, indicating that the specific levels of latexin expression are fundamental in the final cell fate the cells acquire. This evidence helps to understand why certain stimuli able to upregulate latexin expression such as RA or BrdU promote a cellular phenotype that is enhanced when latexin is overexpressed, suggesting that specific levels of latexin expression must be achieved to promote the complete acquisition of a cellular phenotype. In any case, it should be of outstanding interest to ascertain whether an anticancer drug is able or not to promote the expression of latexin since it would be determinant to predict the final outcome these agents will promote. Taken altogether, we can conclude that cell morphology changes promoted by latexin in different cellular contexts mainly consist of larger cell shapes with a more proportion of cytoplasm. In this regard, it is reported that changes in cell morphology are associated, among other processes, to cell division, differentiation, response to physiological stimuli and motility (Lecuit & Pilot 2003). Accordingly, it is feasible that latexin, via modifying cell shape, could be promoting specific cellular roles that will depend on the cellular context and the specific stimuli.

As predicted in the first Chapter, whether latexin is beneficial or detrimental for tumor progression, will depend on the cellular phenotype that will be specified. Certainly, when analyzing different tumor databases we discover that latexin is able to promote differential outcomes depending on the type of tumor. In this regard, latexin mRNA expression positively influences overall survival in neuroblastoma patients. This result is in accordance with the Schwannian phenotype promoted by latexin having less tumorigenic potential than the other subtypes. On the other hand, in glioblastoma tumors latexin mRNA levels do not seem to correlate with

patient survival. Taken altogether, these results turn to indicate that latexin does not behave as a tumor suppressor *per se* but its implication in cell specification processes ultimately leads to different outcomes depending on the cellular context.

VI. CONCLUDING REMARKS

CONCLUDING REMARKS

1. Unlike RARRES, having a heterogeneous pattern of expression in neuroblastoma-derived cells, latexin expression appears commonly absent in neuroblastoma cells.

2. The expression of latexin can be modulated by the DNA demethylating agent 5-aza-dC and by RA in SH-SY5Y cells.

3. Latexin is not directly altering cell proliferation or cell death responses in SH-SY5Y cells.

4. The overexpression of latexin does not promote neuronal-like differentiation upon RA treatment under different cell differentiation conditions.

5. Latexin promotes the early appearance of a population of flattened cells, resembling the S-type phenotype, only detected under high serum conditions (15% FBS).

6. Latexin-overexpressing cells exhibit an enhanced resistance towards high concentrations of RA.

7. Akt and ERK 1/2 are more efficiently activated upon RA treatment in latexin-overexpressing cells.

8. The p42/p44 MAPK pathway is not essential in the promotion of the RA-induced S-type phenotype.

9. The PI3K/PDK1/Akt pathway plays a pivotal role in the RA-induced S-type phenotype facilitated by latexin.

10. Latexin promotes long-term cell survival under unfavorable extracellular conditions allowing cells to senesce.

11. Cellular senescence promoted by latexin in response to BrdU treatment relies on the PI3K/Akt pathway activation.

12. Latexin promotes large changes in gene expression, mainly in genes involved in cell adhesion, cell development and cell differentiation process.

13. Latexin specifically upregulates some genes that are not upregulated by RA treatment, indicating potential divergent functions.

14. Genes upregulated by latexin, either in the presence or in the absence of RA, mainly reside in the ECM.

15. Latexin facilitates BrdU-induced senescence in the I-type cell line SK-N-LP.

16. Latexin promotes cell shape changes comprising cytoplasmic extensions, cell rounding and membrane ruffling in LN-18 upon the appropriate stimuli.

17. Latexin downregulation induces doxorubicin-induced astrocytic-like morphology in U87-MG cells.

18. Latexin mRNA levels correlate with good prognosis in neuroblastoma patients whereas is not statistically associated with patient survival in glioblastoma tumors.

VII. BIBLIOGRAPHY

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