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An unanticipated role for the Phospholipase A2 receptor (PLA2R1) as a novel cellular senescence regulator and tumour suppressor gene

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Arnaud AUGERT

Directeurs de Thèse : Dr Yvan de LAUNOIT & Dr David BERNARD

Pr Corinne ABBADIE	Président
Pr Bertrand FRIGUET	Rapporteur
Dr Jesus GIL	Rapporteur
Dr Stéphane ANSIEAU	Examinateur
Dr Yvan de LAUNOIT	Examinateur
Dr David BERNARD	Examinateur

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INTRODUCTION

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I/ INTRODUCTION

I.1 A short introduction to Cancer

I.1.1 Cancer basics

Although more commonly diagnosed nowadays, cancer is not a modern disease. In fact, human beings have had cancer throughout history and it comes to no surprise that people have written about it for centuries. The oldest descriptions in humans (although the word cancer was not used at the time) were discovered in Egypt and date back to about 3000 BC [1].

The origin of the word cancer comes from the Greek physician Hippocrates of Kos (460-370 BC). Hippocrates considered as the father of medicine used the term *"karcinos"* and "*karcinoma"* to refer to chronic ulcers or growths that seemed to be malignant tumours. These Greek terms refer to a crab, probably because cancerous growths reminded Hippocrates of a moving crab. [1]

Based on GLOBOCAN 2008, there were about 12.7 million cancer cases diagnosed and 7.6 million deaths accounted (around 13% of all deaths) in 2008. In fact, cancer is the leading cause of death worldwide. Although higher incidence and death rates are found in economically developed countries, it also affects developing countries (the second cause of death) [2]. Cancer incidence increases with ageing. It rises exponentially after the age of 50 [3]. It is quickly increasing in developing countries as a result of a "westernized lifestyle" that include changes in reproductive patterns, physical inactivity and obesity [4]. Moreover, in

developing countries cancer mortality rates are very high mainly due to low resources and limited health infrastructure [5].

Cancer aetiology studies suggest that environmental factors (tobacco smoke, obesity, viral, bacteria or parasites infections), inherited genetic (germline mutations) and epigenetic defects are the key determinants of cancer. Additionally, these cancer determinants might interact to increase cancer risk [6-8].

It is believed that continued growth and ageing of the world's population will greatly influence the future cancer burden. By 2030, it is expected that 75 million persons will be alive with cancer. It is predicted that 27 million cancer cases will be diagnosed with 17 million cancer deaths occurring annually [9]. Better/quicker diagnoses, breakthroughs in patient treatments and personalised cancer therapy for more effective ways to treat the diseases will be required to cope with such a burden.

I.1.2 The biology of Cancer (hallmarks)

In the year 2000, two pioneers of cancer research (Douglas Hanahan and Robert Weinberg) published in *Cell* a review listing, what they considered to be, the hallmarks of cancer [10]. At the time, they described and listed the hallmarks of cancer as being six essential alterations in cell physiology. Self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis were the six hallmarks described. Although this view was largely accepted with more than 6000 citations in 2010, an opinion article published in 2010 by Yuri Lazebnik in *Nature Review Cancer*

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suggested that the hallmarks of cancer defined by Hanahan and Weinberg were in fact, at least for five of them, the hallmarks of tumours in general (irrespective of their benign or malignant status) [11]. Importantly, Lazebnik took as an example solid cancers (which constitute the majority of cancers) and reminded that cancer are malignant tumours whereas benign tumours cannot be considered as a cancer.

Although it might not be the case for every benign tumours encountered, it seems that the hallmarks described by Hanahan and Weinberg can be found in some benign stages of tumorigenesis. For example, both benign and malignant tumours are self sufficient in growth signals, as autocrine mechanisms have been implicated in the growth and maintenance of both tumour types [12-15]. Angiogenesis is also a feature of both tumour stages as masses of tissue larger than a few milligrams require angiogenesis to survive [16] and some benign tumours can weight many kilograms [17]. Evasion of cell death programs and resistance to anti-growth factors has also been observed in some cases of benign tumours [18, 19]. Furthermore, as the size on the tumour does not always reflect the stage of the tumour [20, 21], it also seems that limitless replicative potential is shared by some benign and malignant tumours. As stated by Lazebnik, the only hallmark that clearly separates benign from malignant tumours is tissue invasion and metastasis. Benign tumours with exception to very rare cases do not metastasise whereas malignant solid tumours invade tissues and metastasise [11].

Hanahan and Weinberg published in March 2011 the “Hallmarks of cancer: the next generation” in the same journal *Cell*. They stated that it was for them “implicit that as normal cells acquire a succession of capabilities (hallmarks) to progress to a neoplastic state and that multistep process of human tumour pathogenesis could be rationalized by the need of incipient cancer cells to acquire the traits that enable them to become tumorigenic and

ultimately malignant". Therefore the hallmarks of cancer characterise in their terms tumorigenesis from the earliest stages (benign tumours) to the metastatic stages (malignant tumours).

Their vision of cancer hallmarks might not be shared by every scientist in the cancer field. Nevertheless, it provides robust and common traits of both benign and malignant tumours. In the last decade, major progresses were made adding two new "emerging" hallmarks of cancer: the reprogramming of energy metabolism in cancer cells and the evasion from the immune system (escape from immune destruction). These hallmarks have not been included as canonical hallmarks of cancer yet because Hanahan and Weinberg are unsure that they are extensive to all cancer cases. Additionally, enabling hallmarks have been added. Tumour promoting inflammation, gene instability and mutation do not necessary elicit tumour development but surely contribute in the transition. Furthermore, in addition to cancer cells, tumours are much more complex as they are surrounded by various types of cells that contribute to the acquisition of hallmarks: the "tumour microenvironment" (Figure 1).

Figure 1: The hallmarks of cancer as described by Douglas Hahanan and Robert Weinberg.

The six hallmarks proposed in 2000 remain robust hallmarks. They include enabling replicative immortality, evading growth suppressors, sustaining proliferative signalling, resisting cell death, inducing angiogenesis and activating invasion and metastasis. Two new “emerging” hallmarks of cancer: the reprogramming of energy metabolism in cancer cells and the evasion from the immune system (escape from immune destruction). Enabling hallmarks, tumour-promoting inflammation and gene instability and mutation were also added. Finally, tumorigenesis cannot be seen as a cell autonomous mechanism. In agreement, the “tumour microenvironment” has also recently been identified as a crucial regulator of tumour progression. Adapted from [22].

To counteract the acquisition of these hallmarks that will drive cells towards a malignant state, normal cells have developed failsafe mechanism such as apoptosis and cellular senescence. Cellular senescence is a mechanism that I have studied throughout my thesis. In fact the start of my thesis' work was based in identifying and characterising regulators of this cellular response. I therefore analysed cellular senescence in various contexts. For that reason, I have decided to write an introduction covering most of the cellular senescence field.

I.2 Cellular Senescence

Senescence (Latin word *senex*, meaning old age, or advanced in age) was described approximately 50 years ago by Leonard Hayflick and Paul Moorhead while cultivating human diploid cell strains [23]. Although at the time they did not refer to the term cellular senescence, they made the observation that serially cultured diploid strains had a finite lifetime *in vitro*. Four years later, Leonard Hayflick proposed in the same journal "that the finite lifetime of diploid cells strains *in vitro* may be an expression of ageing or senescence at the cellular level" [24]. He observed that primary cells in culture proliferate for approximately 55 population doublings before reaching the "Hayflick limit" which marks the end of their proliferative capacity and the entry into an irreversible growth arrest state. Since then, the term "replicative senescence" has been used to designate this type of cellular senescence.

However, "replicative senescence" is not the only type of cellular senescence that has been observed. Senescence can also be induced "prematurely" in response to cellular insults

such as mitogenic oncogenes, the loss of bona fide tumour suppressor genes, DNA damage, oxidative stress, endoplasmic reticulum stress and/or autophagy [25-33].

Historically, oxidative stress was the first cellular insult associated with "premature senescence" [34-36]. In 1994, DNA damage was also associated with a cellular senescence "like" phenotype [37]. A few years later, in a work published in *Cell*, Serrano and colleagues made what turned out to be a pioneer discovery, showing that cellular senescence could be induced "prematurely" in response to activated oncogenes [31]. From then on, a multitude of publications have provided supplemental evidences, reinforcing the idea that cellular senescence can be activated "prematurely" in response to various cellular insults.

Cellular senescence has been associated with numerous pathologies such as Cancer, Ageing and Ageing related diseases [38-41] and more recently tissue repair [42-44]. In this "cellular senescence" section, I will highlight the major discoveries made to date in the senescence field (in a cancer context) including: the specific markers that characterise cellular senescence, the different types of cellular senescence encountered and the pathways regulating this phenomenon. Since the discovery of a senescence-associated secretory-phenotype (SASP), cellular senescence can no longer be seen as a cell autonomous process. I will therefore comment on the most recent discoveries describing the interconnection that exist between senescent cells and the microenvironment, especially the immune system. Finally, I will describe the intimate relationship that exists between cellular senescence and cancer. Deciphering the role of cellular senescence in cancer is crucial as pro-senescence therapy has been proposed as an anti-tumoral treatment [45-48].

I.2.1 Features of senescent cells

I.2.1.1 Cytological alterations

Cellular senescence is not limited to human primary fibroblast models originally described by Hayflick in 1961 and 1965. It has been observed in human mammary epithelial cells [49], prostatic cells [50], lens cells [51], keratinocytes [52], endothelial cells [53], lymphocyte T cells [54] among others. This cellular response is not limited to human cells as it also takes place in other species. It has been described in Mouse [55], Rat [56], Chicken [57], Caenorhabditis elegans [58], Zebrafish [59] and Yeast [60].

Upon entering senescence, cells lose their original morphology. They become increased in size, have a much larger flattened cytoplasm, bigger nucleus and nucleoli and are sometimes multinucleated [61-63]. Analysis of the cytoplasm reveals the appearance of vacuoles as well as an increase in cytoplasmic microfilaments [64, 65]. Various research articles have also observed an increase in the number of lysosomes and Golgi [66-70]. Figure 2 shows the morphology of normal human fibroblasts (WI38) and normal human mammary epithelial cells (HMEC) prior and during cellular senescence.

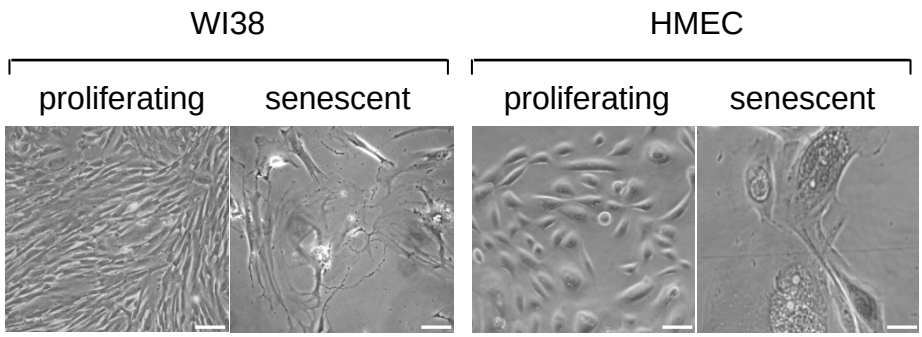


Figure 2: Cellular senescence morphology.

Morphology analysis of primary (WI38 and HMEC) senescent cells versus their proliferating counterparts.

I.2.1.2 Growth arrest

Cellular morphology is a notable feature of cellular senescence. However what is probably even more evident is the cell cycle arrest associated with cellular senescence. As the growth arrest in senescent cells is tightly linked to cell cycle regulation, the various players of the cell cycle have proven to be critical regulators of the senescent growth arrest state.

The cell cycle is a fundamental feature of living organisms that is responsible for maintaining and/or increasing both cell size and number. The eukaryotic cell cycle is divided into four successive phases: G_1 (Gap 1 or growth phase 1), S (synthesis phase), G_2 (Gap 2 or growth phase 2), and M phase (mitotic phase). A fifth phase exists, G_0 or quiescence. During this phase, cells exit the cell cycle and remain in a quiescent state. Cells persist in this state indefinitely or, if required, re-enter the cell cycle upon mitogenic stimulations. Interphase, includes G_1, S, and G_2 phase. It is the period during which the cell prepares for division (M phase) (Figure 3).

At the core of mammalian division are the cyclin dependent kinases (CDK), a family of serine/threonine kinases [71]. In humans, the CDK family is composed of 13 members that interact with 29 cyclins or cyclins related proteins [71]. Although there are many members for each family, only a limited number of CDK-cyclin complexes are involved in cell cycle regulation. Interphase involves CDK2, 4 and 6 whereas mitosis requires CDK1 (Cdc2). These CDKs are regulated by ten cyclins that belong to four classes of cyclins (the A-, B-, D- and E-type cyclins) [72] (Figure 3).

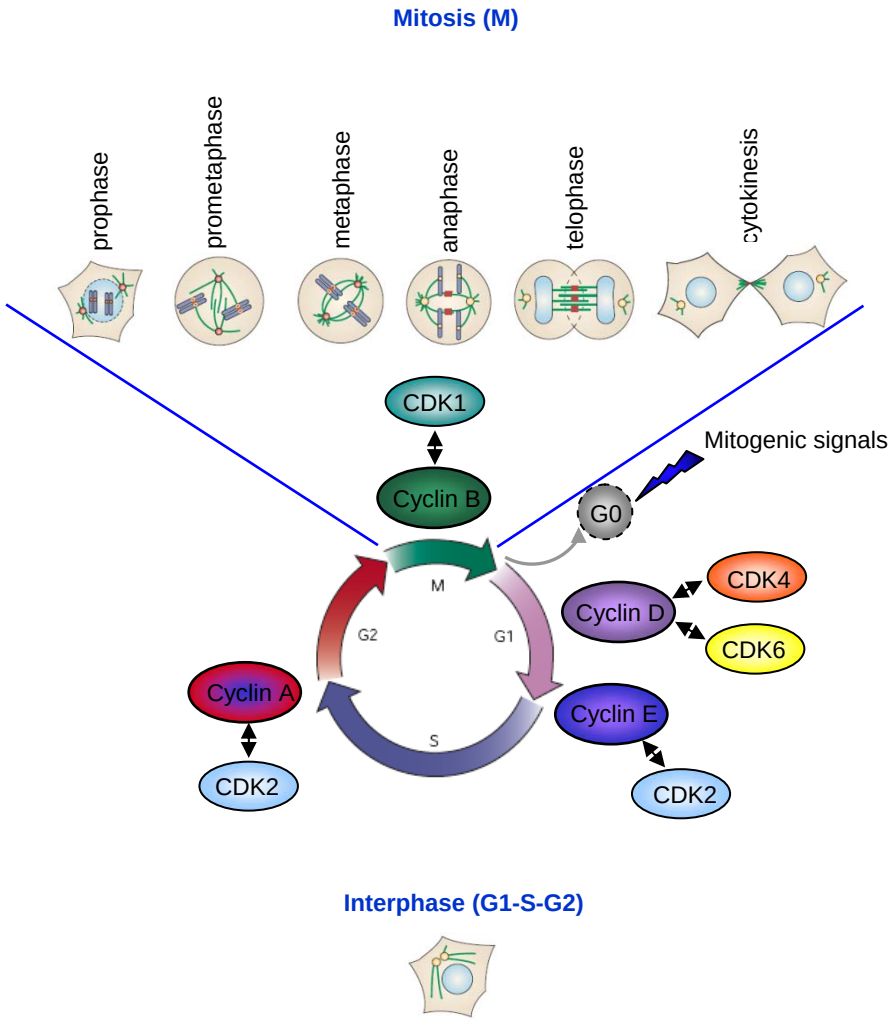


Figure 3: Mammalian cell cycle regulation by the CDK-cyclin complexes (basic information).

The cell cycle is divided into five phases. Interphase, which includes three phases (G_1, S, and G_2 phase). Mitosis (M phase) and quiescence (G_0) are the last two phases. Mammalian cell cycle is controlled by CDK-cyclin complexes. Cyclin expression oscillate during the different phases resulting in active CDK-cyclin complexes at specific stages of the cell cycle. Upon mitogenic stimulation, cells enter the cell cycle and will require specific CDK-cyclin complexes to progress through the different phases. Adapted from [72-75].

The proteins that regulate CDK-cyclin complexes are the cyclin dependent kinase inhibitors (CKIs). They are essential in the establishment of the senescent growth arrest state [76]. CKIs are divided into two families. The first family; INK4, for inhibitors of CDK4, includes $p16^{INK4A}$ [77], $p15^{INK4B}$ [78], $p18^{INK4C}$ [79, 80], $p19^{INK4D}$ [80, 81]. The second family of CIP (CDK-interacting protein) and KIP (cyclin-dependent kinase inhibitor protein) proteins include $p21^{CIP1}$ [82-86], $p27^{KIP1}$ [87-89], $p57^{KIP2}$ [90, 91].

The INK4 family control cell cycle arrest in G1 phase by blocking the formation of the catalytically active CDK4/6-type D cyclins complexes [92-94]. The CDK4/6-type D cyclins complexes are often found to be interacting with KIP/CIP inhibitors. Whether these complexes are active is still a matter of debate [92, 94]. However, when INK4 proteins inhibit CDK4/6-cyclin D formation, KIP and CIP proteins are removed from the CDK4/6-type D cyclins complex and their bioavailability increases (Figure 4). This enables them to interact and inhibit CDK2-type E or A cyclin complexes. INK4 and CIP/KIP proteins therefore regulate G1 cell cycle progression by inhibiting the CDK-cyclin complexes [92].

The CDK-cyclin complexes favour G1 cell cycle progression through the phosphorylation of the RB family members [92, 94]. RB family proteins (Rb, p107, p130) are, among other, transcriptional co-factors that interact with E2F transcription factors (E2F1-5) inhibiting their transcriptional activity required for DNA synthesis. Upon phosphorylation of RB family members by the CDK-type D and E cyclin complexes, E2F transcription factors are released from their interaction with RB proteins leading to cell cycle progression [95, 96] (Figure 4).

During senescence, the CKIs most frequently found up-regulated and associated with a G1 cell cycle arrest are $p16^{INK4a}$ and $p21^{CIP1}$ [76]. However $p15^{INK4B}$ has also been found up-regulated in various senescence settings [97-101] and in absence of $p16^{INK4A}$, $p15^{INK4B}$ can act as a critical tumour suppressor [102]. An increase expression during cellular senescence of the other INK4 family members, $p18^{INK4C}$ and $p19^{INK4D}$, is to my knowledge, not very well documented and/or has not been described. On the other hand the KIP family protein $p27^{KIP1}$ has been found to be up-regulated and to play a critical functional role in a subset of senescence contexts [103-105]. Information on the functional role of the other KIP family member $p57^{KIP2}$ is, to date, limited. However, a couple of studies have suggested a functional role $p57^{KIP2}$ in senescence induction [106-108].

Senescent cells are usually blocked in the G1 phase of the cell cycle [31, 37, 109, 110]. However, in some cases they display (4n) DNA suggesting that cells are either blocked in the late S phase, G2 phase or M phase [29, 111-113]. Finally, cell cycle arrest is not limited to G1 and G2 arrest. Edji Hara's laboratory also provided evidence that the $p16^{INK4a}$/ Retinoblastoma (Rb) pathway could cooperate with mitogenic signals to activate a reactive oxygen species (ROS)-PKCδ signalling leading to an irreversible cytokinesis block (Figure 4) [114].

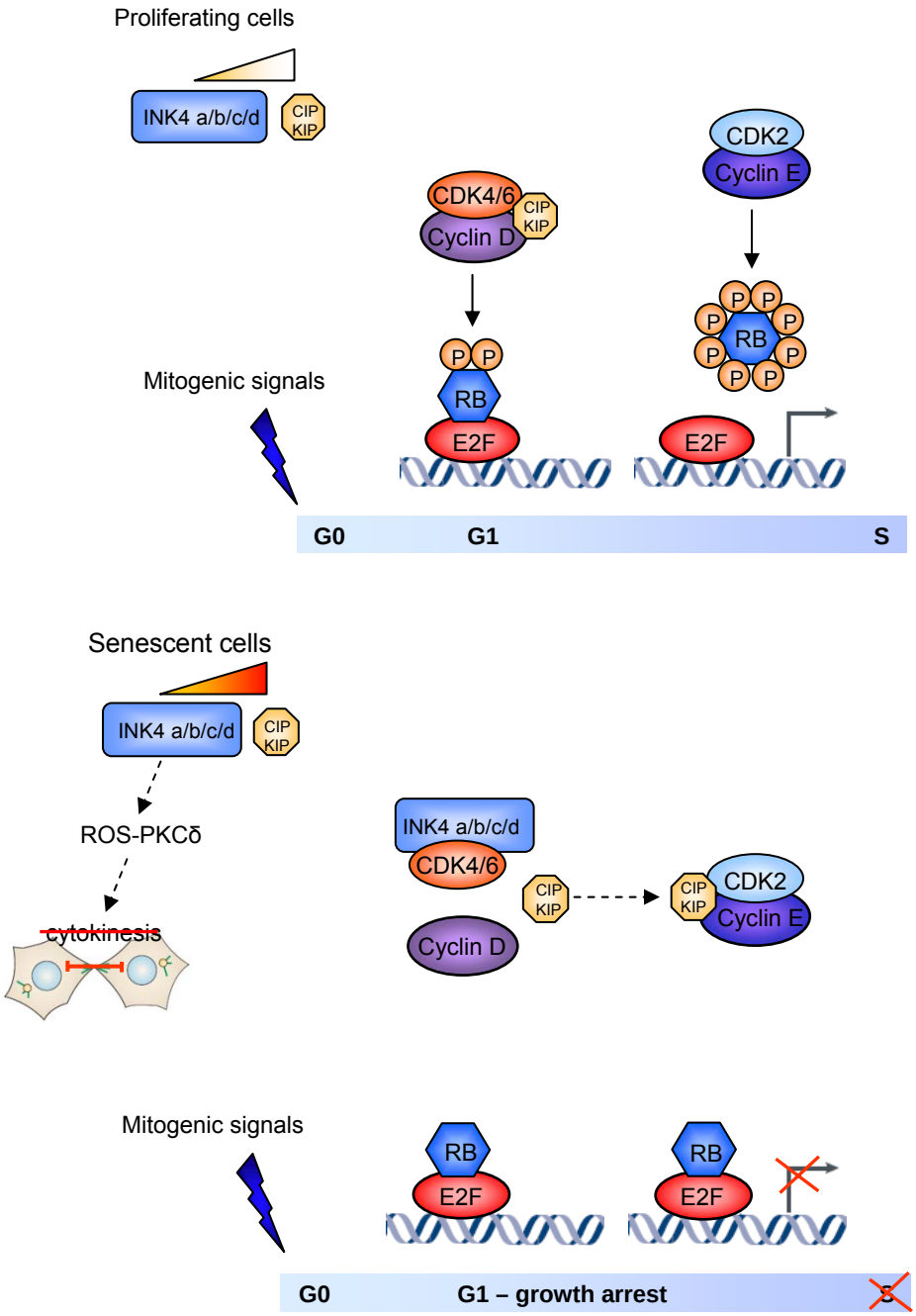


Figure 4: The regulation of the cell cycle growth arrest in senescent cells.

The upper scheme represents proliferating cells. In this context, the levels of CKI is low, CDK-cyclin complexes are activated and Rb is hyperphosphorylated leading to E2F transcriptions factors activation. The bottom scheme represents the typical G1 growth arrest in senescent cells. CKI levels are high, leading to an inactivation of the CDK-cyclin complexes and inhibition of cell cycle progression. The cytokinesis block by the ROS-PKCδ was also illustrated. However the G2 phase arrest was illustrated in the figure for clarity purposes. Adapted from [92, 95].

I.2.1.3 Alterations in gene expression

Various studies have catalogued the alterations of genes and microRNAs expression in senescent cells and their counterparts [115-123]. In this section, I will focus on some of the critical genes that not only have their expression altered during senescence but are also functionally involved the senescent phenotype. A notable example comes from recent studies suggesting that molecules of the senescence-associated-secretory-phenotype (SASP) are not only bystanders of the senescent phenotype but can also be functionally involved in reinforcing the growth arrest state [98, 124-126]. Indeed, the up-regulation of various SASP components such as interleukin 6 (IL-6), interleukin 8 (IL-8), plasminogen activator inhibitor 1 (PAI-1), insulin growth factor binding protein 7 (IGBP7) is required for senescence growth arrest and maintenance as their depletion allows a bypass of cellular senescence [98, 124-126]. However, these proteins do not directly trigger growth arrest but instead activate critical regulators of the growth arrest state.

The two major pathways regulating senescence are the tumour-suppressive pathways governed by the tumour protein 53 (p53) and Rb proteins [76]. These pathways are activated in response to various cellular insults. Once activated, they regulate, through their transcriptional regulator activities, the expression/activity of critical genes involved in the induction and the maintenance of the growth arrest in senescent cells. For example, $p21^{CIP1}$ is a direct target of p53 and is up-regulated in response to p53 activation [76].

Senescence induction is not only associated with gene up-regulation as many genes are also found down-regulated. It is the case for polycomb complex members such as enhancer of zeste homolog 2 (EZH2) and chromobox homolog 7 (CBX7) which are involved in the

repression of the $p15^{INK4B}$/alternative reading frame (ARF)/$p16^{INK4A}$ (INK4B/ARF/INK4A) locus and cellular senescence [50, 127].

I.2.1.4 Senescence-associated-beta-galactosidase (SA-βgal) activity

In the 1990s, Judith Campisi's team was looking for a marker to identify senescent cells *in vivo*. It had been suggested that senescence might exist *in vivo* and accumulate with age [128], however markers to distinguish senescent cells from quiescent or terminally differentiated cells in tissues were lacking. Goberdhan Dimri and colleagues identified in senescent cells a β-galactosidase activity, histochemically detectable at pH 6.0 [129]. Aside from a few cell types (adult melanocytes, sebaceous and eccrine gland cell), this marker was specific to senescent cells as it was absent in pre-senescent, quiescent, terminally differentiated and immortal cells. Looking for evidence of senescence's presence in various skin samples of human donors, Goberdhan Dimri also observed an increase in the β-galactosidase activity in an age dependent manner suggesting that senescent cells exist *in vivo* and accumulate with age [129]. This research article gave raise to the "senescence-associated-beta-galactosidase (SA-βgal) activity".

The method measures an enzymatic activity at pH 6.0 in order to distinguish it from the acidic (lysosomial) β-galactosidase activity, present in all cells and detectable at pH 4.0 [130]. It relies on a chromogenic substrate 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal), that once cleaved by the β-galactosidase enzyme yields an insoluble blue compound specific of senescent cells at pH 6.0 (Figure 5). It has now been demonstrated that the increased β-galactosidase activity observed in senescent cells is in fact due to the lysosomal β-

galactosidase present in all cells [69]. Additionally, it has been demonstrated that this SA-βgal is not functionally involved in the cellular senescence phenotype [131].

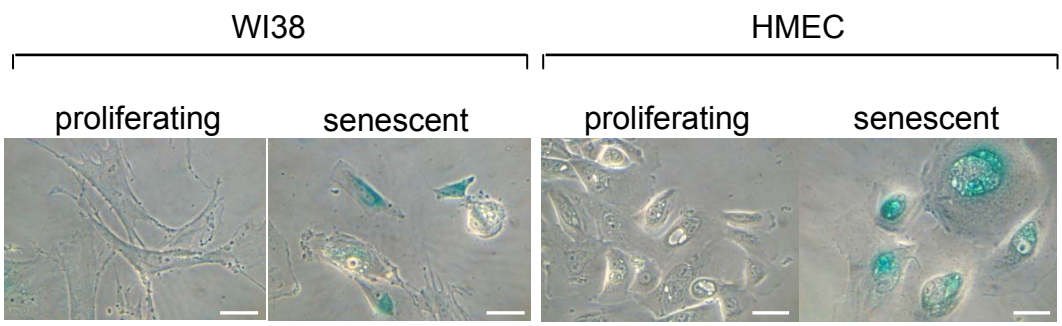


Figure 5: Senescence-associated-beta-galactosidase (SA-βgal) activity.

SA-βgal analysis in proliferating and senescent primary human fibroblast (WI38 cells) and human mammary epithelial cells (HMEC). (Augert et al, unpublished data). Scale bar represents 50 µM.

I.2.1.5 Senescence-associated-heterochromatin-foci (SAHF)

A regulatory role of the senescence phenotype by chromatin alterations was first suggested approximately fifteen years ago [132, 133]. However, specific chromatin structures associated with senescent cells were unambiguously described and characterised in 2003 by the group of Scott Lowe. In a study published in *Cell*, Masashi Narita and colleagues used 4′-6-Diamidino-2-phenylindole (DAPI) to stain the DNA of normal and senescent human cells. As expected, he observed that normal human cells exhibit a diffuse distribution of DNA through the cell nucleus. However, in DAPI-stained replicative and premature senescent cells, brighter and punctuate DNA foci were observed (Figure 6). This feature was specific to senescent cells as it was not observed in quiescent cells or in cells that escaped from senescence [134]. Analysing the structures, they found that the process was regulated by the Rb pathway and the appearance of the foci was associated with E2F target genes repression (e.g inactive transcription sites that contained proliferative genes such as A-type cyclins). They suggested that this feature could be responsible for the stability of the senescent state and decided to name these distinct heterochromatin structures present in senescent cells, SAHF, for senescence-associated-heterochromatin-foci [134].

Since this major discovery, researchers have tried to gain deeper molecular insights underlining SAHF. SAHF are facultative heterochromatin as the constitutive heterochromatin regions such as pericentromeric and telomeric regions are located at the outermost edge of these structures [134-136]. They contain common markers of heterochromatin which include hypo-acetylated histones, methylated histones (H3K9Me) and the presence of heterochromatin protein 1 (HP1). The histone variant macroH2A, and HMGA a non histone

protein, have been identified as crucial regulators in SAHF formation [135, 137, 138]. SAHF establishment is a multistep process that will not be discussed herein. However recent studies suggest that its role and regulation might be more complex than first believed. Indeed, it has recently been proposed that SAHF are dispensable for cellular senescence and that their physiological relevance might be limited [139]. Finally, SAHF could also cooperate with other regulatory pathways of cellular senescence [140]. Altogether, it suggests that even if SAHF have been used as senescence marker and functionally involved in this phenotype, the role of SAHF and the complexity of its molecular determinants are yet to be deciphered.

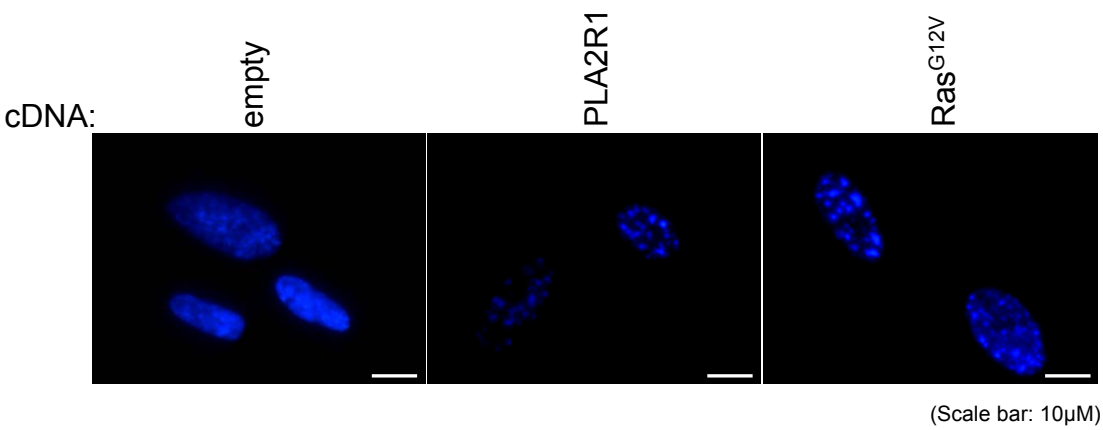


Figure 6: Senescence-associated-heterochromatin-foci (SAHF) activity.

SAHF detection in primary human fibroblast (WI38 cells) expressing a control vector, PLA2R1 or H-Ras^{G12V} as a positive control (Augert et al, unpublished data). Scale bar represents 10 µM.

I.2.1.6 Senescence-associated-secretory-phenotype (SASP)

The first observations suggesting an increased expression of secreted proteins during cellular senescence were made in the early 1990s. Indeed, proteases involved in the extracellular matrix (ECM) degradation such as collagenase, tissue plasminogen activator (t-PA) and metalloprotease (MMPs) were found to be up-regulated both in senescent cells and in their supernatant [141-145]. In the late 1990s and early 2000s various studies confirmed these observations [146-149]. From then on, Judith Campisi's laboratory and others have made big efforts identifying the secreted factors associated with cellular senescence, which has been termed the senescence-associated-secretory-phenotype (SASP).

Since its discovery, the SASP has provided novel insights to the senescence field. Cellular senescence can no longer be seen as a cell autonomous suppressor of tumorigenesis, but as a process that can modulate the microenvironment through the secretion of proteases, cytokines and other secreted factors. The idea behind this, as described by Judith Campisi, is that senescence does not only have a role in inhibiting tumour progression, but can also act as a "double edge sword" [150]. Indeed, the mobilisation of the microenvironment by senescent cells can in some cases benefit the organism, but also be detrimental [151].

Various publications have described paracrine effects of SASP molecules on neighbour cells [146-149, 152, 153]. What was however unexpected was the role of these secreted proteins in reinforcing the growth arrest state of senescent cells. Indeed, as stated by Judith Campisi and d'Adda di Fagagna in *Nature Review Molecular and Cellular Biology* in 2007, "many changes in gene expression appear to be unrelated to the growth arrest. Many

senescent cells over-express genes that encode secreted proteins that can alter the tissue microenvironment" [76].

The first article identifying a functional role of these factors in senescence phenotype came from the René Bernard's laboratory in a work published in *Nature Cell Biology* in 2006 [125]. Kortlever and colleagues demonstrated that plasminogen activator inhibitor-1 (PAI1) was not just simply a senescence marker as previously described by others [31, 154, 155] but could instead act as a critical regulator of the senescent growth arrest [125]. By performing a loss of function genetic screen they showed that PAI1, a p53 target gene [156], was essential for the establishment of "replicative senescence" [125].

In 2008 followed a series of articles published in *Cell* demonstrating that the functional role of secreted proteins was not only limited to PAI1 but could be extended to other members of the SASP. The first publication was the work of Michael Green's lab [126]. Wajapayee and colleagues identified in an unbiased genome wide genetic screen 17 components required for B-Raf^{V600E} induced senescence [126]. Among these genes was identified a secreted protein, IGFBP7. Interestingly other members of this family had previously been associated with cellular senescence [157]. The authors demonstrated that IGFBP7 was both highly synthesized and secreted during cellular senescence in response to B-Raf^{V600E} [126]. The extracellular role of this protein was highlighted using IGFBP7 recombinant protein. Indeed, the recombinant protein was able to induce senescence in primary melanocytes and apoptosis in tumour cell lines [126].

During the same year were published back to back in *Cell*, two research articles by the teams of Jesus Gil and Daniel Peeper identifying critical functional role of pro-inflammatory

cytokines in the establishment of senescence growth arrest state [98, 124]. Acosta and colleagues through the use of an unbiased shRNA library screen targeting 8000 human genes identified the chemokine (C-X-C motif) receptor 2 (CXCR2) as a crucial inducer of cellular senescence [124]. Analyzing the expression of CXCR2 ligands, they went on to identify some of its ligands (interleukin 8 (IL-8) and Gro alpha (Gro-α)) which turned out to be functionally involved in cellular senescence [124]. Daniel Peeper's lab identified, using a genome wide transcriptomic approach, an interleukin dependent inflammatory network associated with oncogene-induced-senescence (OIS) [98] in which interleukin 6 (IL-6) plays a central role. Both studies identify the transcription factor CCAAT/enhancer binding protein beta (C/EBPβ) as a crucial regulator of the cytokine pro-inflammatory network. Additionally, Acosta and colleagues highlighted a role of nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-kβ) as critical in the up-regulation of CXCR2 binding chemokines during OIS [124].

Since these major discoveries, numerous new SASP components have been identified. The year that followed, Campisi's laboratory identified interleukin 1 α (IL-1 α), a membrane bound cytokine, as the up-stream regulator of both IL-6 and IL-8 cytokine inflammatory network associated with the senescent phenotype [158]. During cellular senescence, an interconnection between the DNA damage response and the pro-inflammatory network has also been described [153]. Additionally, in the context of persistent DNA damage associated with cellular senescence, specific damage foci were identified. The foci, termed DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS), maintain cytokine production [159] and associate with promyelocytic leukemia (PML) bodies which are positive regulators of cellular senescence [160-162]. Additional studies should be performed to characterize the functional role of DNA-SCARS in cellular senescence. Nonetheless, it appears that it could be a good senescence marker *in vitro* and *in vivo* [159].

Finally, the SASP is not only observed in primary human cells. Indeed, it has recently been demonstrated that mouse fibroblasts, melanoma and breast cancer cells conserve this feature [163,353]. A recent work from Corinne Bertolloto's team has highlighted a new axis, the PARP/NF-kβ secretory phenotype (PNAS), as a critical axis in the induction of the SASP in cancer cell lines. The authors show that following diverse stimuli including anti melanoma drugs, cancer cell lines develop a SASP [163]. This secretory phenotype could affect the microenvironment in different ways. Indeed, it has been demonstrated that the SASP can activate the immune system leading to the destruction of the senescent cells that might be detrimental for the organism [164] but might also have detrimental effects on neighbour cells [151] (Figure 7).

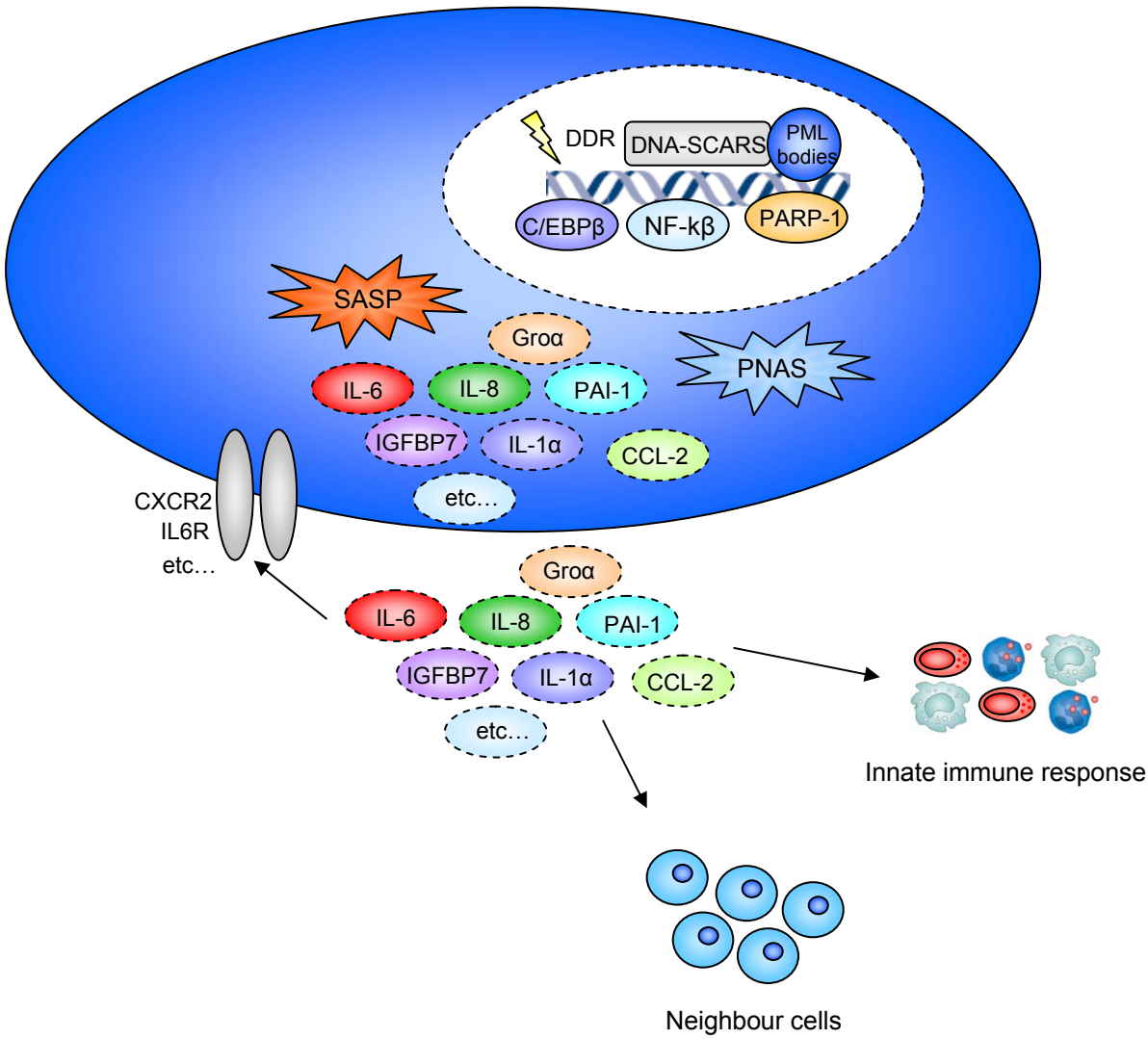


Figure 7: Senescence-associated-secretory-phenotype (SASP).

Secreted proteins associated with the SASP can have both autocrine and paracrine actions on neighbour cells and the innate immune response. The SASP is regulated by the activation of a DDR response, DNA-SCARS associated with PML bodies and the transcriptional factors NF-kβ and C/EBPβ. A new axis, PNAS for PARP/NF-kβ secretory phenotype has also recently been identified and could play an important role in the SASP of cancer cell lines especially melanoma and breast cancer cells. Adapted from [98, 163, 165].

I.2.2 Replicative senescence

Even if "replicative senescence" was first observed in 1961, it took 35 years to gain major compelling evidences of the molecular mechanisms involved in the growth arrest. We now know that the end point of this proliferative limit is largely due to telomere lengths and structures. In the next section, I will introduce the telomeres and telomerase (the enzyme involved in maintaining the telomere length) and their role in "replicative senescence" mostly thought the activation of the DNA damage pathway.

I.2.2.1 Telomeres and Telomerase

The first half of the 20th century saw studies from Herman Muller working on fruit fly and Barbara McClintock working on maize (both Nobel Prize winner 1945 and 1983 respectively), proposing the existence of specialised structures at the chromosomes ends [166]. They hypothesised that these structures, by protecting from chromosome fusion, could ensure correct segregation of the genetic material into daughter cells during each cell division.

In 1978, Blackburn and Gall identified tandemly repeated sequences of six nucleotides at the termini of the extra chromosomal ribosomal RNA genes in Tetrahymena [167]. Various research works from Blackburn and Szostak published in the 1980s proposed that these repeat sequences could have a role in stabilising chromosomes. Blackburn and Szostak also predicted the existence of an enzyme that could regulate telomeres. Carol Greider a graduate student in Blackburn's team purified the telomerase protein and demonstrated its enzymatic activity [168].

Mutations of the yeast telomerase component first demonstrated the role of telomerase in telomere maintenance [60]. The telomere maintenance by the telomerase was also demonstrated in a mouse model [169]. These data suggested a conserved role of telomerase in mammals. In 2009 was awarded to Elizabeth Blackburn, Carol Greider, and Jack Szostak the Nobel Prize in Physiology or Medicine for their discovery of telomeres and the catalytic subunit of the telomeres, the telomerase [168].

Due to the limits of the DNA replication machinery, chromosome DNA ends are not entirely replicated. Each DNA replication cycle reduces the number of telomere repeats. Telomeres are protective structures that cap the end of all eukaryotic chromosomes. They are long double stranded DNA sequences composed of TTAGGG repeats, oriented 5'-to-3' towards the end of the chromosome (Figure 8a). Their sizes vary from 9-15kb long in humans to 100kb in rodents. The end of the telomere is a 3'overhang that contains a sequence of single stranded G rich 50-300 nucleotides termed the G tail or G-overhang. This G tail or G overhang is presumably the results of post-replicative processing of the C-rich strand. Telomere lengths are maintained by telomerase, a ribonucleoprotein complex that includes a RNA template (known as TERC) and the reverse transcriptase catalytic subunit (TERT) (Figure 8a) [170-173]. Telomerase activity is mainly dictated by the TERT expression, as TERC seems to be ubiquitously transcribed [174] (Figure 8a).

Telomeric DNA contains a complex composed of six shelterin proteins. This complex comprises telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), TRF1-interacting nuclear factor 2 (TIN2), transcriptional repressor/activator protein (RAP1), TIN2-interacting protein (TPP1) and protection of telomeres 1 (POT1). The last two components, TPP1 and POT1 regulate the access of the telomeric substrate for the telomerase

(Figure 8a) [170-173]. A protective (t) loop can be formed between single stranded overhang and double stranded region of the telomere. The loop that is formed from the invasion of the single G strand overhang is called the (D) loop (Figure 8b).

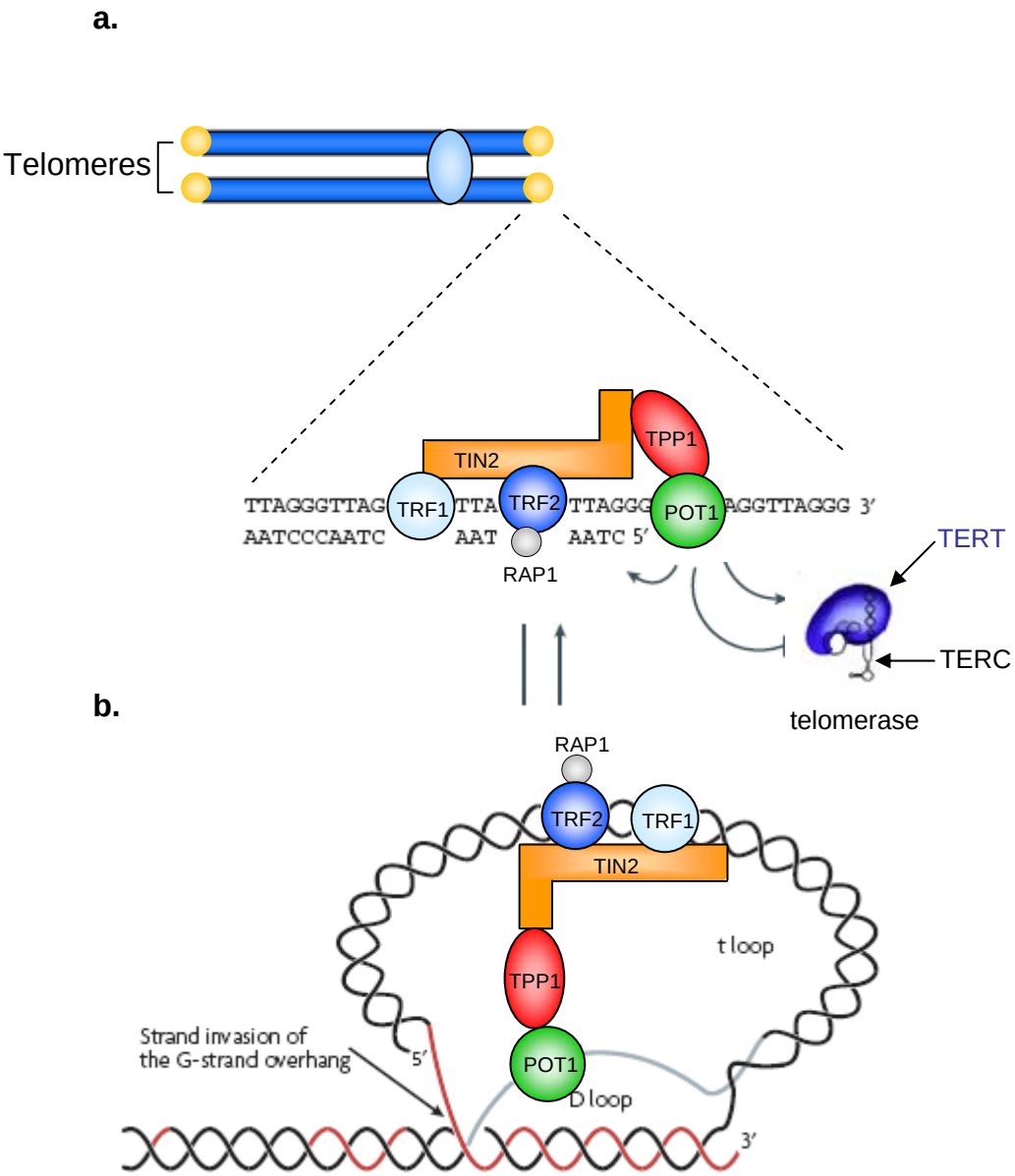


Figure 8: The structure of the mammalian telomeres and telomerase.

Figure 8a illustrates the telomeres, TTAGGG repeats, oriented 5'-to-3' towards the end of the chromosome. The telomeres structures are maintained by six proteins (TRF1, TRF2, TIN2, RAP1, TPP1 and POT1) that make up the shelterin complex. Telomere lengths are maintained by telomerase, a ribonucleoprotein complex that includes a RNA template (known as TERC) and the reverse transcriptase catalytic subunit (TERT). Figure 8b shows the protective't' and the 'D' loop structures formed from the strand invasion of the G-strand overhang. Adapted from [171, 175, 176].

I.2.2.2 Telomeres and Telomerase in "replicative senescence"

In the mid 1970s, Hayflick's team, using enucleation and fusion techniques that allowed to switch nuclei from senescent to proliferating cells, postulated that the "replicometer" of "replicative senescence" might be located in the nucleus [177-179]. In 1989, Lundblad and collegues made the observation that a mutant with defect in telomere elongation could trigger senescence in yeast [60]. The year that followed, it was suggested that telomere length might be responsible for "replicative senescence" in primary human cells [180]. Although not knowing whether it was the cause or a consequence of "replicative senescence", Harley and colleagues noticed that both the amount and length of telomeric DNA decreased during serially passage human primary fibroblasts [180]. However, functional experiments in human cells were still missing.

It took another eight years to get compelling evidences that telomere erosion was the critical trigger of "replicative senescence". In 1998, two articles by the group of Woodring Wright and Samuel Benchimol published in *Science* and *Current Biology* respectively showed for the first time that re-introducing telomerase expression in normal primary cells led to elongated telomeres, lifespan extension and abrogation of "replicative senescence" [181, 182]. Since then, telomerase re-expression alone [183-186] or in combination with other alterations [187-189] has been associated with the immortalisation of various human cells types. Although preventing telomere erosion, the dogma that the telomerase was able to immortalise cells through the maintain of the telomere size was questioned when various scientist observed that, telomerase immortalised cells displayed in some cases shorter telomeres than senescent cells [185, 190]. In order to explain the discrepancy, Elisabeth Blackburn proposed that the telomeres structure might be a critical regulator of "replicative senescence" [191]. In accordance

with this model, in a work published in *Science* in 2002 by the group of Titia de Lange, TRF2, a component of the shelterin complex, allowed cells to proliferate although their telomeres were much shorter than control senescent cells [192]. In agreement, the group of Robert Weinberg showed in a work published in *Nature Genetics* in 2003, that senescent cells showed an erosion of the single stranded 3' overhang, a key component of telomere structure [193]. Interestingly, the ectopic expression of the telomerase impeded the erosion of the 3' overhang structure potentially altering the T loop and the proper telomere structure [193].

From these studies, it seemed that the telomere structures were the major determinants. However, a study of Carole Creider's group published in *Cell* suggested that the major determinants of cellular senescence were in fact the shortest telomeres and not the average telomere length of a cellular population [194]. Additionally, it had also been noted that the ectopic expression of the telomerase preferentially elongates short telomeres and that it was still accompanied with a decrease in the average telomere size [194-196].

Although different hypotheses were made with strong evidences to back them up, it seems that both telomere length and structure are keys regulators of "replicative senescence". When telomeres are unable to exert their end protective functions they can be defined as dysfunctional. Dysfunctional telomeres can result in the activation of the DNA damage pathway. Indeed, many DNA damage repair proteins localise to dysfunctional telomeres and activate a DNA damage response (DDR). In the next section I will describe how dysfunctional telomeres can activate "replicative senescence".

I.2.2.3 Cellular senescence in response to dysfunctional telomeres

Two pioneers papers from the lab of Steve Jackson and Titia de Lange published in *Nature* and *Current biology* in 2003 presented evidences that telomere shortening was associated with a DDR [197, 198]. More specifically, their research demonstrated that dysfunctional telomeres were associated with DDR proteins, resulting in a DDR activation functionally required for "replicative senescence" induction [197, 198]. DNA repair and DNA damage proteins such as 53BP1, ATM, MDC1 and NBS1 were found associated with uncapped telomeres and senescent cells contained phosphorylated active forms of DNA damage checkpoint proteins CHK1 or CHK2 [197, 198] (Figure 9).

The DDR response induced by dysfunctional telomeres is believed to be ATM, p53, $p21^{CIP1}$ dependent but $p16^{INK4A}$ independent [109]. However, the role of $p16^{INK4A}$ in telomere induced senescence could be greater than first believed. In the absence of p53, the inhibition of $p16^{INK4A}$ rendered cells irresponsive to telomere induced DNA damage suggesting that $p16^{INK4A}$ could have a role in p53 independent response to DNA damage [199] (Figure 9). The role and the pathways regulating $p16^{INK4a}$ in telomere induced senescence remain to be clarified. Interestingly, a recent publication of Ronald DePinho's team published in *Nature* in 2011, demonstrated that telomere dysfunction had an impact on mitochondrial biogenesis and increased ROS levels [200]. Interestingly, Sahin and colleagues suggested that alternative pathways might exist between telomere dysfunction and mitochondria altered function [200]. Dysfunctional telomeres could therefore alter mitochondrial function leading to increase generation of ROS, $p16^{INK4A}$ activation and cellular senescence in a p53 independent manner (Figure 9). To conclude, although the DNA damage response plays a critical role in response to dysfunctional telomeres, the role of the p16 ^{INK4A}/Rb pathway remains to be clarified.

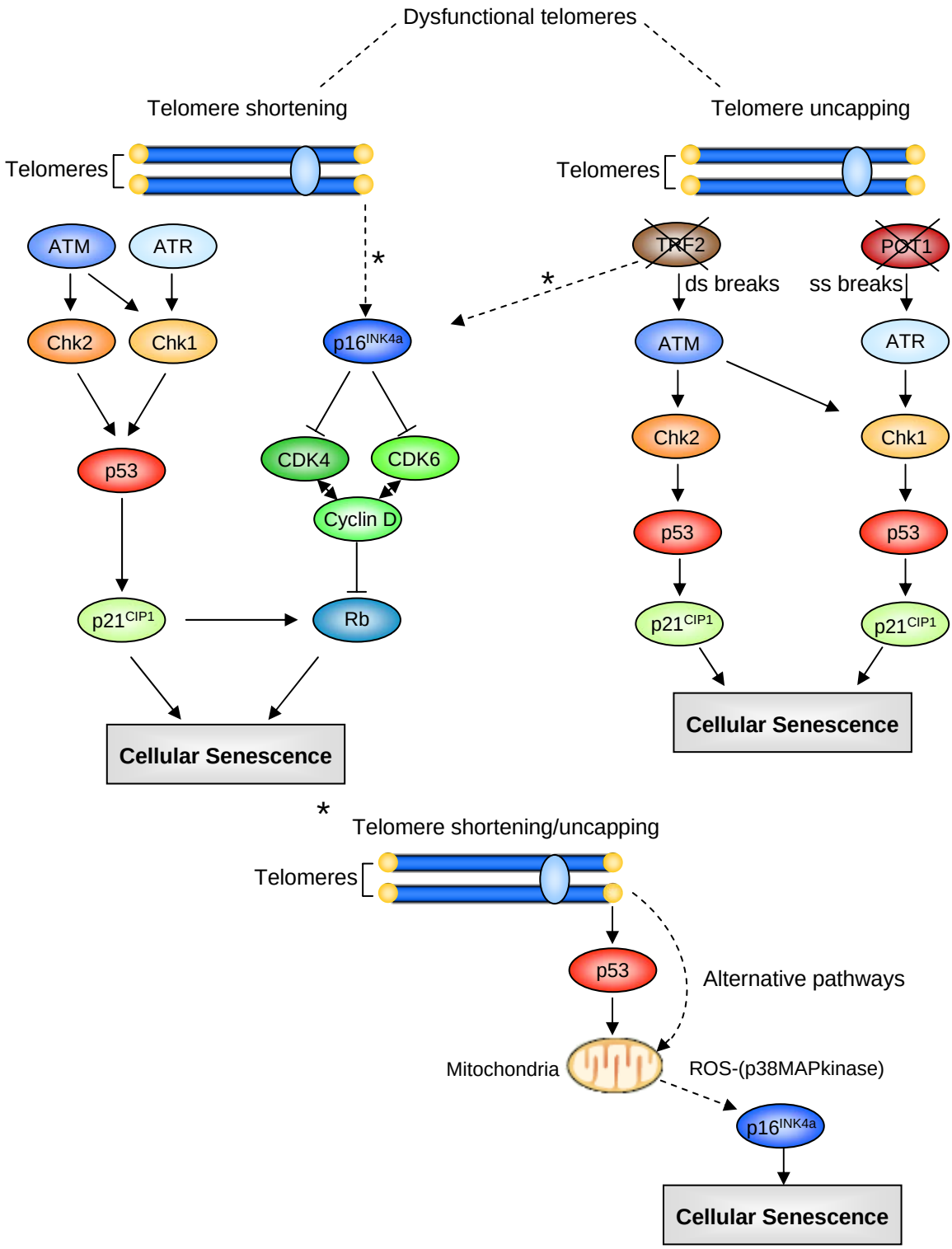


Figure 9: Telomere dysfunction and the activation of a DNA damage response (DDR).

Both telomeres shortening and uncapping has been associated with DDR activation leading to p53 activation, an upregulation of $p21^{CIP1}$ and cellular senescence. The role of the $p16^{INK4a}$/Rb pathway in telomere induced senescence remains to be clarified but the pathway could be activated by alternative routes (ROS-p38MAPkinase). For clarity, DNA damage associated proteins such as NBS1, 53BP1 and MDC1 were left out of the picture.

I.2.3 Premature senescence

1.2.3.1 Oncogene-induced-senescence (OIS)

Serrano and colleagues made in a work published in *Cell* in 1997 what turned out to be a pioneer discovery. They observed that in response to oncogenic Ras (H-Ras^{G12V}) primary cells entered a "premature senescence" state [31]. The following year, two research articles published in *Genes and Development* provided evidence that the establishment of premature senescence was not limited to Ras but could be extended to most of the actors of the MAPK pathway (Raf, Mek) [201, 202]. OIS could be clearly separated from "replicative senescence". Indeed, cells expressing the catalytic subunit of the telomerase (TERT) still underwent OIS, suggesting a telomere independent mechanism [203].

OIS has been observed in response to the over-expression of various mitogenic signalling proteins (Ras, Raf, Rac, Mek, β catenin…). It has been proposed, at least in some contexts, that OIS results from DNA hyper-replication leading to DDR activation [25, 29].

Furthermore, OIS can also occur following loss of tumour suppressor genes that restrain the proliferative effect of oncogenes. Indeed, the loss of function of phosphatase and tensin homolog (PTEN), Rb, von Hippel-Lindau tumor suppressor (VHL), and neurofibromin 1 (NF1) has been associated with "premature senescence" and is also referred as OIS [26, 27, 32, 105] (Figure 10).

The view suggesting that senescence might be induced in response to mitogenic stress to limit excessive proliferation was an attractive idea. However, whereas senescence in ageing tissues had been observed since 1995 [129], the demonstration and physiological role of OIS *in vivo* was lacking. Four research articles published in *Nature* in 2005, demonstrated for the first time that OIS occurs *in vivo* in response to numerous physiological oncogenic activation.

Three studies used mouse models to confirm the presence of OIS *in vivo*. The team of Manuel Serrano used a mouse model carrying a knock-in conditional oncogenic K-Ras^{G12V} activated in the presence of the enzyme cre recombinase [97]. Following K-Ras^{G12V} activation, they observed senescence staining in premalignant tumours as judged by SA-βgal staining and other senescence markers [97]. The team of Clemens Schmitt used N-Ras^{G12D} (a constitutively active version of N-Ras) expressed in lymphoid tissue (Eμ-N-Ras^{G12D}) [204]. In this study, Braig and colleagues reported a critical regulatory role of the chromatin remodeling enzyme suppressor of variegation 3-9 homolog 1 (Suv39h1) in cellular senescence [204]. Pier paolo Pandolfi's group used a PTEN knockout mouse model and provided the first demonstration of tumour suppressor loss induced senescence *in vivo* [26]. They observed senescence in both mouse and human prostate intraepithelial neoplasia (PIN) samples [26]. These findings suggested for the first time that OIS occurred *in vivo*. A work in the same issue from Daniel Peeper's laboratory demonstrated that OIS observed in human was not limited to prostate intraepithelial neoplasia (PIN) but could also be observed in human naevi (moles), which harbor, B-Raf^{V600E}, a B-Raf constitutively active oncogene [205]. The year that followed the group of Jiri Bartek published a work in *Nature* demonstrating that OIS could elicit a DDR leading to the appearance of "premature senescence" *in vivo* [25]. Since then, OIS has been observed in various *in vivo* contexts [55]. To avoid exhaustive listing, Figure 10 illustrates the pathways underlining OIS that have been identified *in vivo*.

It is noteworthy to mention that OIS has not always been observed in mouse models [206-208]. It seems that levels of oncogene expression are key determinants. It has been proposed that oncogene expression levels could be regulated by several feedback loops underlining cellular senescence [27, 209]. Another determinant could be the levels of ROS [210]. It has recently been demonstrated that Nrf2, a transcriptional activator of an antioxidant response involved in ROS detoxification, is activated by oncogenes to promote senescence escape and favour tumorigenesis [210].

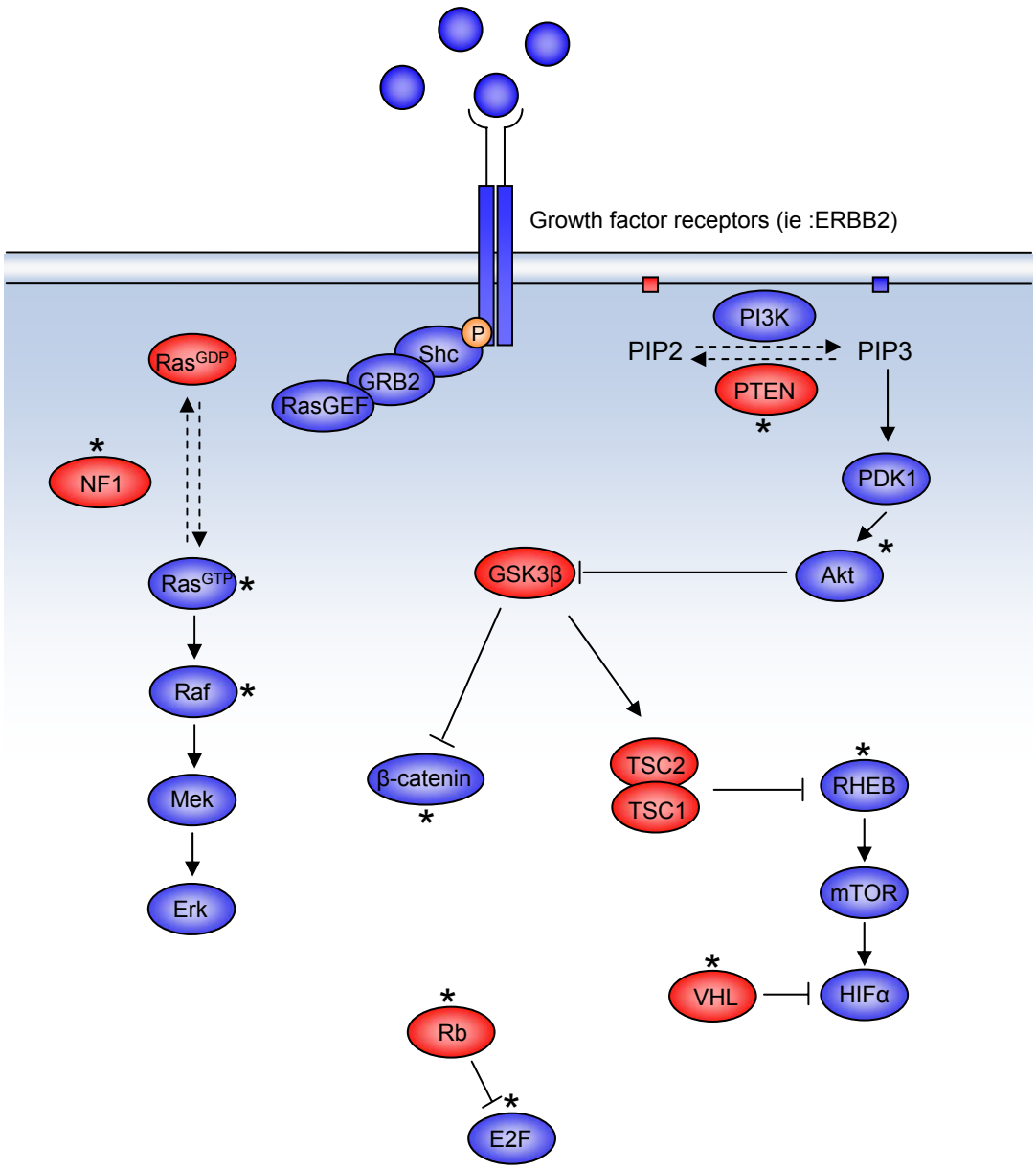


Figure 10: Pathways regulating Oncogene-induced-senescence (OIS) (*in vivo*).

Oncogene-induced-senescence (OIS) can be induced following a hyperactivation of an oncogenic pathway. It results from at least two mechanisms. The loss of bona fide tumour suppressors that control the activity of the pathway or a mutation that renders the oncogene hyperactive. The tumour suppressor genes are display in red. The oncogenes are presented in blue. The asterisk corresponds to the OIS cases reported *in vivo*. Feedback loops and alternative pathways (for example the antioxidants pathway) have not been included. Adapted from [55].

I.2.3.2 Oncogene-invalidation-induced-senescence (OIIS)

Senescence is not limited to primary cells. It can also be triggered in cancer cells [45-48, 163, 211-213]. Cancer cells develop oncogene addiction [214] and various studies using synthetic lethal screening have attempted to identify critical pathways involved in cancer cell growth and survival [215, 216].

Interestingly, the knockdown of various oncogenes in cancer cells can result in cellular senescence. It is for example the case for v-myc myelocytomatosis viral oncogene homolog (c-Myc), a bHLH-LZ transcription factor involved in various biological processes such as proliferation, angiogenesis and cell metabolism [217]. Interestingly, a subset of cancer cells depend on Myc activity to proliferate and its inhibition greatly blocks cancer cell proliferation [218-221]. Even if the p53 and Rb tumour suppressive pathways that regulate senescence are often altered in cancer cells, it seems that they can remain in a latent state in which they can still be activated. Additionally, alternative pathways regulating cellular senescence can also be activated [46].

Interestingly, c-Myc inhibition in cancer cell lines and also in c-Myc transgenic mice that had developed lymphomas or osteosarcomas led to senescence induction [222-225]. Myc inhibition can also alter cancer cell proliferation when the tumour initiator is another oncogene. Senescence is induced in K-Ras^{G12D} initiated lung tumours following inactivation of the three myc paralogs (Myc, Mycn, Mycl1) whereas the same inactivation in normal tissue had little or no effect after a month of treatment [219].

OIIS is not limited to Myc inhibition. Indeed, the inhibition of CDK4 in K-Ras^{G12V} driven non small cell lung carcinoma is associated with tumour regression and cellular senescence induction [226]. Moreover, the inhibition of several embryonic factors (twist homolog 1/2, T-box 2 (TBX2)) in cancer cells can also result in cellular senescence [227, 228].

Finally, small molecules inhibitors have recently been successfully used to trigger cellular senescence in cancer cell lines. It has recently been demonstrated by the team of Pier Paolo Pandolfi in a work published in *Nature* in 2010 that SKP2 pharmacological inhibition results in cellular senescence in PTEN deficient cancer cells lines [103]. In line with this, Bruno Amati's group in a work published in *Nature Cell Biology* in 2010, induced cellular senescence in Myc addicted human leukaemia cells by targeting CDK2 with two small molecule inhibitors [229]. Altogether, these studies demonstrate that cellular senescence can be re-induced in tumours upon oncogene inactivation (Figure 11).

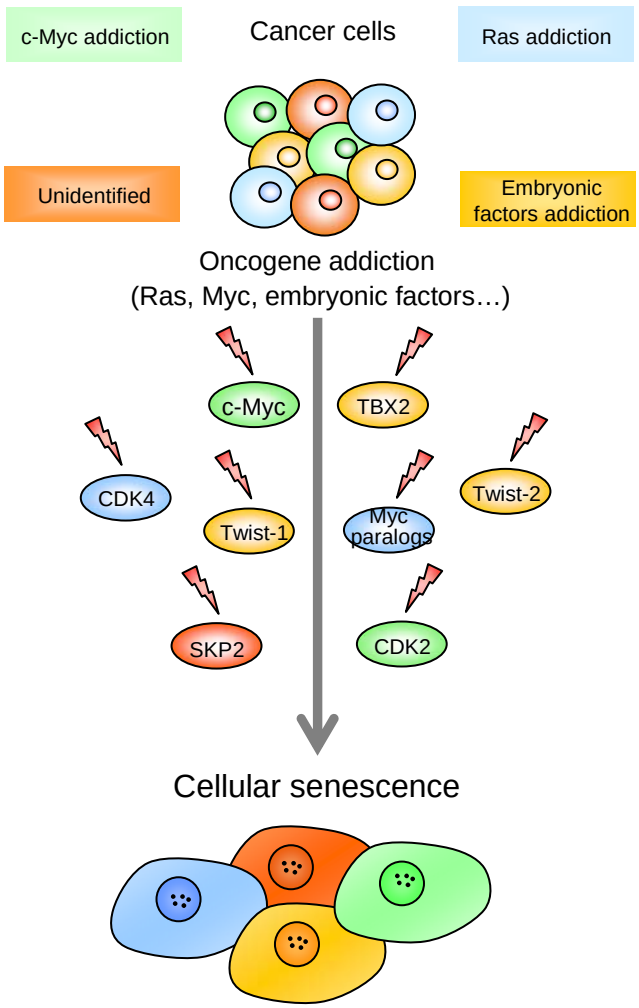


Figure 11: Oncogene invalidation induced senescence (OIIS).

Oncogene-invalidation-induced-senescence (OIIS) has been described in several cases of oncogene addiction. Myc inactivation in Myc transgenic mice driven tumours leads to cellular senescence and similar results were obtained from cancer cell lines. The inhibition of various embryonic transcription factors (twist1/2, TBX2) can also restore cellular senescence in cancer cells. Interestingly, in K-Ras driven tumours, the inhibition of several “effector genes” can results in cellular senescence. For example, CDK4 and Myc paralogs can restore cellular senescence. Finally, small inhibitory molecules have been successfully used to trigger cellular senescence. It is the case for CDK2 inhibition in Myc overexpressing leukaemia cells and SKP2 inhibition in PTEN deficient prostatic cells. Personal illustration.

I.2.3.3 Stress induced premature senescence (SIPS)

Stresses are major determinants of cellular senescence [67]. In 1999 at an EMBO workshop of molecular and cellular gerontology in Switzerland, the term SIPS for stress-induced-premature-senescence was introduced by Christine Brack [230]. Although OIS and OIIS also generate stresses and can be classified in the stress induced cellular senescence category, SIPS, to my knowledge, includes different types of stresses.

One of the major determinants of stress-induced-premature-senescence (SIPS) is oxygen. Oxygen is the essence of cellular respiration and a crucial requirement for aerobic life. Oxygen singlet (O_2), itself, is not toxic for cells; however O_2 consumption is transformed into ROS [67]. ROS can be classified into two groups. The free radical group contains one or more unpaired electrons in their outer molecular orbitals. It includes reactive species such as superoxide and hydroxyl radicals. The second group comprises non-radical ROS, which do not have unpaired electrons but are chemically reactive and can be converted to radical ROS. This group includes hydrogen peroxide, ozone, peroxinitrate and hydroxide [231].

Since the free radical theory of Harman [232], in which was postulated that free radicals produced by metabolic pathways can generate cellular damage and contribute to ageing, evidences demonstrating that ROS can trigger cellular senescence have accumulated. When Hayflick and Moorhead first observed “replicative senescence” in 1961, they were cultivating cells at 20% O_2 levels, the atmospheric oxygen level conditions [23, 24]. Interestingly, when the same primary human cells were cultured in 3% O_2 levels, which is closer to the physiological conditions (normal physiological conditions vary from 14% in the lungs to 1-2% in some parts of the brain, skin, heart and kidney) [233], the cells achieved at least 20 supplemental

populations doublings before reaching cellular senescence [234]. Conversely, raising the oxygen levels over 20% induced a premature growth arrest [34, 35, 235]. Furthermore, exposure to sub-lethal doses of ROS such as H_20_2 also led to a premature senescence like state [236]. SIPS in response to oxidative stress is not limited to H_20_2 and can also be induced by tert-butylhydroperoxide [237].

SIPS is not restrained to oxidative stress triggers. It can be induced in response to additional inadequate culturing conditions such as abnormal concentrations of nutrients, growth factors, the absence of neighbour cells and extracellular matrix components [183, 238, 239]. Finally it can be induced by other physical, chemical and cellular stressors such as ethanol, ionizing radiation and mitomycin C in a subset of proliferating cells [240-242] (Figure 12).

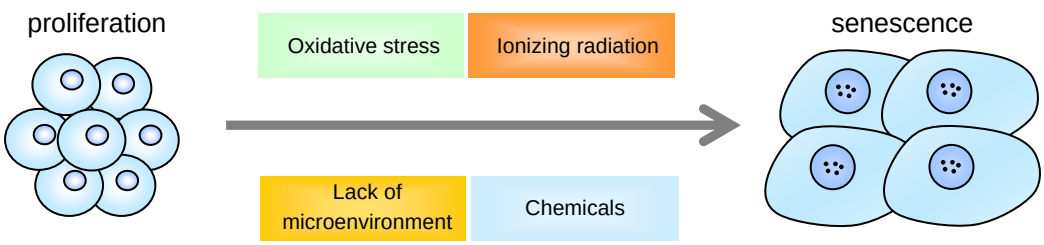


Figure 12: Stress induced premature senescence (SIPS).

“Premature senescence” can be induced in response to various types of stresses. SIPS represents a form of “premature senescence” that is induced in response to oxidative stress, ionizing radiation, lack of microenvironment and various chemicals. Personal illustration.

I.2.4 Pathways regulating cellular senescence

I.2.4.1 The DNA damage/p53 pathway in cellular senescence

The p53 pathway is a tumour suppressive pathway critical in the regulation of senescence. In many cases, it is activated following stimuli that induce a DDR such as dysfunctional telomeres, OIS, ionising radiation and ROS among others [175] (Figure 13).

Activation of a DNA damage response consists in the recruitment of DNA damage sensors at the sites of damage. Various DNA damage sites associated with cellular senescence have been described. These include telomere-dysfunction-induced-foci (TIFs) [198], senescence-associated DNA damage foci (SDF) [197] and DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) [159].

Two large protein kinases ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), are recruited at the DNA damage sites [243], phosphorylate and activate the histone variant H2AX [244]. When damages are double stranded breaks (DSBs), they are sensed by the MRE-11/NSB1/RAD-50 complex and NSB1 recruits ATM. ATM is activated upon autophosphorylation and phosphorylates H2AX (γH2AX). γH2AX recruits additional ATM, amplifying ATM local activity. This positive feedback loop leads to an increase of active H2AX forms [175]. This step is mediated by the DDR mediators, mediator of DNA-damage checkpoint 1 (MDC1) [245-247] and p53-binding protein 1 (53BP1) [248] which facilitates the recruitment of ATM to γH2AX [249, 250] (Figure 13).

The molecular events involved in single stranded breaks differ from DSBs. Replication protein A (RPA) binds to damaged sites and favors ATR recruitment [251]. ATR activity is increased by a trimeric complex composed of RAD1-RAD9-HUS-1 [252] and by topoisomerase II-binding protein 1 (TOPBP1) [253]. ATM is therefore engaged in DSBs while ATR in single stranded breaks, however it has been observed that both kinases can be recruited to the same lesions [254]. Once activated, if the level of activation is sufficient, ATM and ATR activate DDR factors that can exert their function far from the original site of damage. CHK2 is activated by ATM phosphorylation [255], while ATR and ATM activate CHK1 [256]. Once phosphorylated and activated, both CHK1 and 2 engage a DDR signaling in distant nuclear regions from the DNA damage site [257] (Figure 13).

The activation of the DDR kinases results in the phosphorylation and activation of p53 which in turn activates $p21^{CIP1,}$ one of its transcriptional targets crucial in the growth arrest (Figure 13). The important role of the DNA damage pathway in senescence induction has been highlighted by invalidating various component of the DDR. Indeed, inactivation of DDR proteins as well as p53 and its transcriptional target $p21^{CIP1}$ is sufficient to abrogate cellular senescence in various settings [109, 197, 258]. The DNA damage is not the only pathway activating the p53 pathway to trigger senescence. For example, ARF plays a crucial role in the activation of p53. Activated in response to oncogenic stimulations, it activates p53 by sequestering the E3 ubiquitin protein ligase mouse double minute 2 (MDM2 or HDM2 in humans), an inhibitor of p53 [259] (Figure 13).

The activation of a DDR is not a universal feature of cellular senescence. In mouse cells, it has been observed that the induction of senescence following an oncogenic stress did not require DRR proteins nor showed any sign of DDR activation [260]. Furthermore, OIS does

not always require p53 activation [98, 103, 112, 261]. These results suggest that the DDR response is not universally associated with cellular senescence and that the DNA damage/p53 pathway activation might dependent on the cellular context and the cell types.

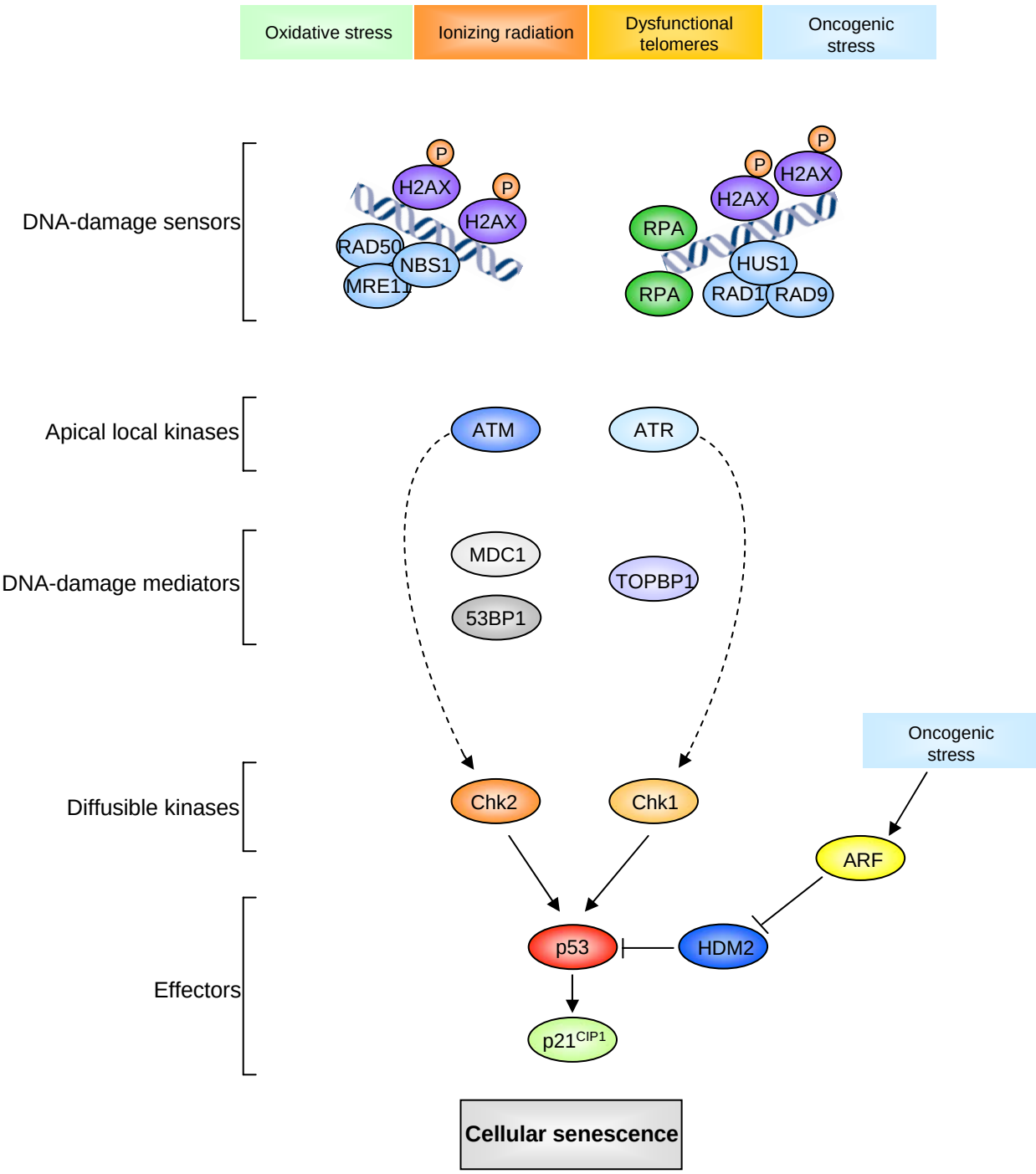


Figure 13: The DNA damage/p53pathway in cellular senescence.

The DNA damage/p53 pathway can be activated in response to dysfunctional telomeres, OIS, ionising radiation and ROS among others. A DNA damage response (DDR) is induced (see the text for more details), leading to the activation of p53, the transcription of $p21^{CIP1}$ and cellular senescence. An alternative pathway involving ARF can be activated in response to various oncogenes leading to the inhibition of HDM-2 and hence the activation of p53 and cellular senescence. Adapted from [76, 175].

I.2.4.2 The INK4B/ARF/INK4A locus in cellular senescence

The INK4B/ARF/INK4A locus (also known as cyclin-dependent kinase inhibitor 2a and 2b (*CDKN2a* and *CDKN2b)*) encodes three tumour suppressor genes that play critical regulatory roles in cellular senescence and tumour suppression [92, 262-264]. Two of these genes, $p15^{INK4B}$ and $p16^{INK4A}$, are cyclin dependent kinase inhibitors (CKIs) and therefore play an important role in inducing the cell cycle arrest associated with cellular senescence [265]. As previously mentioned, INK4 family members inhibit CDK4 and CDK6 complexes leading to a G1 cell cycle arrest [72]. The third gene, ARF is, as previously mentioned, a critical regulator of the p53 tumour suppressor pathway [266]. ARF and $p16^{INK4A}$ share common exons but are encoded in alternative reading frames leading to the production of completely different proteins in both amino acid sequence and function [92, 262-264].

Major regulators of the INK4B/ARF/INK4A locus are the polycomb repressive group complexes (PRCs) [264]. The polycomb family is composed of two repressive complexes PRC1 and PRC2. The PRC2 establishes the repressive mark (tri-methylation on the lysine 27 of histone 3; H3K27met3) through EZH2 leading to the recruitment of the maintenance polycomb complex (PRC1). A functional role of polycomb proteins in the regulation of the INK4B/ARF/INK4A locus and cellular senescence was first described by the team of Martin van Lohuizen [267]. Jacobs and colleagues showed in a work published in *Nature* in 1999, that the over-expression of the Bmi1 polycomb protein was associated with the repression of the locus and senescence bypass [267]. This work was followed by a work of David Beach's laboratory in which Jesus Gil and colleagues demonstrated that CBX7, another polycomb protein, could repress the locus and regulate cellular senescence in a Bmi1 independent fashion [50]. The regulation of the locus and cellular senescence is not limited to Bmi1 and

CBX7 as other polycomb members have been associated with the repression of the locus and cellular senescence [101, 268, 269] (Figure 14).

During cellular senescence, the INK4B/ARF/INK4A locus can be activated in response to various stimuli. First, it has been demonstrated that polycomb proteins cannot exert their repressive function during cellular senescence partly due to a decrease in gene expression and the recruitment of key enzymes involved in the modulation of the repressive marks [264]. For example, it has been observed that EZH2 expression decreases during cellular senescence [127, 270, 271]. Furthermore, it seems that activators of the locus are recruited during cellular senescence. The H3K27 demethylase JMJD3 has recently been found to be up-regulated, recruited and involved in the activation of the locus in response to OIS [270, 271]. Moreover, the removal of polycomb repressive complexes can be mediated by MAPKAP, a protein kinase activated in response to OIS [272]. The activation of the locus can also be mediated by chromatin remodeling SWI/SNF complex which are involved in the repression of the polycomb proteins [273] (Figure 14).

The regulation of cellular senescence by the INK4B/ARF/INK4A locus might not be limited to the polycomb proteins. The $p16^{INK4A}$ gene product is regulated by the stress activated kinase p38MAPKinase. Activated in response to various stresses such as high levels of ROS, dysfunctional telomeres and OIS, it has been shown to activate $p16^{INK4A}$ and cellular senescence in various contexts [274-277]. The regulation by p38MAPKinase of the locus encoding proteins is not limited to $p16^{INK4A}$ as it can also affect $p15^{INK4B}$ and ARF [278]. To date, it is unclear how p38MAPKinase activates $p16^{INK4A}$. However it is likely that it acts at least in part by modulating the epigenetic regulators of the locus. Interestingly, p38MAPKinase regulates MAPKAP, a protein kinase that has recently been implicated in the

inhibition of the polycomb proteins [272]. It phosphorylates polycomb proteins leading to their dissociation and a de-repression of the locus [272] (Figure 14). However, whether MAPKAP regulates cellular senescence is currently unknown.

The locus products can also be regulated by post-translational modifications. For example, TGF-β stabilises $p15^{INK4B}$ [279], $p16^{INKA4}$ is stabilised in response to UV treatment through the inhibition of a SKP2 related degradation [280] and ARF stability has recently been shown to be regulated by the E3 ubiquitin ligase TRIP12/ULF [266, 281] (Figure 14).

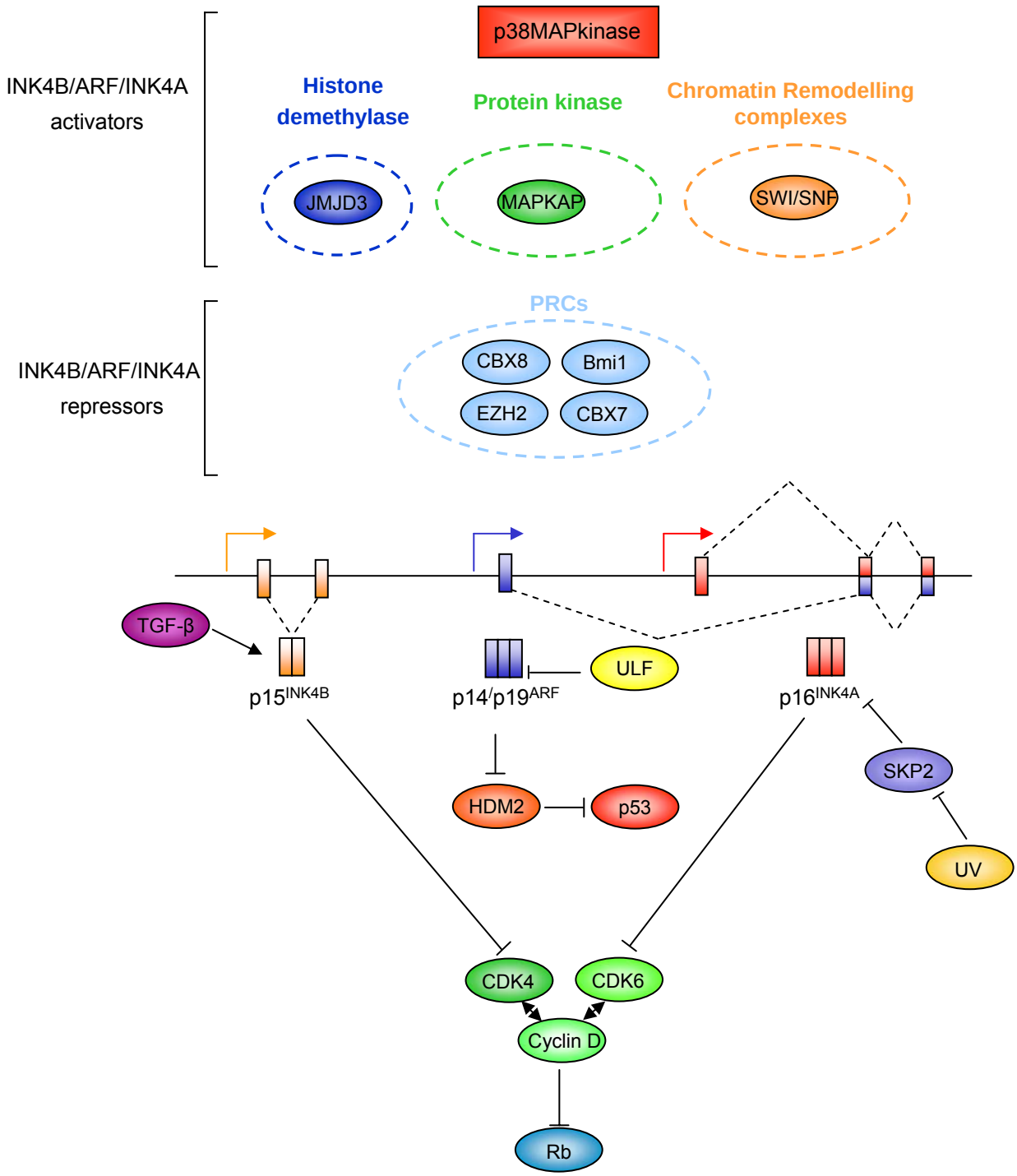


Figure 14: The INK4B/ARF/INK4A locus in cellular senescence.

The locus is regulated by epigenetics mechanism. Repressive regulation implicates members of the polycomb repressive complex (PRCs). The activation of the locus requires histone demethylase (demethylating repressive marks), chromatin remodelling complexes and might involve protein kinases activated by p38MAPKinase. The regulation of the locus products is regulated by the TGF-β, ULF and SKP2 among others. Adapted from [92, 263, 264].

I.2.4.3 Reactive oxygen species (ROS) in cellular senescence

Cellular senescence, both replicative and OIS have been associated with increased ROS compared to their counterparts [282, 283]. ROS are not only bystanders but also critical regulators of cellular senescence. Oxygen levels modulate cellular senescence in both human and mouse cells [34, 35, 234, 235, 284]. In 1999, the group of Toren Finkel demonstrated in a work published in the *Journal of Biological Chemistry* that ROS were required for OIS in primary fibroblast [283]. In accordance with these observations, ROS regulated proteins such as seladin-1 (modulators of peroxiredoxines, a class of antioxidants) have also been involved in OIS [285]. Oncogenic Ras is believed to regulate ROS production, at least in part by activating 5-lipooxygenase (5LO), an enzyme that generates ROS by converting arachidonic acid (AA) to leukotrienes [286]. The regulatory role of ROS in OIS is not limited to oncogenic Ras. Akt was recently identified as a major determinant of various types of cellular senescence by modulating oxygen consumption and down-regulating ROS scavengers [287].

ROS have also been functionally linked to “replicative senescence” induction. In 1995, it had been proposed that ROS could modulate telomere length [36]. The group of Thomas von Zglinicki was looking for an explanation to why such heterogeneity was observed in the population of cells undergoing “replicative senescence”. They suggested that ROS produced from dysfunctional mitochondria induce DNA damage at telomeric ends leading to a telomere dependent cellular senescence [288]. In accordance with ROS regulating telomere length, antioxidant proteins modulation such as the extracellular superoxide dismutase (SOD) increases the lifespan of primary cells in a telomere length dependant manner [289]. This observation is not limited to cell lines. Indeed, targeted over-expression of the antioxidant enzyme catalase to the mitochondria increase mice lifespan [290]. The ROS regulatory role of

cellular senescence has also been highlighted by modulating ROS induced damages. For example, the continuous elimination of oxidised nucleotides (8-oxoguanine, a major oxidative DNA lesion) prevents cellular senescence [291, 292].

ROS can either directly or indirectly activate the p53 and/or Rb pathways. For example, ROS can activate the p38 MAPKinase pathway which has been associated with the activation of the p53 and/or Rb pathway [261, 293-296]. Additionally, feedback loops may exist between these pathways and ROS production. $p21^{CIP1}$ can induce mitochondrial dysfunction and the production of ROS and cellular senescence [297]. In agreement with this observation, it was demonstrated that inhibition of $p21^{CIP1}$ mediated ROS accumulation could inhibit p21 mediated cellular senescence [298]. Finally, alternative ROS mediated pathways exist to control cellular senescence. The ROS-PKCδ also plays a crucial in the maintenance of cell cycle arrest during cellular senescence [114]. Altogether, theses studies demonstrate a pivotal role ROS during cellular senescence.

I.2.4.4 Small non coding RNAs in cellular senescence

Since the discovery of *lin-4* and *let-7*, two small non coding RNAs [299, 300], the miRNAs (micro RNAs) have been extensively studied [301]. miRNAs are small (approximately 23 nucleotides) RNAs that play a pivotal role in gene regulation by modulating the translation and/or stability of mRNA [301]. miRNAs bind 3'UTR and sometimes 5'UTR of the mRNA. miRNAs are conserved among animals, viruses and plants [301] and more than 700 have already been identified in humans [302]. It is believed that approximately 20-30% of genes can be regulated by miRNAs [303].

The regulatory role of miRNAs during cellular senescence has recently been addressed [304]. Expression profiling studies indicated an altered expression among various miRNAs during cellular senescence [116, 120, 305, 306]. However, such expression analysis studies, although very interesting, do not provide information on the functional role of miRNAs.

In 2006 was published in *Cell* a work of Reuven Agami's team identifying and characterising miR-372 and miR-373 as critical repressors of OIS and putative oncogenes [307]. The year that followed, several articles identified a family of miRNAs (miR-34a-b/c), regulated by the p53 pathway to trigger apoptosis, growth arrest and senescence [308]. This role was later confirmed during "replicative senescence" [309]. More recently, miRNAs have also been implicated in the regulation of the SASP. Indeed, the team of Judith Campisi published a work in *Aging*, in which they identified miR-146a/b as a novel regulator of the SASP. miR-146a/b was found up-regulated during cellular senescence and repressed both IL-6 and IL-8 [310]. In line with this study, Li and colleagues described similar results [311].

miRNAs might not only be involved in the SASP by regulating the expression of its components. Indeed, an elegant research article published by Chen-Yu Zhang laboratory in *Molecular Cell* in 2010 provided evidence that miRNAs can be secreted, transported into microvesicles (MVs) and serve as signalling molecules to mediate intracellular communication [312]. The secretion of miRNAs was very recently observed in exosomes transferred from T cells to antigen presenting cells [313]. It is therefore plausible that such secreted miRNAs could also play a role in regulating the cellular senescence phenotype.

Finally, the functional role of miRNAs is not limited to the regulation of cellular senescence in primary cells. Indeed miR-22, miR-29 and miR-30 trigger cellular senescence

in cancer cell lines [314, 315]. Table 1 presents a non exhaustive list of miRNAs that have been functionally involved in cellular senescence.

miRNAs involved in cellular senescence (non exhaustive list)

miRNAs	+/- senescence regulation	targets	Refs
miR-34 a	positive	E2F, Myc	{Christoffersen, #501; Tazawa, 2007 #499}
miR-34 a-b/c	positive	cyclin E, CDK4	{He, 2007 #496}
miR-372/373	negative	LATS2	{Voorhoeve, 2006 #500}
miR-20a	positive	LRF	{Poliseno, 2008 #502}
miR-(15b-24-25-141)	negative	MKK4	{Marasa, 2009 #503}
miR-128a	positive	Bmi-1	{Venkataraman, #504}
miR-146a/b	negative	IRAK1	{Bhaumik, 2009 #497}
miR-(34a-217)	positive	SIRT-1	{Ito, #505; Menghini, 2009 #506}
miR-106a	negtive	p21	{Li, 2009 #192}
miR-(29-30)	positive	B-Myb	{Martinez, #507}
miR-(22)	positive	CDK6, SIRT-1, Sp1	{Xu, #508}

Table 1: miRNAs in cellular senescence

A non exhaustive list of miRNAs regulating cellular senescence is presented. Interestingly, miR-34 family members are positive regulators of cellular senescence by regulating the expression of various targets. Adapted from [304].

I.2.4.5 The autophagic pathway in cellular senescence

Autophagy was first characterised as a recycling process, involved in catabolism, sustained metabolism and viability in conditions of nutrients deprivation. The process is mediated by autophagosomes, which are double membrane vesicules that engulf cytoplasmic contents and then fuse and deliver their content to lysosomes. Lysosomes contain hydrolases that digest the material which ultimately leads to a breakdown of the vesicules and their constituents [316, 317].

The first research article suggesting a functional role of autophagy in cellular senescence came from a study in which incubation of human umbilical vein endothelial cells (HUVEC) with glycated collagen I (GC) resulted in senescence induction [318]. A functional role of autophagy was suggested because cellular senescence induction upon GC treatment was reverted in the presence of an autophagosome inhibitor the 3 methyl-adenine (3MA) [318].

More recently, it was suggested that autophagy could regulate OIS. The group of Masashi Narita published in *Genes and Development* in 2009 demonstrated that senescent cells displayed an increase in autophagic vacuoles and an upregulation of autophagy regulators such as the ATG related genes (ULK1 and ULK3). Functional experiments confirming the role of autophagy in cellular senescence came from the knockdown of two ATG proteins (ATG-5 and ATG-7) which was sufficient to induce an escape from cellular senescence [33]. Conversely, the over-expression of ULK3 reduced cell growth [33].

Autophagy does not seem to be only associated with OIS acute generated stress. It has also been associated with “replicative senescence” a process that involves modest but long

term stress. The group of Christian Behl published in 2009 a work in *the EMBO journal* suggesting that during "replicative senescence" there is a gradual shift from the proteasome pathway to autophagy for polyubiquitinated protein degradation [319]. They suggested that this shift is critical to maintain protein homeostasis in the presence of stressful conditions associated with "replicative senescence" such as high levels of ROS [319].

Finally, autophagy is not only involved in cellular senescence establishment. Indeed, Corinne Abbadie's laboratory, in a work published in *American Journal of Pathology* in 2009, demonstrated that senescent keratinocytes do not die of apoptosis but instead die of autophagy [320]. Senescent keratinocytes display an altered expression in macroautophagy regulators such as beclin-1 and a high quantity of autophagic vacuoles. The use of 3 methyladenine (3MA) functionally confirmed the role of autophagic cell death in senescent cells [320].

The role of autophagy in senescence induction is not fully understood but seems to have a pleiotropic role. The first role could be to maintain energy production in conditions where great amount of energy are required (for example during OIS) and therefore provide raw material for protein synthesis of critical regulators of senescence such as the SASP. Alternatively, it could also have a critical role in recycling damage proteins in order to maintain protein homeostasis. Furthermore, Masashi Narita has made the hypothesis that autophagy could control protein expression at post transcriptional levels [321] (Figure 16). Finally, in some cell types at least, autophagy might also be able to mediate senescence cell death. Figure 16 highlights some of the pleiotropic effects autophagy exert to regulate cellular senescence.

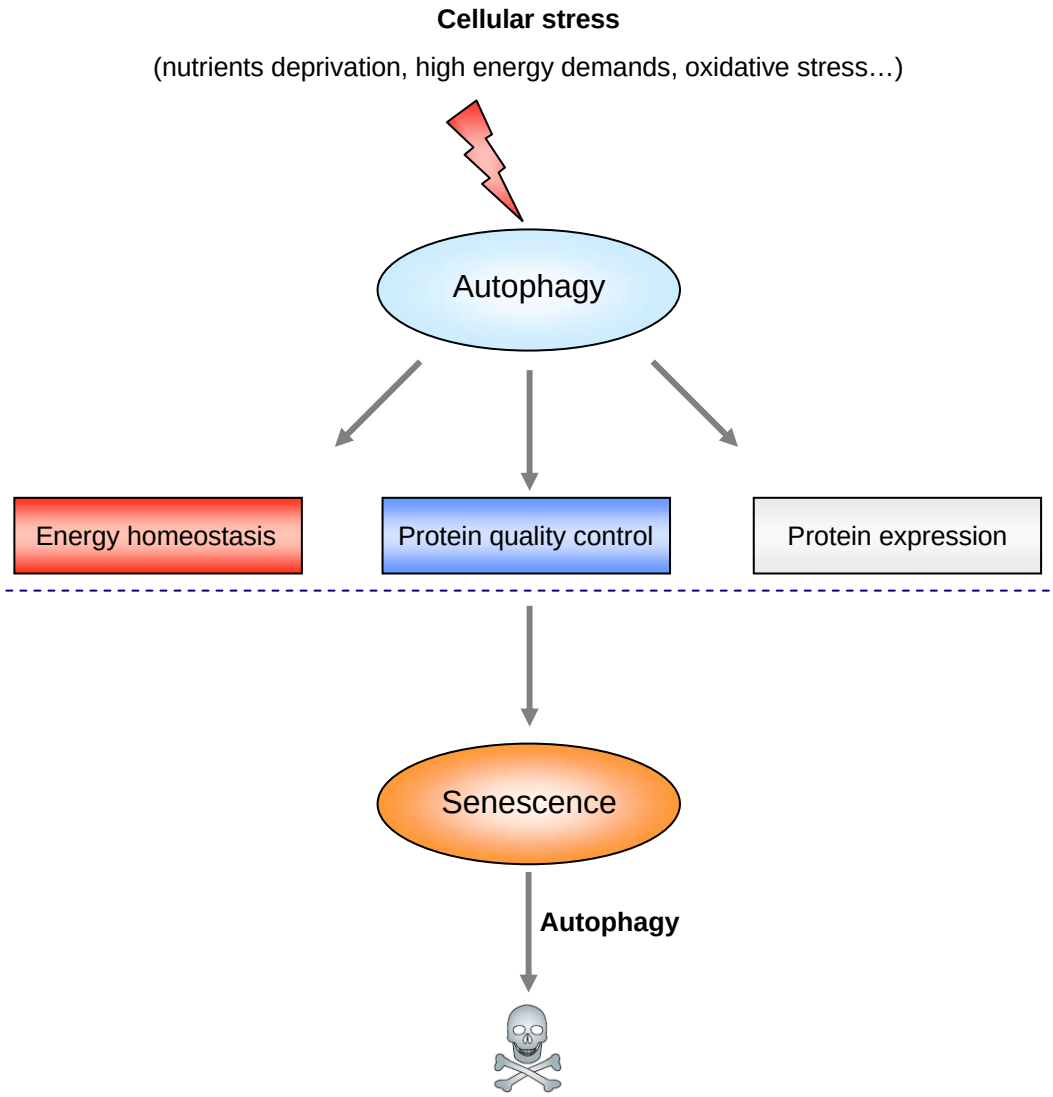


Figure 16: The autophagic pathway in cellular senescence.

Following different types of stresses autophagy could have several roles in regulating cellular senescence. It could be necessary for energy production providing raw material for protein synthesis required for critical regulators of cellular senescence. It could alternatively, it could recycle damaged proteins. Additionally, a recent hypothesis has emerged. Autophagy could control protein expression at post transcriptional levels. Adapted from [321-323].

I.2.5 Cellular senescence in tumour suppression

I.2.5.1 "Replicative senescence" as a physiological tumour suppressor mechanism

Ever since the discovery and the term "cellular senescence" introduced by Hayflick in 1965 [24], it has been proposed that senescence can act as a barrier against tumorigenesis. A simple fact can be used to illustrate this hypothesis. Primary normal cells are said to be "mortal". They have a finite number of population doublings before entering a state of growth arrest term "replicative senescence". In contrast, cancer cell lines are immortal. Therefore, escape from "replicative senescence" is a critical stage that has to be overcome.

In accordance with a critical role of telomerase in tumour growth, targeting telomerase activity in cancer cells lines limits their growth potential and triggers apoptosis [324-326]. Furthermore, telomerase expression in combination with other pro-oncogenic alterations is enough to create a human tumour cell from a primary human cell [327]. Finally, "replicative senescence" is associated with an increase in expression/activity of major tumour suppressor pathways such as p53 and the Rb pathway [76].

Despite multitude *in vitro* observations, evidence that "replicative senescence" could be a limiting barrier to tumorigenesis progression *in vivo* were still lacking. In 2007 were published two articles by the group of Sandy Chang and the group of Carole Greider (Nobel Prize winner in 2009) published respectively in *EMBO reports* and *Cancer Cell* demonstrating for the first time that "replicative senescence" in response to dysfunctional telomeres could limit tumour progression [328, 329]. Using mouse models in which the RNA component of the telomerase was knockout ($TERC^{-/-}$), they demonstrated that when apoptosis

was inhibited by either expressing a mutant form of p53 ($p53^{R172H}$) or inactivating Bcl2, dysfunctional telomeres induced cellular senescence and tumour regression [328, 329]. In accordance with these results, late generation $TERC^{-/-}$ deficient mice that have short telomeres and are telomerase deficient have been shown to be resistant to multistage skin carcinogenesis [330] and are more resistant to tumour formation in a subset of cancer mouse models [331-333]. In line with a crucial role of "replicative senescence" in tumour suppression, the status of the DNA damage/p53 pathway, a critical regulator of cellular senescence, appears to be critical in tumour suppression in response to dysfunctional telomeres [171]. Indeed, in an inactivated DNA damage/p53 pathway background dysfunctional telomeres are implicated in genomic instability and cancer progression [172].

I.2.5.2 "Oncogene-induced-senescence" as a physiological tumour suppressor mechanism

Since its discovery *in vitro* [31], there has been debates to know whether growth arrest and cellular senescence induction in response to an aberrant oncogenic stimulation could be physiologically relevant and represent a barrier against neoplastic expansion. Interestingly, the inactivation of potent tumour suppressor genes such as p53 and its upstream regulator ARF is sufficient to allow an escape from OIS in murine cells [31, 334]. In human cells, the inactivation of tumour suppressive pathways such as the p53 pathway [25, 29, 335], the Rb pathway [27, 134, 336, 337] or both [27, 338, 339] leads to OIS bypass.

OIS might not only act as a barrier against tumorigenesis by inducing a cell cycle arrest. Indeed, a series of six research articles published in *Nature* and *Genes and*

Development in 2009 suggested for the first time that, in the context of oncogene activation (kruppel-like factor 4 (KFL4) and c-Myc), cellular senescence could impede/reduce the efficiency of induced pluripotent stem (iPS) cells [340-345]. One could therefore hypothesise that cancer stem cells arise from the same reprogramming process that could be counteracted by cellular senescence [346]. In agreement, cellular senescence could not only inhibit tumour formation by limiting the proliferation of aberrant cells but it could also restrain cancer stem cells generation.

Although many indications suggested that OIS might reflect a failsafe program against oncogenic signalling, *in vivo* demonstrations were lacking. The first evidences of a tumour suppressive role of OIS *in vivo* were published in 2005 in *Nature* [26, 97, 204, 205]. The team of Pier Paolo Pandolfi and Clemens Schmitt demonstrated that the loss of the tumour suppressor PTEN and the activation of N-Ras led to the development of pre malignant tumours composed of many senescent cells [26, 204]. Interestingly, the inactivation of critical senescence regulators such as p53 or SUV39H1 led to aggressive tumours and most importantly, an absence of cellular senescence demonstrating a functional role of cellular senescence as a physiological barrier against tumour development [26, 204].

In accordance with these results, cellular senescence is associated with early stages of tumorigenesis in human tissue whereas it is absent in later stages [25-27, 98, 124, 205, 309]. Finally, since 2005, various cancer mouse models have provided additional information demonstrating that OIS occurs *in vivo* and acts as a tumour suppressor mechanism. For example, in H-RAS^{G12V} driven mammary tumours or B-Raf^{V600E} induced lung tumours, the genetic deletion of p53 or the ARF/INK4A locus, led to progression towards malignant stages and full blown tumours [347, 348].

Finally, two very elegant research articles published by the group of Tyler Jacks and Scott Lowe in *Nature* in 2007 provided evidences that in established tumours, the re-activation of p53 *in vivo* was associated with cellular senescence and tumour regression [164, 349]. Senescence associated tumour suppression might also require the tumour environment. Indeed, Xue and colleagues observed an activation of the innate immune response that they believed was responsible for the clearance of the senescent tumours [164]. Additionally in 2010 the Clemens Schmitt group, in a research article published in *Cancer Cell*, demonstrated that the failsafe mechanisms cellular senescence and apoptosis can act together via the innate immune response to inhibit tumour progression. In a model of OIS *in viv*o resulting in apoptosis, macrophage were attracted and required to engulf apoptotic reminders. In turn, activated macrophages secreted various cytokines such as TGF-β to induce cellular senescence of malignant cells [350] (Figure 17).

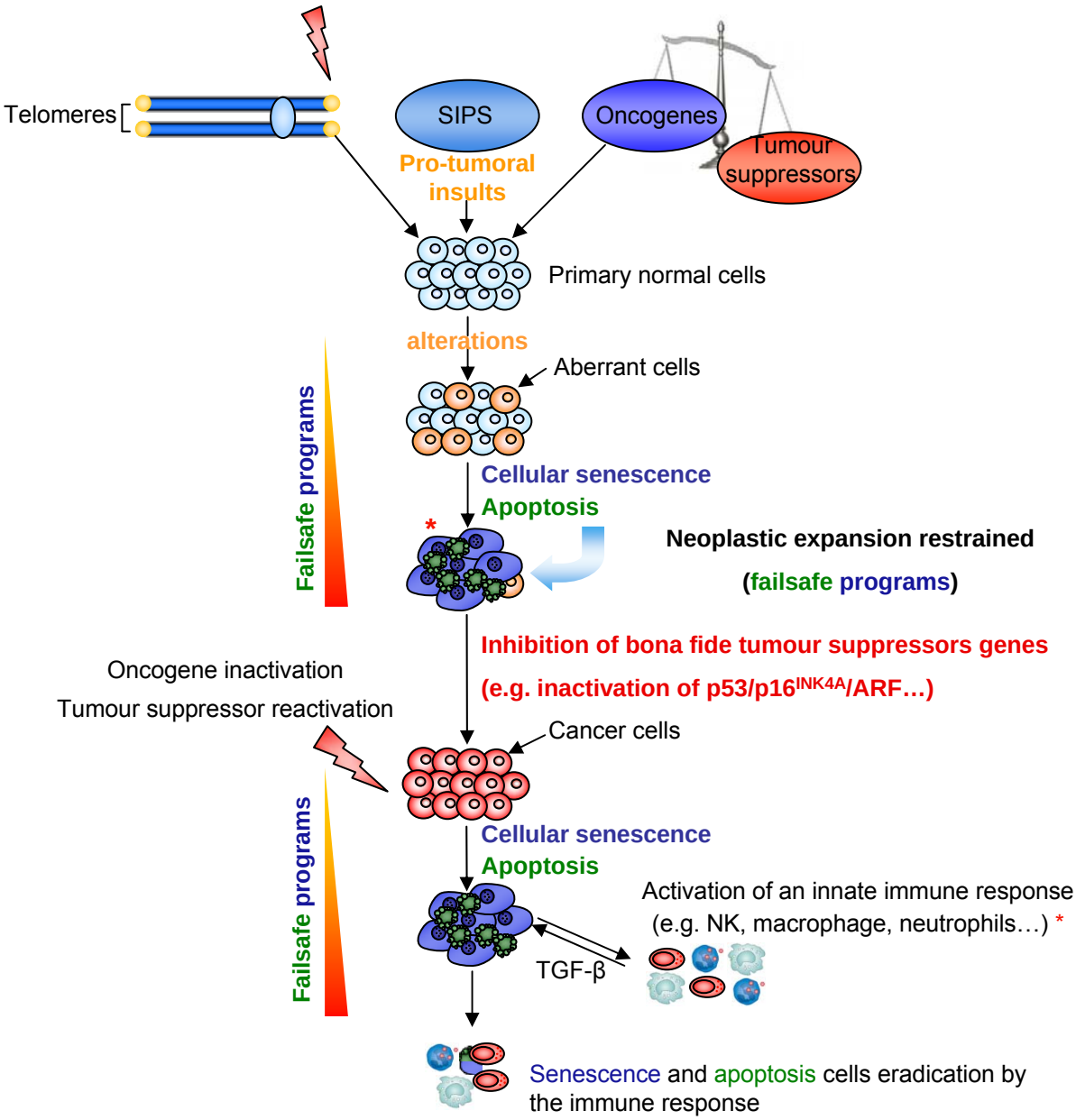


Figure 17: Cellular senescence as a physiological tumour suppressor mechanism. Cellular senescence can play a tumour suppressive role during different stages of tumour development. First, in response to pro-tumoral insults (OIS, dysfunctional telomeres, SIPS…), cellular senescence just like apoptosis is activated to restrain neoplastic expansion. The antitumoral barrier is stable and senescent cells can restrain neoplastic expansion for long period of time (e.g naevi, [205]) and additional mutations are required (inhibition of bona fide tumour suppressors) for cells to escape cellular senescence. Cellular senescence can also be induced in late stages of tumour development in response to the activation of tumour suppressors or the inhibition of oncogenes. The activation of the innate immune response might take place at different stages of tumour development (asterisk). It is critical to eradicate senescent and apoptosis cells and might also be important in the induction of cellular senescence via the production of various cytokines (TGF-β). Personal illustration.

I.2.6 Cellular senescence as a physiological tumour promoting mechanism

I.2.6.1 The SASP as a tumour promoting mechanism

Since the discovery of the SASP, cellular senescence can no longer be seen as a cell autonomous response. Components of the SASP include pro-inflammatory cytokines [98, 124, 157]. Cancer is a disease tightly controlled by inflammatory responses. Such inflammatory responses can modulate early (initiation, promotion) and late stages (invasion and metastasis) of tumour progression [351]. It was previously highlighted that the SASP could activate the immune system to induce senescent tumours destruction [164]. However, inflammation can also cause immuno-suppression. Additionally cancer cells, in an positive feed back loop can attract immune/inflammatory cells to maintain the tumour associated inflammation [351]. Altogether, the recent data provides additional complexity to the actual picture of cellular senescence as a tumour suppressive mechanism.

In line with this, secreted molecules from senescent such as Gro-α and amphiregulin cells can increase the proliferation of pre-malignant cells in a paracrine fashion [352, 353]. The effect of SASP molecules on neighbour cells is not limited to proliferation. Vascular endothelial growth factor A (VEGF), a critical determinant of endothelial cells migration and invasion, is expressed by senescent cells [146]. Senescent cells also secrete matrix metalloproteinase (MMPs) involved in the degradation of the extracellular matrix, facilitating cell invasion [141, 142, 353, 354]. Additionally, cytokines such as IL-6 and IL-8, which have been involved in the reinforcement of cellular senescence in an autocrine fashion [98, 124], have also been associated with an increased invasion of epithelial premalignant neighbour cells [152] (Figure 18).

Various research articles have provided compelling evidences that paracrine effects of SASP molecules can fuel neighbour premalignant cells to acquire additional characteristics of tumour cells *in vitro*. However, can such phenotype really favour tumour progression *in vivo*? The answer is yes, at least in xenografts models. The injection of senescent cells with premalignant cells favours the apparition of tumours [148] whereas the injection of senescent cells with cancer cells accelerated tumour formation [353, 355-357]. However, it seems that the co-injection of senescent cells with premalignant cells does not always lead to increased tumorigenesis. Co-injection of senescent and cancer cells in an internal organ (kidney), did not result in an increase in cancer cells aggressiveness, suggesting that cancer promotion by senescence might be organ and tissue specific [358]. Additionally, not all senescence secreted molecules have paracrine pro-tumoral effects and some can even, in a paracrine way, inhibit tumour progression [126, 359]. Given the crosstalk previously described between senescent cells and the immune system [164], it is important to keep in mind that the xenografts experiments were performed in immune deprived mice.

I.2.6.2 Senescence cell autonomous features as a tumour promoting mechanism

Cellular senescence as a physiological tumour promoting mechanism might not be limited to secreted factors associated with cellular senescence. It has been suggested that under different types of stresses, normal and cancer cells can enter senescence which can be associated in some cases to the emergence of aneuploid neoplastic cells with a strong genomic instability. This mechanism, formally termed neosis, is the result of an unusual budding mitosis [360-364]. Although providing very interesting insights into a senescence cell autonomous pro-tumoral process, additional studies will be required to identify the molecular mechanisms

underlining this phenomenon. Additionally, its physiological relevance *in vivo*, although hardly feasible to date, will have to be assessed in the future.

Since pro-senescence therapy to treat cancer has been suggested by many scientific researchers in the cellular senescence field, it will be crucial to identify the potential negative effects of such therapies considering the negative side of cellular senescence. Nonetheless, despite the potential negative effects, to date the physiological role of cellular senescence remains tumour suppression [46]. Compelling evidences suggest that cellular senescence could be part of the anti-tumoral therapy. Determining the negative effects should help limit the "side effects" of a putative therapy.

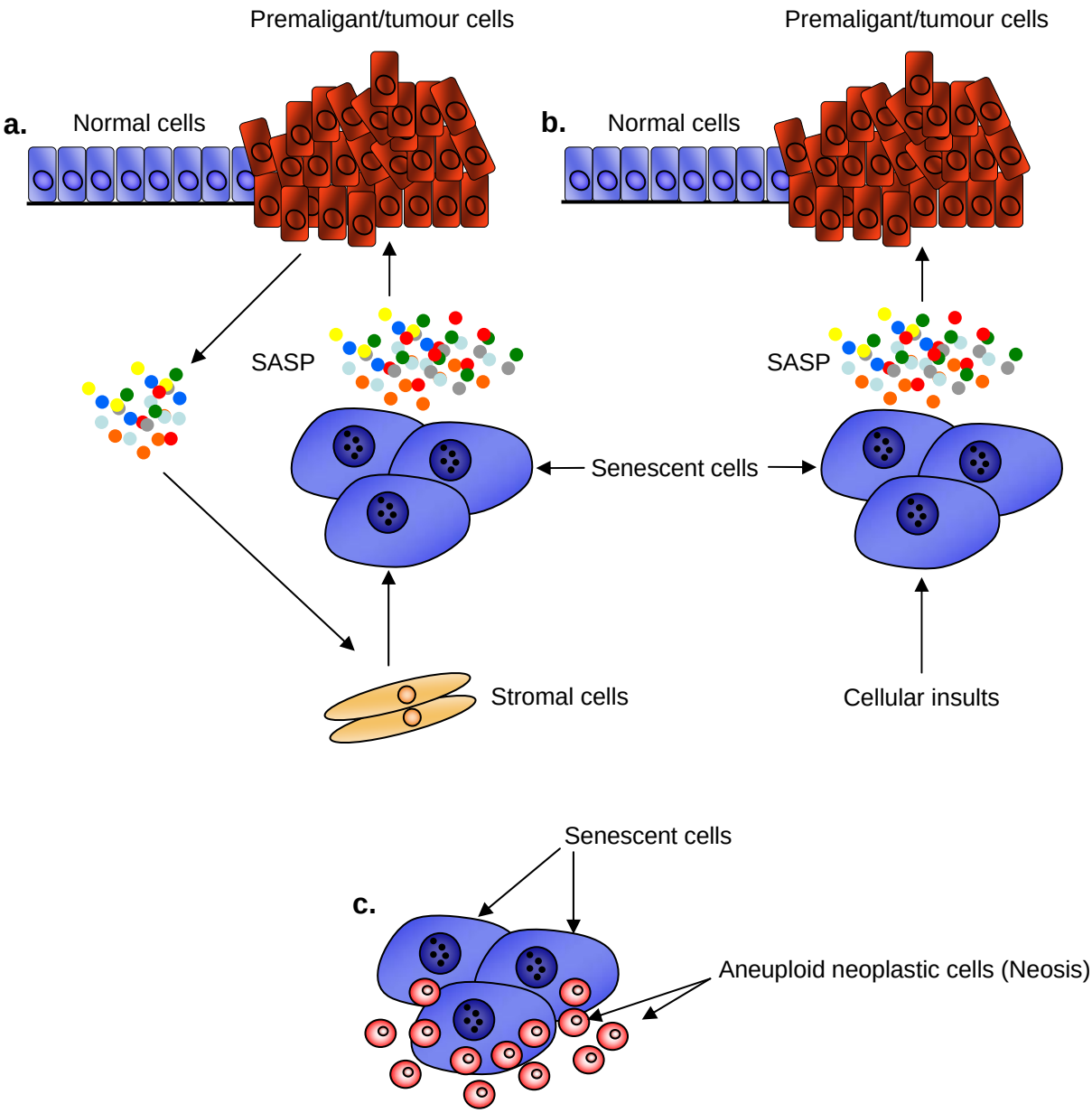


Figure 18: Cellular senescence in promoting tumour progression.

Various hypotheses can be formulated to explain the role of the SASP as a tumour promoting mechanism. (a) First, cancer cells by secreting molecules of the SASP could induce cellular senescence of stromal cells. Senescent stromal cells could in turn provide SASP molecules required for tumour growth. (b) Another possibility is that cellular insults could contribute to increasing the number of senescent cells which in turn would provide SASP molecules for premalignant and cancer cells. It is probable that both hypotheses co-exist. (c) Alternatively, cell autonomous mechanism might also favour tumour progression. It has been proposed that senescent cells might generate aneuploid neoplastic cells in an unusual budding mitosis process termed neosis. Adapted from [157].

Cellular senescence is not a response specific to cancer. Indeed, cellular senescence can also be associated with other biological responses. I will therefore shortly introduce cellular senescence in other contexts.

I.2.7 Cellular senescence and ageing

It was speculated by Leonard Hayflick in 1965 "that the finite lifetime of diploid cells strains *in vitro* may be an expression of ageing or senescence at the cellular level" [24]. In 1995 Judith Campisi's laboratory first demonstrated, what had been speculated since 1965, the presence of cellular senescence in ageing tissues *in vi*vo. Since then, senescent cells have been observed in various tissues [129, 365-367]. Cellular senescence has also been associated with age related diseases such as renal tubulointerstitial fibrosis and glomerulosclerosis, osteoarthritis and atherosclerosis [368-370] and premature ageing syndromes such as Hutchinson-Gilford progeria syndrome [38-41].

Two main hypotheses have been formulated to try to explain how cellular senescence contributes to ageing and ageing related disease. The first suggests that the accumulation of senescent cells in ageing tissue might reach a threshold that could compromise tissue functions. Comprised tissue functions might be the result of senescent cells being physically present and/or of the SASP. Indeed, secreted factors could also modulate normal tissue homeostasis [371].

The other hypothesis that has emerged implicates stem cells. It has been demonstrated that adult stem cells can undergo cellular senescence. As a consequence, in adult organisms,

cellular senescence may account for a decrease number and/or function of stem cells subtypes that could be required for tissues repair and renewal (Figure 19) [262, 372-375].

The two hypotheses might be non exclusive or could synergise to result in ageing. The causal role of cellular senescence in organismal ageing is intuitive. However a recent publication has provided precious tools to test the functional role of cellular senescence in premature ageing syndromes at least *in vitro*. In a work published in *Nature* in 2011, Juan Carlos Izpisua Belmonte's laboratory was able to generate iPS cells from the fibroblasts of Hutchinson-Gilford progeria syndrome patients (HGPS-iPSCs). Hutchinson-Gilford progeria syndrome is a fatal premature ageing syndrome characterized by premature arteriosclerosis and degeneration of vascular smooth muscle cells (SMCs) [41]. Interestingly, the differentiation of HGPS-iPSCs into SMCs was associated with cellular senescence. It might therefore be possible in this context to test the functional role of cellular senescence during the differentiation of HGPS-iPSCs into SMCs.

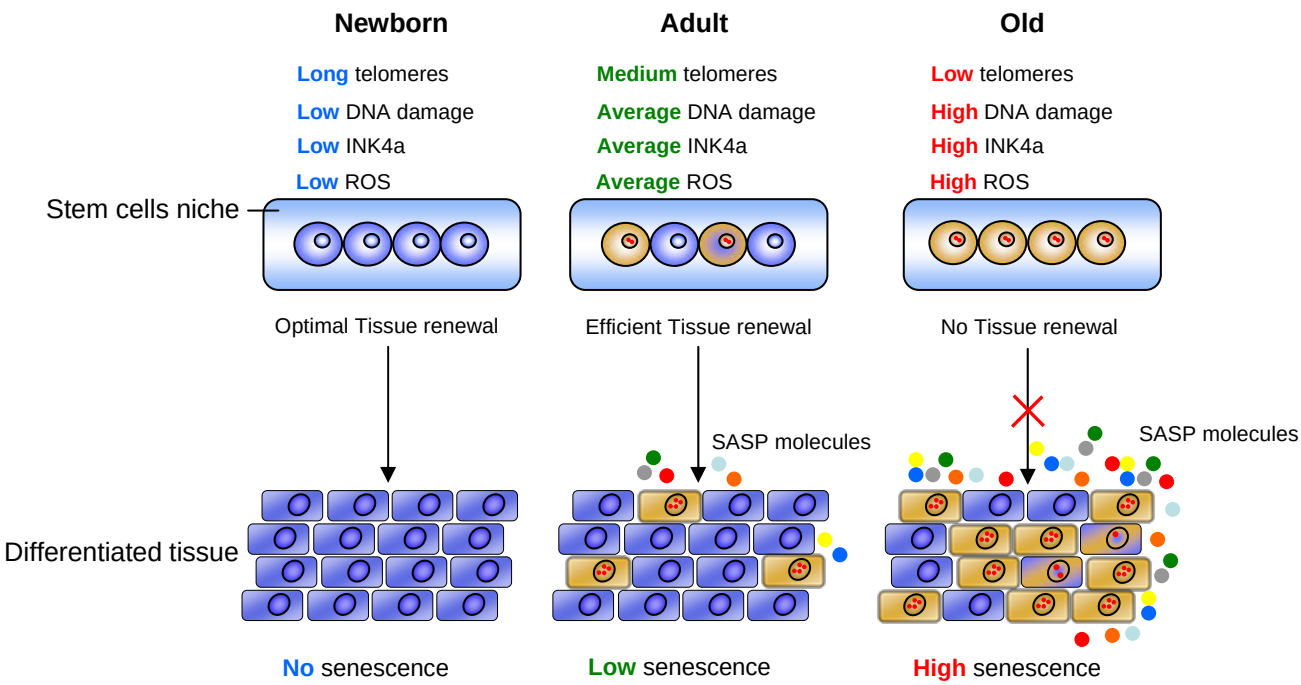


Figure 19: Cellular senescence and ageing.

Two hypotheses have emerged to try to explain the role of cellular senescence in organismal ageing. The first, suggest that the accumulation of senescent cells and the SASP could compromise differentiated tissue functions. The second hypothesis involves stem cells niches. In response to accumulated stress (ie: telomere dysfunction, DNA damage, ROS) and an accumulation of $p16^{INK4A}$ stem cells can could enter cellular senescence. This might lead to a decrease/abrogation of tissue renewal and accumulation of senescent cells in differentiated tissue. These two hypotheses could be non exclusive or synergise to contribute to ageing. Adapted from [262, 376].

I.2.8 Cellular senescence and tissue repair

Cellular senescence has recently been functionally involved with tissue repair [43, 44]. Wound healing is composed of several overlapping phases. The first, an inflammatory phase is associated with the infiltration of neutrophils and macrophages. This phase is followed by a proliferative phase that includes ECM deposition. Finally, a maturation phase is required for resolution of the granulation tissue and matrix remodelling [377].

Scott Lowe's laboratory in a work published in Cell in 2008 was the first to demonstrate a role of cellular senescence in wound healing in response of liver tissue to injury [44, 378]. Krizhanovsky and colleagues observed that upon acute liver injury in mice, hepatic stellate cells (HSC) initially proliferate and secrete extracellular matrix (ECM) components. This leads to the production of a fibrotic scar that eventually resolves [44]. Interestingly, they observed that hepatic stellate cells (HSC) rapidly underwent cellular senescence. It was accompanied by a decrease in ECM components and an increased in metalloproteinase (MMPs) which are ECM proteases. They concluded that the senescence response could contribute in resolving scar tissue. In line with the hypothesis, (p53/ARF or $p16^{INK4a}$/ARF) knockout mice, which have stellate cells (HSC) unable to enter cellular senescence, developed severe fibrosis after acute liver injury [44].

In 2010, a work published in *Nature Cell Biology* by the Lester Lau's laboratory demonstrated that the regulatory role of cellular senescence might not limited to liver wound healing. Jun and colleagues showed that the secreted protein CCN1 was able to induce cellular senescence in resident myofibroblasts following a cutaneous wound healing [43]. The cellular senescence induced by CCN1 was crucial to limit fibrosis as mice expressing a

mutant form of CCN1 that was unable to bind fibroblasts and induce cellular senescence, developed excessive fibrosis during cutaneous wound healing. [43]. The authors suggested a role of the matrix metalloproteinase in the phenotype observed. These two research articles provide new insights into the role of cellular senescence suggesting that this cellular process is not limited to cancer and ageing (Figure 20).

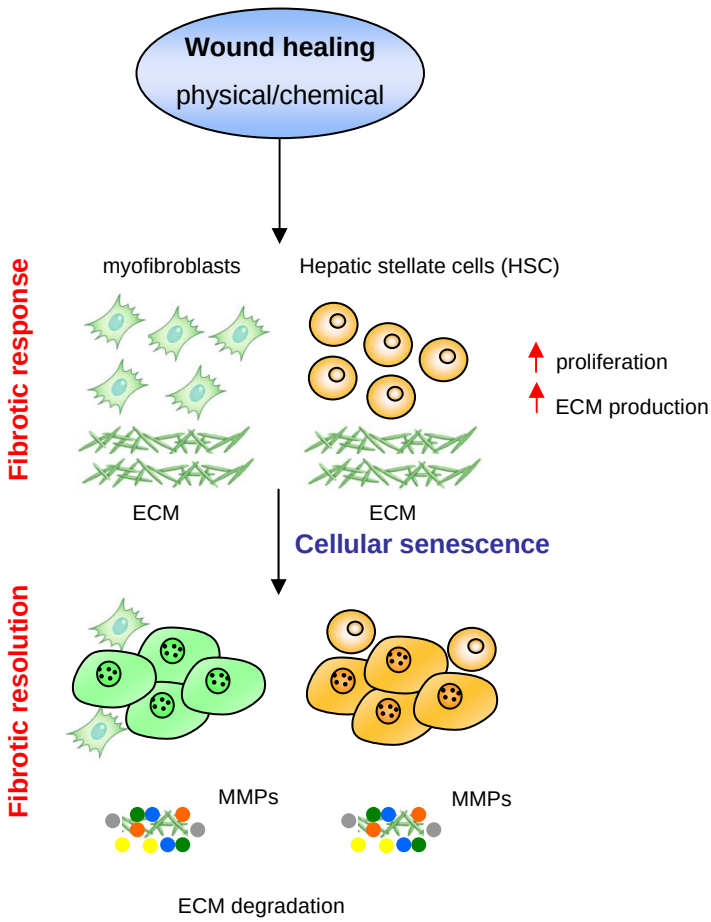


Figure 20: Cellular senescence and tissue repair.

Following wound healing that can be the result of physical and/or chemical insults, a fibrotic response is engaged. Fibrosis is associated with increase cell proliferation and extra cellular matrix (ECM) production which helps restraining the site of injury. Following the fibrotic response, the cells involved in the process undergo cellular senescence. As a consequence, ECM is degraded and fibrosis is restrained. Adapted from [378, 379].

I.3 Research objectives and scientific context

I.3.1 Identifying new regulators of cellular senescence using a shRNA library

We have seen that cellular senescence is regulated by bona fide tumour suppressor genes such as p53, RB, and the products of the INKAB/ARF/INK4A locus [380]. However many cellular senescence regulators are yet to be discovered. Identifying novel regulators of cellular senescence can serve multiple purposes. For example, it might reveal unexpected regulatory networks [98, 124] but also lead to the discovery of new tumour suppressor genes [381]. Such discoveries can also lead to new therapeutic tools [126]. In order to identify new regulators of "replicative senescence", we used the reverse genetic technique of RNA interference.

The mechanism of RNA interference (RNAi) was first described in *Caenorhabditis elegans (C.*elegans) by Andrew Fire and Graig Mello (Nobel Prize Winners in 2006) [382]. Three years later, a similar mechanism was described in mammalian cells although with much smaller (21 nucleotides) RNAs duplex [383]. From then on, genetic tools taking advantage of this biological process have been constructed to uncover novel mechanisms underlining biological responses. In 2003, the group of Reuven Agami published in *Cancer Cell* a new retroviral based method that would allow stable gene inhibition. Using a retroviral vector system stably inserted in the genome and expressing functional siRNAs they were able to induce stable gene repression [384].

The retroviral based system expresses short hairpin RNAs (shRNAs) containing double-stranded stem of 19–29 base pairs connected by a loop of 6–9 bases that is cleaved into functional siRNAs [385, 386]. Such vector constructs turned out to be powerful technologies in the study of phenotypes that develop over long period of time [381]. Retroviral based shRNA

libraries targeting several thousands human or mouse genes were constructed and successfully used in large scale RNAi screens [387, 388]. Figure 21 highlights the mechanism of RNA interference in mammalian cells.

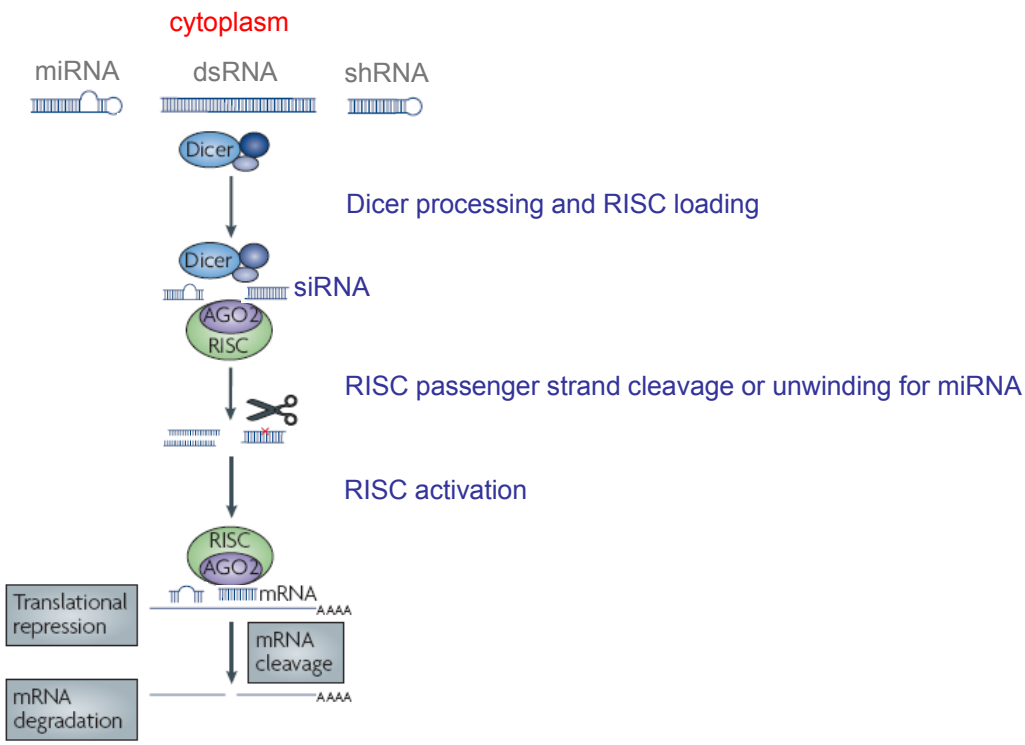


Figure 21: Mechanism of RNA interference in mammalian cells.

RNA interference (RNAi) pathways are guided by small RNAs. The siRNA pathway begins with cleavage of long double-stranded RNA (dsRNA) by the Dicer enzyme complex into siRNA. They are then incorporated into Argonaute 2 (AGO2) and the RNAi-induced silencing complex (RISC). AGO2 cleaves the passenger (sense) strand so that active RISC containing the guide (antisense) strand is produced. The siRNA guide strand recognizes target sites to direct mRNA cleavage. Presumably, shRNAs are converted to siRNA-like molecules in the cell through both dicer dependent and independent pathways [385, 386]. Once produced, they are processed like siRNA molecules. microRNAs (imperfect sequence complementarity) are loaded onto RISC. The passenger (sense) strand is unwound leaving a mature miRNA bound to active RISC. It recognizes target sites (in the 3′ and/or 5'UTR of the mRNA) leading to direct translational inhibition or degradation. Adapted from [389].

We took advantage of this technology to perform a loss of function genetic screen in order to identify putative new regulators of cellular senescence. The goal was to identify new pivotal regulators of cellular senescence that, when knockdown, would allow an extension of cellular lifespan (an extension of population doublings). As mentioned earlier, "replicative senescence" is a barrier against immortalisation [327], can be induced *in vivo* to limit tumour progression [328, 329] and is regulated by bona fide tumour suppressor genes [380]. We were hoping that such genetic screen would lead to the identification of new cellular senescence regulators and/or unexpected networks underlining this mechanism. Among the genes identified was the gene coding for the phospholipase A2 receptor (PLA2R1). After performing the genetic screen and identifying several new regulators, I focused a big part of my thesis' work trying to characterise the role of this gene in regulating cellular senescence as well as analysing its function as a tumour suppressor candidate. In the following chapter, I will introduce PLA2R1. The retroviral based shRNA library used, the detailed genetic screening strategy employed and the "hit" list of genes discovered will be described in the results section.

I.4 The M-type Phospholipase A2 receptor (PLA2R1)

I.4.1 PLA2R1 historical discovery

In the late 1980s, Gérard Lambeau, student in the group of Michel Lazdunski, identified in rabbit skeletal muscle cells a binding site for two neurotoxic monochains phospholipase A2 (OS1) and (OS2) isolated from the venom of Taipan snake [390]. Lambeau purified the phospholipase A2 binding protein from myotubes and identified a protein of approximately 180 kDa [390].

Basically at the same time, Arita and colleagues, looking in mouse cells for specific binding sites of the pancreatic-type phospholipase A2 (PLA2-I, nowadays sPLA2-IB) identified a binding site of approximately 200 kDa. They suggested, in a work published in the *Journal of Biological Chemistry* in 1991, an pro-proliferative effect of PLA2-IB on swiss 3T3 cells via this specific binding site [391]. In 1992, Hanasaki under the supervision of Arita, analysed the cellular and tissue distribution of PLA2-I high binding site in rat. They identified its presence in a subset of rat cells such as vascular smooth muscle cells (SMCs), vascular endothelial cells, synovial cells, chondrocytes and gastric mucosal cells [392]. This high affinity binding protein was not limited to mouse, rat and rabbit as it was also found on the membranes of bovine corpus luteum [393].

In 1994, the team of Michel Lazdunski and Hitoshi Arita published in the *Journal of Biological Chemistry*, the cDNA cloning of the rabbit and bovine receptor for secretory phospholipase A2 [394, 395]. Lambeau and colleagues first published their work and named the receptor, the M-type (muscle-type) Phospholipase A2 receptor (PLA2R1), as it was purified from rabbit skeletal muscle. Following its discovery these two teams published from the mid to the late 1990s several research articles providing information on PLA2R1 such as genetic and biochemical properties as well as putative biological mediated responses (*in vitro* and *in vivo*).

Despite its cloning in 1994, in contrast to the great amount of information that can be found on its ligands (sPLA2), still little is known on the biology of the receptor. sPLA2 biological functions have been linked to PLA2R1 even if, as we will see, PLA2R1 mediated sPLA2 biological functions has not always been convincingly demonstrated. Additionally, biochemical properties as well as biological responses can be species dependent. So far, most

of the research in the topic has been performed on Rabbit, Mouse or Bovine PLA2R1. Very little is known on the biological role of human PLA2R1. In this chapter, I will describe PLA2R1 genetic and biochemical properties and its biological mediated responses. Before describing PLA2R1 properties, I will succinctly introduce the mannose family receptor which it belongs to and I will introduce basic information on its ligands, the sPLA2.

I.4.2 The mannose receptor family

PLA2R1 belongs to the mannose receptor family. This family is composed of four members: the mannose receptor (MR), the M-type phospholipase A2 (PLA2R1), DEC 205 (gp200-MR6) and Endo 180/uPARAP [396] (Figure 22). Despite their overall structure, the four receptors have evolved separately. As a result, they use different interacting domains to bind with specific ligands and mediate unique biological responses. Furthermore, their ability to mediate endocytosis, phagocytosis and even their intracellular destinations vary depending on the family member [396]. Therefore the receptors have specific independent functions. For example, the MR is known to be involved in both innate and adaptive immune responses whereas Endo180 is involved in the remodelling of the extracellular matrix, DEC-205 in the internalisation of antigen for the presentation to T lymphocytes and PLA2R1 is known to mediate sPLA2 signalling [396].

The mannose receptor family belongs to the superfamily of C-type like lectin (CTLD) [397]. The C-type like lectin is a large family divided into 17 subfamilies, composed of transmembrane receptors but also soluble proteins [397]. The mannose receptor family has a characteristic structure organisation. The extracellular domain is composed of an N terminal cysteine-rich domain (Cys-R) followed by a fibronectin like domain (FN-II) and multiple C-

type lectin like domains (CTLD) aligned on a single polypeptide chain (8 for the MR, PLA2R1 and Endo 180 and 10 for DEC-205). The transmembrane domain is single pass and the family members contain a short cytoplasmic tail with an endocytosis motif [396]. This motif is involved in the internalisation of extracellular ligands through the interaction with the cellular endocytosis machinery (Figure 22).

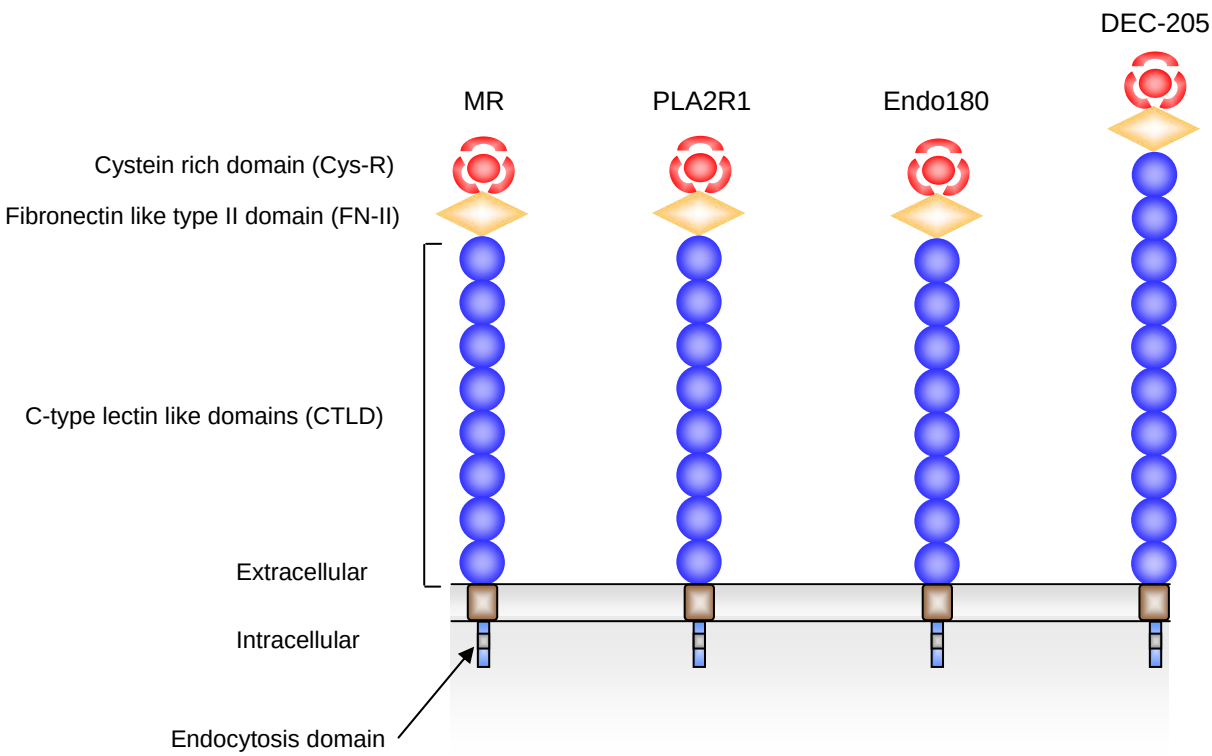


Figure 22: The mannose receptor family.

The mannose receptor family shares mainly structure characteristics. They contain an N terminal cysteine rich domain (Cys-R), a fibronectin like domain II (FN-II), C-type lectin like domains (CTLD) (8 or 10) aligned on a single polypeptide chain, a single pass transmembrane domain and a short cytoplasmic domain with an endocytosis motif. Adapted from [396].

I.4.3 The secretory phospholipase A2 (sPLA2)

Phospholipases A2 (PLA2s) are esterases that hydrolyze the *sn*-2 ester of glycerophospholipids. Through their esterase activity, they have been involved in various biological processes such as host defence against bacterial infection, the digestion of dietary phospholipids but also arachidonic acid (AA) release for the biosythesis of eicosanoids during inflammation [398]. The mammalian contains 10 enzymatically active sPLA2 with the most studied being sPLA2 (IB, IIA, V, X and III). They exhibit unique tissue and cellular localizations suggesting a non redundant role [398]. They can bind to membrane bound receptors but also soluble receptors. Among the receptors identified to date are the M-type phospholipase A2 receptor but also factor Xa, integrin, heparan sulfate proteoglycans, integrins, VEGF receptor, calmodulin among others [399]. So far, sPLA2 binding properties have essentially been studied for sPLA2-IB and IIA. It remains to be determine whether the other mammalian sPLA2 can bind the same receptors or will bind a different set of receptors [399].

I.4.4 PLA2R1 genetic, biochemical properties and tissue distribution

The gene encoding the M-type phospholipase A2 receptor is located on chromosome 2 [400] and genomic DNA blotting studies demonstrated that it was encoded by a single copy gene [401]. Genomic blast analysis suggested that there were no closely related genes in the mammalian genome [401]. Structural analysis of the receptor revealed a conserved structure with the mannose receptor family, although their homology is only 29% [402]. PLA2R1 is a receptor of approximately 180-200 kDa made of a large extracellular domain composed of a cysteine rich domain (Cys-R), a fibronectin like domain II (FN-II) and eight carbohydrate recognition

domains also known as C-type lectin like domains (CTLD). It contains a single pass transmembrane domain and a short intracellular cytoplasmic tail (40 amino acids) including a consensus sequence for casein kinase II (CK-II) phosphorylation [400] and a coated pit mediated endocytosis domain (NPYAA) [403]. Finally, the receptor can be found as a membrane bound and soluble form. The soluble form is believed to result from an alternative splicing, protein shedding or both [400, 404] (Figure 23).

PLA2R1 contains several putative sites of N-glycosylation, believed to be important for optimum recognition of its ligands or at least one of its ligand: sPLA2-IB [405]. The CTLD are the main ligand interacting domains [394] (Figure 23). Nicolas and colleagues demonstrated that the CTLD4-5-6 were the binding domains for sPLA2s. More specifically, CTLD5 was critical for the binding of sPLA2 (OS1), with a putative regulatory role of the surrounding CTLD4 and CTLD6 [406].

PLA2R1 CTLDs are not only involved in the binding of sPLA2s. In the same way as the CTLDs of the mannose receptor or Endo-180 [407], PLA2R1 CTLDs can bind several sugars such as mannose and galactose with a requirement of the CTLD5-6-7-8 [395, 406]. sPLA2s and sugar binding to PLA2R1 is believed to be Ca^{2+} independent [406, 408]. Finally, the CTLD are not the only ligand binding domains. Indeed, the FN-II domain has been shown to bind type I and IV collagen [409] (Figure 23). So far, the cystein rich domain (Cys-R) has not been described as a site of ligand binding.

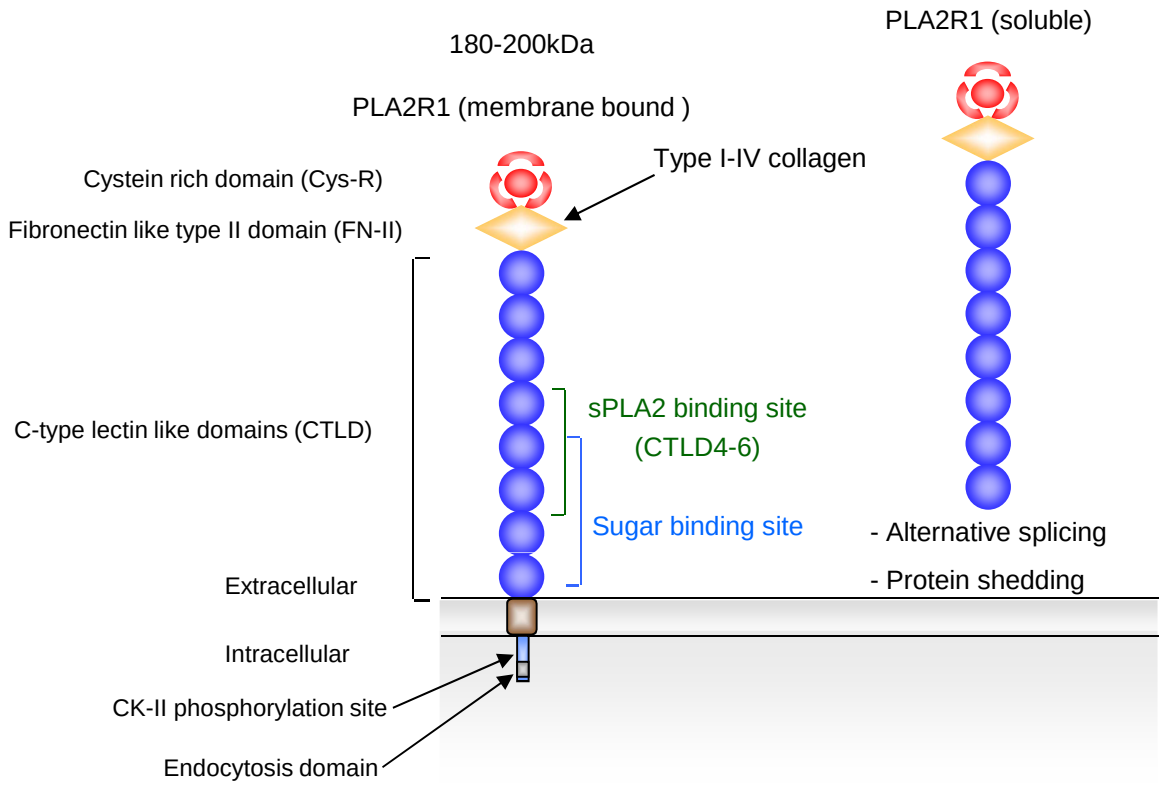


Figure 23: The M-type Phospholipase A2 receptor (PLA2R1).

PLA2R1 is a receptor of approximately 180-200 kDa made of a large extracellular domain composed of a cysteine rich domain (Cys-R), a fibronectin like domain II (FN-II) and eight carbohydrate recognition domains also known as C-type lectin like domains (CTLD). It contains a single pass transmembrane domain and a short intracellular cytoplasmic tail (40 amino acids) including a consensus sequence for casein kinase II (CK-II) phosphorylation and a coated pit mediated endocytosis domain (NPYAA). It can be found as a membrane bound and soluble form. The soluble form is believed to result from an alternative splicing, protein shedding or both. CTLD (4-6) mediate sPLA2 binding whereas the CTLD (5-8) are believed to mediate sugar binding. Finally type I-IV collagen bind the FN-II domain. It is important to mention that binding properties are highly species dependent. Personal illustration.

PLA2R1 expression analysis revealed tissue specific expression. Northern blot analysis revealed that in mice, mouse PLA2R1 (mPLA2R1) expression is detected in several tissues including the lung, the kidney and the spleen [401]. Interestingly, human PLA2R1 (hPLA2R1) mRNA has also been detected in kidney, lung and additionally in pancreas [400]. Finally, rat PLA2R1 (rPLA2R1) mRNA was also found in the lung and the liver and testis [392].

I.4.5 PLA2R1 mediated biological responses

sPLA2-IB has been associated with pro-proliferative, chemokinetic migration and cell invasion [410]. Interestingly, sPLA2-IB in human cancer cells was shown to activate the MAPK pathway to regulate proliferation [411]. A work of Kundu and colleagues using PLA2R1 specific anti-sense oligonucleotide first convincingly demonstrated a sPLA2-IB/PLA2R1 dependent phenotype [412]. The effects of sPLA2-IB suggested to be mediated by PLA2R1 are not limited to cell proliferation. Indeed, sPLA2-IB leads to hormone secretion in endocrine organs [413]. Finally, sPLA2-IB regulates the production of lipid mediators such as arachidonic acid (AA), PGD_2, and PGE_2 via COX-2 [414] and pro-inflammatory cytokines [415, 416]. sPLA2-IIA also mediates biological responses via PLA2R1. Indeed, in RAW 264.7 (mouse leukaemic monocyte macrophage cell line), sPLA2-IIA activates inducible nitric oxide synthetase (iNOS) in a PI3K/AKT dependent manner. Interestingly, PLA2R1 increased iNOS levels in a sPLA2-IIA dependent manner [417]. Altogether, these results suggested a role of PLA2R1 in sPLA2 mediated biological response.

In 1997 Hanasaki and colleagues published, in the *Journal of Biological Chemistry*, the results from the PLA2R1 knockout mice they had generated [416]. The $PLA2R1^{-/-}$ mice were

viable, sterile and necropsy and microscopic examination of major tissues revealed no evident histopathological abnormalities [416]. However upon a bacterial lipopolysaccharide (LPS) mimicking an endotoxic shock, PLA2R1 deficient mice were more resistant than control mice and exhibited longer survival. Looking at the plasma levels of various pro-inflammatory cytokines, the authors found that following the endotoxic shock, tumour necrosis factor alpha (TNFα) and interleukin 1 beta (IL1-β) were significantly reduced in PLA2R1 knockout mice. Additionally, the authors suggested a role of sPLA2-IB in mediating the endotoxic shock. Indeed, PLA2R1 knockout mice treated with a sublethal LPS dose and an exogenous dose of sPLA2-IB survived whereas control mice did not [416]. Altogether it suggests that PLA2R1 can mediate some sPLA2 biological response *in vitro* and *in vivo*.

I.4.6 PLA2R1 regulation of sPLA2

It has been proposed that various negative regulations could control the activity of sPLA2 in order to avoid excessive sPLA2 enzymatic activities [418]. As previously mentioned, PLA2R1 can also exist as a soluble form (sPLA2R1). It was demonstrated that the sPLA2R1 could be a negative regulator of sPLA2. Indeed, sPLA2R1 possesses the same sPLA2 binding properties as PLA2R1 and it can block both the enzymatic activity of sPLA2 and its ability to bind PLA2R1 [419].

Additionally, an alternative mechanism that involves a negative regulation of sPLA2 implicates endocytosis. It has been demonstrated that upon sPLA2 binding, PLA2R1 rapidly internalises its ligands via an endocytosis process [420]. Interestingly, upon binding to the receptor, sPLA2-X was internalised and degraded [421]. Analysis of the endocytosis process revealed that sPLA2-X was found with PLA2R1 in lysosomes [421].

Endocytosis mediated by PLA2R1 might also play alternative functions. Indeed, sPLA2-IB was found to be internalised and transported to the nucleus [422]. Therefore, endocytosis might also target sPLA2 to specific cellular compartments where sPLA2 enzymatic activity could have additional roles [422].

RESULTS

II/ RESULTS

II.1 Genetic screen overview

II.1.1 Retroviral based shRNA library

We were kindly provided with a fraction of the NKI (Netherlands Cancer Institute) "first generation" shRNA library [381]. The library is based on a retroviral vector construct (pRetro-Super) that contains a H1 (RNA polymerase type III) promoter driving shRNA transcription (Figure 24a). The library theoretically targets 8000 human genes and is composed of approximately 24000 vectors each containing a single shRNA. It generally contains three vectors targeting a single gene [381]. Once inserted in the cell's genome the shRNA is continually transcribed theoretically leading to specific gene silencing (Figure 24b). For more details refer to Figure 24 legend and the material and methods of the articles.

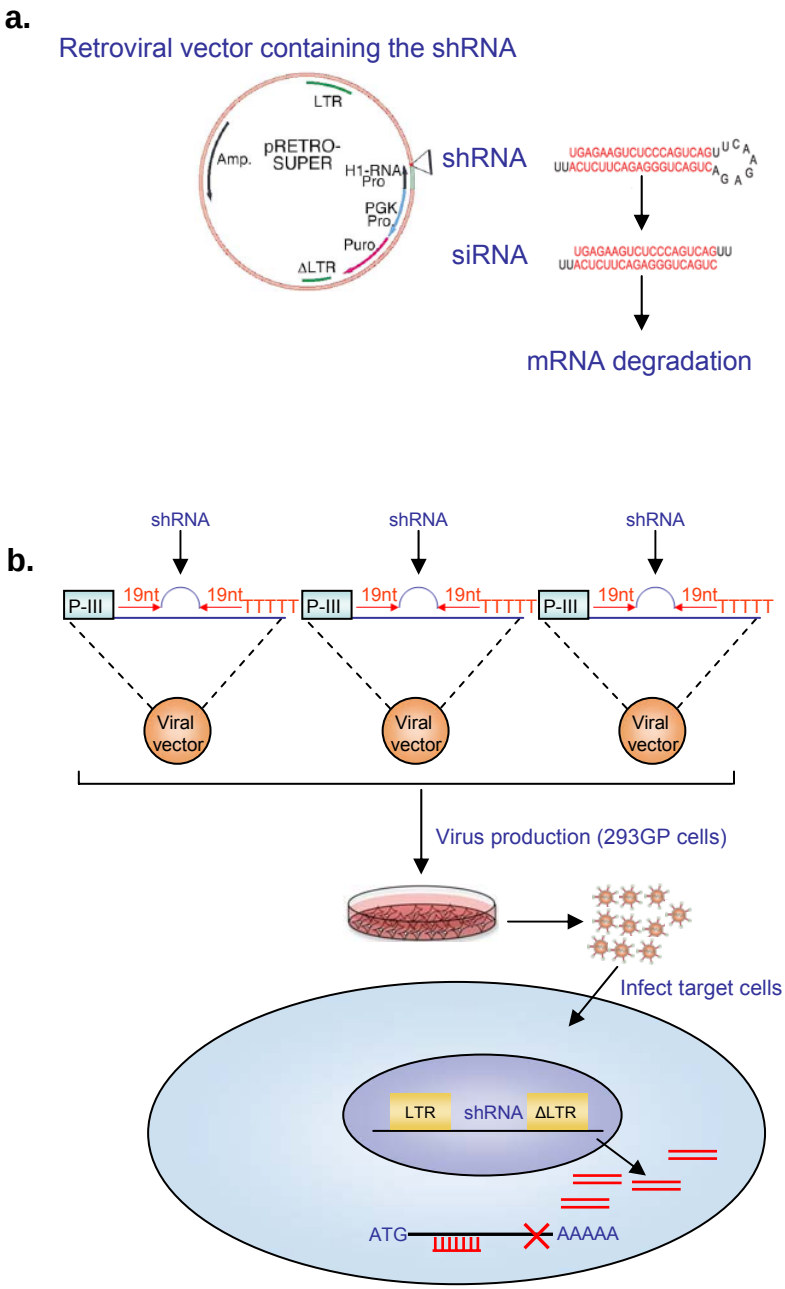


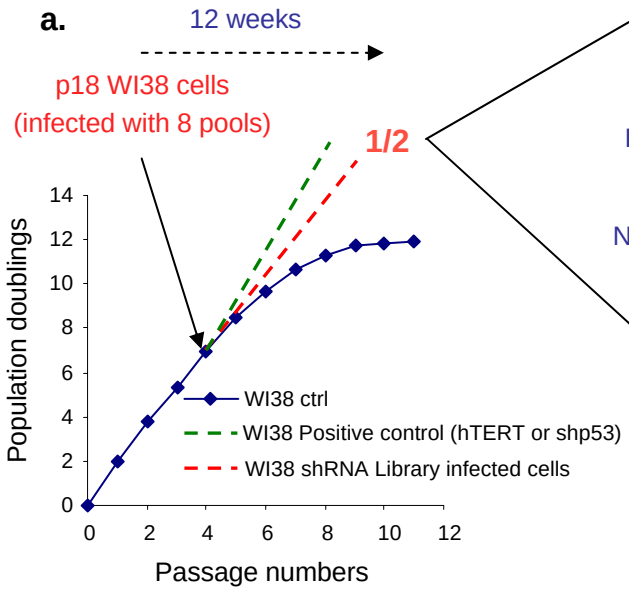
Figure 24: Loss of function genetic screen with the shRNA library.

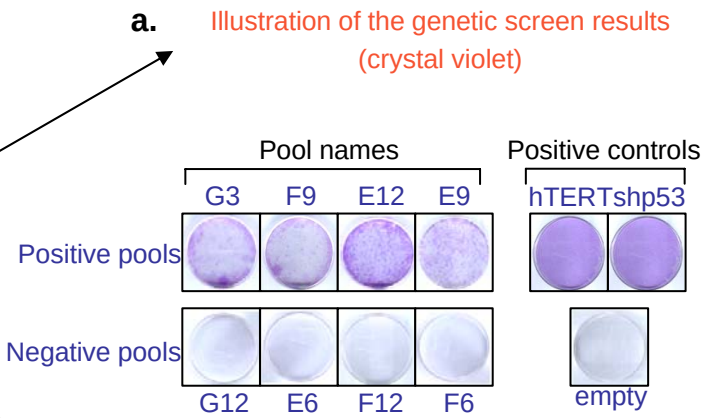
(a) The retroviral vector (pRetro-Super) contains a H1 promoter which drives shRNA transcription in a self-inactivating retroviral backbone based on a mouse stem cell virus (MSCV). It contains an ampicillin bacterial resistance marker and a puromycin-selectable marker for mammalian cells. LTR stands for the retroviral long terminal repeat whereas the Δ'LTR contains a self-inactivating deletion. The retrovirus produced are in theory non replicative. (b) The NKI library theoretically targets 8000 human genes and is made of approximately 24000 vectors each containing a single shRNA. There are two to three vectors targeting a single gene [381]. The vectors are transfected as pools in cells producing virus (293GP cells). The supernatant of virus producing cells is used to infect cells of interest. Once the cell is infected, the retrovirus is stably inserted in the cell genome and its shRNA is continually transcribed theoretically leading to specific gene silencing. Adapted from [384, 423].

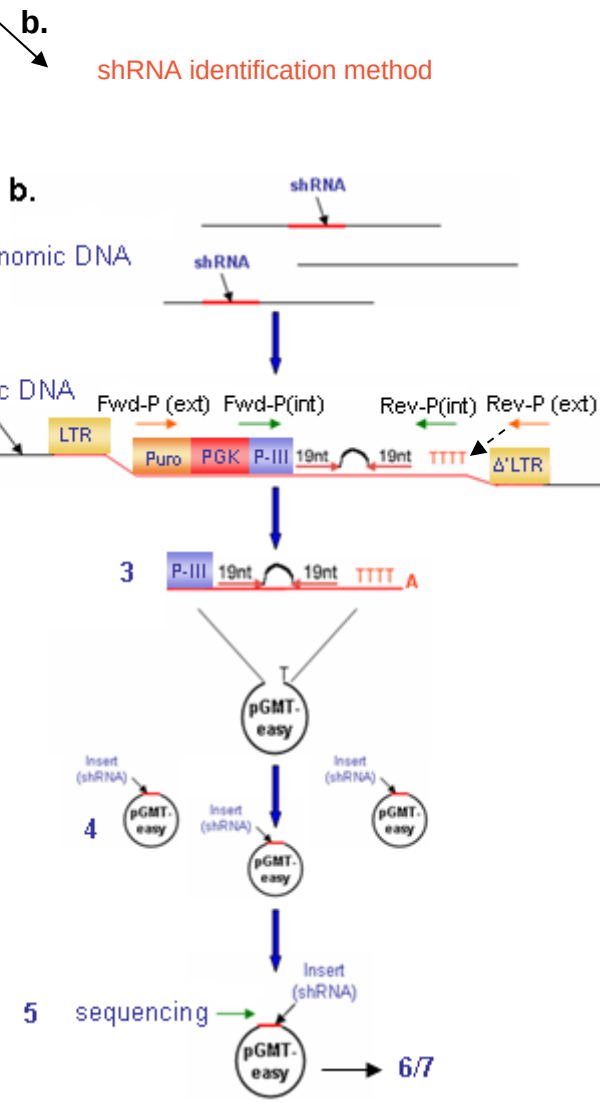
II.1.2 Genetic screening and identification of several new cellular senescence regulators

We decided to perform a loss of function genetic screen in primary human fibroblasts (WI38 cells). The goal was to identify critical regulators of "replicative senescence" that, when knockdown, would allow an extension of the cellular lifespan. This cellular model was adequate because a single genetic event (e.g. the inactivation of p53 or the ectopic expression of hTERT) is sufficient to extend WI38 cells lifespan [424]. Additionally, an identical genetic screen in the same cellular model (with a small part of the shRNA library targeting ~ 600 genes) had been successfully performed by David Bernard. From David's screen was identified the AMPK-related protein kinase 5 (ARK5 or NUAK1) and Topoisomerase (TOP1) and their role as new regulators of cellular senescence was characterised to an extend [425, 426]. As I was not the main investigator, the role of the two genes will not be introduced/discussed in the results section. However, the research articles can be found in the annexe section.

For the genetic screening I used 8 different pools of shRNAs each targeting 288 genes (a total of 2304 genes). Each pool was used to infect a 10cm Petri dish containing WI38 cells (at passage 18) before the "replicative senescence" plateau (Figure 25a). Post infection cells were amplified until control cells (infected with a control vector) entered in a growth arrest state (Figure 25a). In several pools of shRNA infected cells, we were able to observe emerging clones. Importantly, we did not detect emerging clones in control cells. The emerging clones from the various positive pools were amplified and divided in two dishes. One dish was used for a colony assay illustration (Figure 25a). The other dish was used for shRNAs identification (Figure 25b). Figure 25 illustrates the detailed genetic screen and the method used to identify the shRNAs.







1- Genomic DNA extraction

2- PCR amplification/recovery of shRNAs

3- Cloning into pGEMTeasy vector/bacteria plates spreading

4- Vector production containing single shRNA

5- shRNA sequencing

6- Nucleotide blast

7- Identification of the targeted gene

Figure 25: Genetic screening and the shRNA identification method.

(a) The genetic screen was performed in WI38 cells. Passage 14 (p14) WI38 cells were thawed and amplified to p18. p18 WI38 cells were infected with eight shRNA pools (G3/G12/F6/F9/F12/E6/E9/E12). Each pool contains approximately 862 shRNAs targeting a total of 288 genes. The emerging clones from each positive pool were split into two dishes. One dish was used for illustration purposes. The cells were stained using crystal violet coloration. As positive controls we used a shRNA targeting p53 or ectopically expressed the catalytic subunit of the telomerase (hTERT). As a negative control, we used the vector containing no shRNA. Four different pools yielded positive results (G3/F9/E12/E9). From these pools, the other half of the emerging clones was used for the shRNA identification. (b) The genomic DNA was extracted (for details see material and methods of the articles). We recovered and amplified shRNAs by sequential poly chain reactions (PCR). A first reaction was performed using external primers (amplifying parts of the vectors between LTR sequences) and a second nested PCR was used to recover the polymerase III promoter (P-III) and the shRNA. The PCR products were cloned into pGEMT-easy vectors and spread on bacteria plates. Single colonies were amplified and plasmidic DNA was extracted using minipreparation extraction protocol (see material and methods of articles). The different clones were sequenced and the shRNA sequences were blasted against human nucleotides. This enabled us to identify the mRNA of interest targeted by the shRNA. Personal illustration.

The genetic screen enabled us to identify 11 potential new cellular senescence regulators (Table 2). We only considered "hits" as potential new regulators when the shRNA sequences from sequencing process at least came up twice. We then re-designed new shRNAs (3 shRNAs per gene) and tested individually each shRNA for its potential to increase cellular lifespan in WI38 cells. This is a crucial step as shRNAs can trigger 'off target' responses [427], meaning that shRNAs can sometimes target other mRNAs than the ones they were designed to target or elicit non specific responses [427]. Additionally, we could not exclude that, during the genetic screen, more than one shRNA was expressed per cell. In the case where many shRNAs are present per cell, it is possible that the shRNA identified is in fact a "passenger" and not a "driver" of the phenotype observed. Finally, it is also possible that the simultaneous knockdown of two genes per cell cooperates to induce replicative potential extension. For all theses reasons, extensive follow up is required for every "hit" that comes out of the genetic screen. A list of genes identified during the genetic screen is detailed in Table 2. The gene name, the NCBI accession number, the description and the validation status is listed for every "hit". In the "validation" column of table 2, the sign (-) qualified eight genes that were not validated and that corresponds to genes for which (i) we were unable to observed a premature senescence when ectopically expressed and/or (ii) we could not find a second shRNA that induced the phenotype and/or (iii) we did not find a second shRNA that induced the down-regulation of the mRNA of interest and/or (iv) the retesting of the shRNA identified did not produce the phenotype expected and/or (v) research articles were published at the same time and it made the discovery less innovative (so we decided not to focus of these genes). Finally the last "hit" ubiquitin specific peptidase 3 (USP3) was an 'off target' as we were unable to find other shRNAs that down-regulated the gene of interest and reproduced the phenotype.

List of putative new regulators of 'replicative senescence' identifed from the genetic screen

Gene name	Acc No.	Description	Validation
PLA2R1	NM_007366.3	The phospholipase A2 receptor	**validated**
FBXO-32	NM_058229.2	F-box protein 32	-
FKBP-14	NM_017946.2	FK506 binding protein 14	-
PPP1R2	NM_006241.4	protein phosphatase 1 regulatory subunit 2	**validated**
MED12	NM_005120.1	mediator of RNA polymerase II transcription	-
USP3	NM_006537.2	ubiquitin specific peptidase 3	**off-target**
TGM1	NM_000359.1	transglutaminase 1	-
ADRBK2	NM_005160.2	adrenergic beta receptor kinase 2	-
HSPA4L	NM_014278.2	heat shock 70kDa protein 4-like	-
FGF19	NM_005117.2	fibroblast growth factor 19	-
HCLS	NM_000411.4	holocarboxylase synthetase	-

Table 2: List of putative new regulators of "replicative senescence" identified from the genetic screen.

These criteria validated two new regulators namely the phospholipase A2 receptor (PLA2R1) and the Inhibitor-2 (also known as PPP1R2 or Inh-2). The work on inhibitor-2 being too preliminary, I decided to focus on PLA2R1. In the next section, I will introduce the work published and in preparation that accomplished on the phospholipase A2 receptor (PLA2R1) and will briefly introduce our results published in *EMBO reports* in 2009. For more details, the reader should refer to the article that follows this brief introduction. The various discussions and perspectives that arose from this work will either be discussed in the introduction of the second work in preparation or in the discussion and perspectives section.

II.2 Article 1

The M-type receptor PLA2R regulates senescence through the p53 pathway

Resume

Cellular senescence is now considered as a major barrier against tumorigenesis [46]. Activated in response to various types of stresses (e.g. oncogenes, ROS, dysfunctional telomeres), it engages a response that ultimately leads to cell cycle arrest [150, 265, 380]. Involved at various stages of tumour development, it can be activated to restrain neoplastic expansion and also be re-activated in cancer cells lines leading to tumour regression [46, 55, 380]. Cellular senescence is regulated by bona fide tumour suppressor genes [380]. Many mechanisms underlining this biological response have recently emerged, however there is no doubt that new cellular senescence regulators are yet to be identified.

By performing a loss of function genetic screen in a model of "replicative senescence" in human primary fibroblast cells, we were able to identify the phospholipase A2 receptor (PLA2R1) as new critical senescence regulator. To date, little is known on the receptor, not only in a cancer context but also as a whole. Indeed, the only biological responses associated with PLA2R1 to date have not been very convincingly demonstrated [398, 399].

In the present study, we demonstrated that PLA2R1 is a critical cellular senescence regulator. We found that PLA2R1 was a regulator of both replicative and SIPS [424]. Both its over-expression and down-regulation have major impacts on cellular senescence. Trying to determine how PLA2R1 regulates cellular senescence, we found that modulating PLA2R1 expression has an impact on ROS levels. Next, we demonstrated that the PLA2R1-ROS axis activate the DNA damage/p53pathway to trigger cellular senescence. Finally, we highlighted that sPLA2-IIA can also induce cellular senescence in a PLA2R1 dependent manner. Altogether, we have identified a new regulator of the p53 pathway and cellular senescence.

EMBO reports

scientific report

The M-type receptor PLA2R regulates senescence through the p53 pathway

Arnaud Augert[1], Christine Payré[2], Yvan de Launoit[1], Jesus Gil[3], Gérard Lambeau[2] & David Bernard[1+]

[1]UMR 8161, Institut de Biologie de Lille, CNRS/Universités de Lille 1 et 2/Institut Pasteur de Lille, IFR 142, Lille, France,
[2]UMR 6097, Institut de Pharmacologie Moléculaire et Cellulaire, CNRS/Université de Nice-Sophia Antipolis, Valbonne, France, and
[3]Cell Proliferation Group, MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, London, UK

Senescence is a stable proliferative arrest induced by various stresses such as telomere erosion, oncogenic or oxidative stress. Compelling evidence suggests that it acts as a barrier against tumour development. Describing new mechanisms that favour an escape from senescence can thus reveal new insights into tumorigenesis. To identify new genes controlling the senescence programme, we performed a loss-of-function genetic screen in primary human fibroblasts. We report that knockdown of the M-type receptor PLA2R (phospholipase A2 receptor) prevents the onset of replicative senescence and diminishes stress-induced senescence. Interestingly, expression of PLA2R increases during replicative senescence, and its ectopic expression results in premature senescence. We show that PLA2R regulates senescence in a reactive oxygen species–DNA damage–p53-dependent manner. Taken together, our study identifies PLA2R as a potential new tumour suppressor gene crucial in the induction of cellular senescence through the activation of the p53 pathway.

Keywords: senescence; PLA2R; p53

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INTRODUCTION

Senescence is a permanent form of cell-cycle arrest that was first described in primary human fibroblasts (HDFs) that had reached their proliferative lifespan (Hayflick & Moorhead, 1961). It can also be induced by other stimuli such as oxidative or oncogenic stress (Serrano & Blasco, 2001). Senescing cells remain metabolically active and show characteristic changes in their gene expression and morphology (Campisi & d'Adda di Fagagna, 2007). Flattened and enlarged, they show positive senescence-associated β-galactosidase (SA-β-gal) activity (Dimri *et al*, 1995).

[1]UMR 8161, Institut de Biologie de Lille, CNRS/Universités de Lille 1 et 2/Institut Pasteur de Lille, IFR 142, 59021 Lille, France
[2]UMR 6097, Institut de Pharmacologie Moléculaire et Cellulaire, CNRS/Université de Nice-Sophia Antipolis, 06560 Valbonne, France
[3]Cell Proliferation Group, MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Campus, London W12 0NN, UK
[+]Corresponding author. Tel: +33 3 2087 1127; Fax: +33 3 2087 1111;
E-mail: david.bernard@ibl.fr

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Activated in the early stages of tumorigenesis, senescence has recently been described as a tumour suppression mechanism that prevents malignant transformation. This suggests that escape from senescence leads to a progression of malignancy (Braig *et al*, 2005; Chen *et al*, 2005). Understanding why a cell under different stresses enters a senescent state and what genetic events might impede this phenomenon therefore seems to be a necessary step towards understanding tumour development.

Various intracellular proteins are known to regulate cellular senescence mainly through the perturbation of the p53 and/or p16/Rb (Rb for retinoblastoma protein) pathways (Pearson *et al*, 2000; Gil *et al*, 2004; Sun *et al*, 2007). Recently, secreted factors such as insulin-like growth factor binding protein 7 (IGFBP7) and chemokines have been reported to be crucial regulators of senescence (Acosta *et al*, 2008; Kuilman *et al*, 2008; Wajapeyee *et al*, 2008), but so far few receptors have been identified as potential regulators of senescence. To our knowledge, only the chemokine receptors CXCR2 (chemokine (CXC) receptor 2) and IL6R (interleukin 6 receptor) are known to control senescence in primary human cells (Acosta *et al*, 2008; Kuilman *et al*, 2008). Here, we have identified another kind of receptor, the type I transmembrane glycoprotein receptor PLA2R (phospholipase A2 receptor), as a regulator of senescence. PLA2R is also known as the multifunctional M-type 180-kDa receptor, which belongs to the C-type lectin superfamily and specifically binds to several secreted phospholipase A2 (sPLA2) enzymes (Lambeau & Gelb, 2008).

RESULTS AND DISCUSSION

Downregulation of PLA2R bypasses senescence

We performed a loss-of-function genetic screen using the Netherlands Cancer Institute's retroviral short hairpin RNA (shRNA) library that targets approximately 8,000 human genes (Berns *et al*, 2004). The screen was designed to identify genes that, when downregulated, extend the lifespan of near senescent primary HDFs. Indeed, after a growth phase, HDFs, with a limited growth potential, enter a senescent state. In some cases, outgrowing colonies were observed among cells that had been exposed to library pools. The genomic DNA was isolated from these colonies and the inserted shRNA was sequenced. By using this strategy, an shRNA directed against the M-type receptor

PLA2R was identified, along with four other hits that are now under investigation. Positive controls such as shRb and shp53 were also identified. The shRNA identified during the screen was cloned into the pRS vector (shPLA2R) along with two other shRNAs targeting PLA2R messenger RNA (mRNA) at different regions (shPLA2R-6 and shPLA2R-9). In HDFs stably infected with shPLA2R, shPLA2R-6 or shPLA2-9, PLA2R mRNA levels were found to be knocked down by approximately 90% compared with control infected cells (Fig 1A). Then, we assessed cell growth to confirm the effect of the different shRNA-targeting PLA2R. Control, shPLA2R-, shPLA2R-6- or shPLA2R-9-infected cells were seeded at low densities and a colony formation assay was performed. Although control HDFs entered growth arrest, the shRNA-infected cells (shPLA2R, shPLA2R-6 and shPLA2R-9) continued to grow (Fig 1B).

To confirm the growth difference observed, we performed a growth curve analysis. Control and shPLA2R-infected HDFs were seeded at the same density, split and counted every week. Control cells proliferated at a much slower rate than the shPLA2R-infected cells (Fig 1C). Finally, to determine whether downregulation of PLA2R causes a bypass of senescence, we checked the SA-β-gal activity. The proportion of senescing cells was higher among control cells than among the different shPLA2R-infected cells, confirming that the shPLA2R-containing cells escaped senescence (Fig 1D).

Next, we wondered whether the effect of PLA2R down-regulation was cell dependent or could be reproduced in other primary human cells. Post-stasis primary human mammary epithelial cells (HMECs) and other primary HDFs (IMR90) were infected with the shPLA2R construct or with a control construct. Although control cells were unable to form colonies, both shPLA2R-expressing primary cells tested continued to proliferate (Fig 1E). Interestingly, all these cells (IMR90, WI38 and post-stasis HMEC) were immortalized by hTERT (human telomerase reverse transcriptase) expression showing that the depletion of PLA2R affected telomere-induced senescence (supplementary Fig 1 online; Acosta *et al*, 2008).

These results suggest that PLA2R knockdown has important effects on replicative senescence. Next, we investigated whether the depletion of PLA2R could favour bypass from stress-induced senescence. Various studies indicate that reactive oxygen species (ROS) participate in the induction of replicative senescence (Parrinello *et al*, 2003), as well as in the induction of oncogene-induced senescence (Lee *et al*, 1999), by eventually triggering a DNA damage response resulting in a premature senescence state (d'Adda di Fagagna, 2008). shPLA2R, shp53 (positive control) and young WI38 control cells were seeded at low density, subjected to H_2O_2 treatment and stained 2 weeks later. For this experiment, untreated young control cells were also used. Although untreated control and H_2O_2-treated shPLA2R or shp53 cells continued to proliferate, control H_2O_2-treated cells entered a growth arrest state (Fig 1F). In parallel, an SA-β-gal activity experiment indicated that shPLA2R cells escaped stress-induced senescence (Fig 1G).

Taken together, these results reveal that the downregulation of PLA2R both delays the onset of replicative senescence and diminishes stress-induced senescence in primary human cells.

Ectopic PLA2R expression induces premature senescence

In the light of these results suggesting that PLA2R might be important in controlling senescence, we sought to determine whether the endogenous PLA2R level increases during senescence. PLA2R transcript levels were measured in young proliferating (passage 22) HDFs and compared with old senescing (p29) HDFs. They were found to peak at p29 when most cells were senescing (Fig 2A).

To corroborate the function of PLA2R in senescence, we tested the effect of ectopic PLA2R expression on cellular senescence. A retroviral vector expressing PLA2R was generated and young HDFs were transduced with this vector. Ectopic overexpression of PLA2R was verified by reverse transcription–PCR (RT–PCR; Fig 2B). Then, we assessed the growth of control and PLA2R-overexpressing WI38 cells (Fig 2C,D). Cell growth blockade was observed in growth curve analysis and colony formation assay when PLA2R was overexpressed (Fig 2C,D), and was mainly due to senescence induction, as PLA2R-overexpressing cells showed a strong SA-β-gal activity (Fig 2E).

To examine whether some sPLA2 (Lambeau & Gelb, 2008) could be involved in the effect observed when manipulating PLA2R levels, we first assayed the expression levels of various human sPLA2 during senescence. The sPLA2 PLA2G2A mRNA expression was found to increase by more than 20-fold in senescent cells (Fig 2F). Interestingly, constitutive expression of PLA2G2A induced premature senescence (Fig 2G), an effect reminiscent of PLA2R overexpression. To confirm further that PLA2GA could act through PLA2R to induce senescence, we analysed the effect of PLA2G2A constitutive expression in PLA2R-depleted WI38 cells. Interestingly, the growth inhibition induced by PLA2G2A overexpression was reverted in PLA2R-depleted WI38 cells (Fig 2G). These results indicate that PLA2R mediates the effect of PLA2G2A. Murine PLA2G2A seems to be a ligand of murine PLA2R, but this does not hold for human PLA2G2A and human PLA2R (Cupillard *et al*, 1999). Hence, we propose that PLA2G2A regulates senescence through a pathway that remains to be elucidated and that might involve PLA2R, at least partly, through direct or indirect interplay.

Taken together, these results indicate that genetically modifying the expression of PLA2R has an important impact on the senescence of primary human cells. Next, we went on to investigate how PLA2R might regulate the outcome of senescence.

PLA2R activates ROS production to induce senescence

Senescence induced by the CXCR2 receptor is thought to possibly rely on the production of ROS (Acosta *et al*, 2008). Previous results also suggest that arachidonic acid can be produced in a PLA2R-dependent manner (Fonteh *et al*, 2000), and it has been shown that arachidonic acid leads to the production of ROS (Muralikrishna Adibhatla & Hatcher, 2006). Therefore, we investigated whether PLA2R could produce ROS and, if so, whether the production of ROS was necessary for the induction of senescence by PLA2R.

By using H_2DCFDA (2′,7′-dichlorodihydrofluorecein diacetate), a cell-permeant indicator for ROS, we examined whether PLA2R could induce the production of ROS. Young HDFs were infected with either an empty control vector or a PLA2R-encoding vector. After selection, cells were loaded with H_2DCFDA and the fluorescence was examined. PLA2R-overexpressing cells produced greater fluorescence than control cells (about five times more), indicating a higher concentration of intracellular ROS (Fig 3A). We also infected near senescing HDFs with an empty

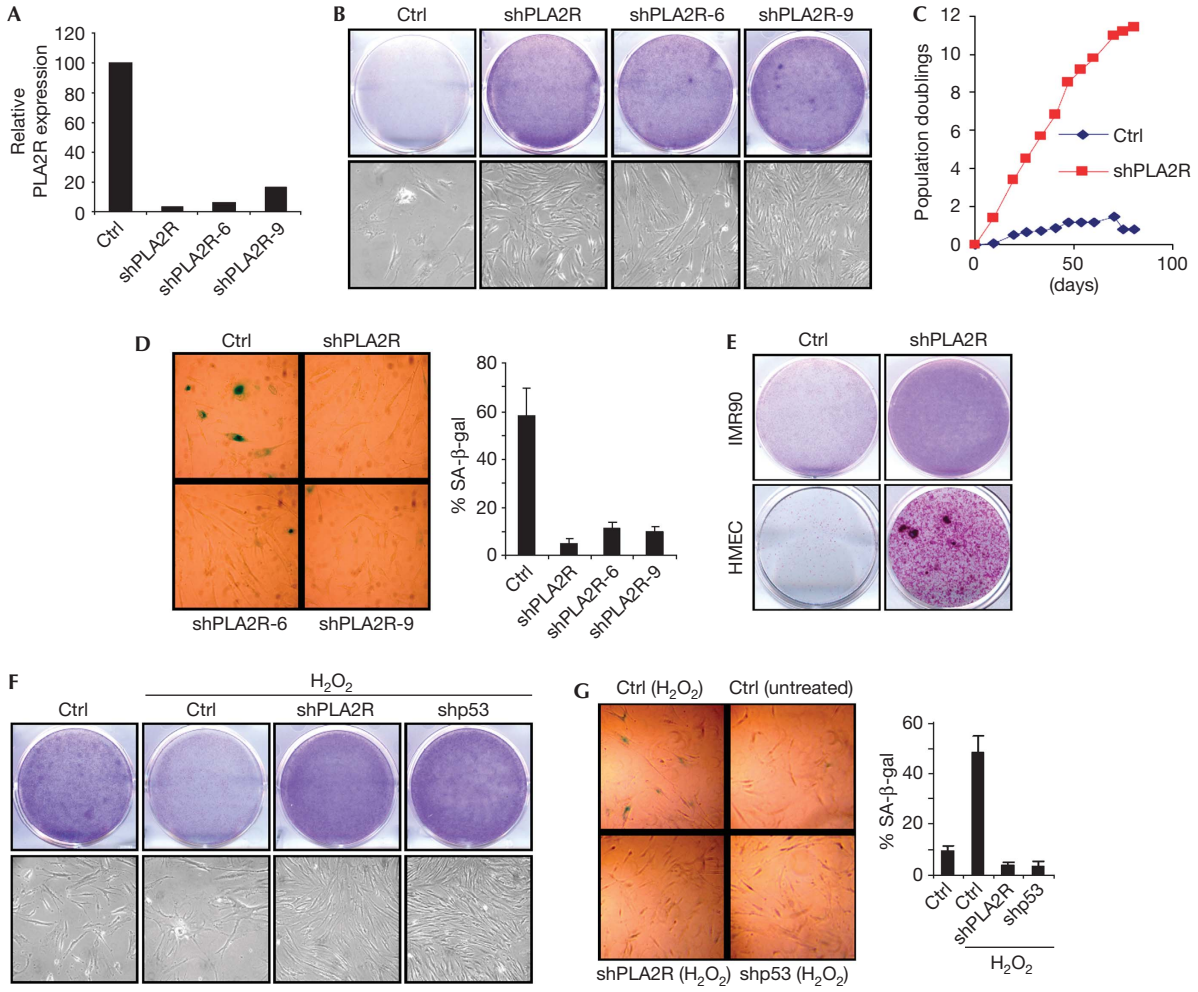


Fig 1 | The downregulation of PLA2R induces a bypass of replicative- and stress-induced senescence. (**A**) After infection of WI38 cells by control (Ctrl) or shPLA2R-encoding vectors and selection, RNAs were prepared. PLA2R mRNA levels were analysed in control cells and in various shPLA2R-infected cells by using QRT–PCR. (**B**) Colony formation assay. Control, shPLA2R-, shPLA2R-6- or shPLA2R-9-expressing WI38 cells were seeded at low densities. After 2 weeks, the cells were fixed and stained with crystal violet. (**C**) Growth curve analysis. WI38 cells were seeded at the same density, split every week and counted. The population doublings were calculated at each passage. (**D**) Control and shPLA2R WI38 cells were analysed for their SA-β-gal activity and the percentage of positive cells in each condition was calculated. (**E**) A colony formation assay of control versus shPLA2R-infected cells was performed in various primary human cells. The cells were seeded at low densities and stained 2 weeks later with crystal violet. (**F**) Control, shPLA2R or shp53 WI38 cells were plated at low densities, pulsed every 2 days with H_2O_2 (50 μM during 30 min) and stained 2 weeks later with crystal violet. (**G**) Control, shPLA2R or shp53 cells were treated with H_2O_2 as described above, until control cells entered a senescence-like morphology after which the cells were subjected to SA-β-gal analysis. For both experiments, untreated controls were also used in parallel. HMEC, human mammary epithelial cells; mRNA, messenger RNA; PLA2R, phospholipase A2 receptor; QRT–PCR, quantitative reverse transcription–PCR; SA-β-gal, senescence-associated β-galactosidase; sh, short hairpin.

control or an shPLA2R-encoding vector. When control cells started to enter senescence, the fluorescence of both populations was analysed. The shPLA2R-infected cells were found to contain ROS in lower amounts than senescing control cells (three times less), indicating that PLA2R has an impact on the intracellular levels of ROS (Fig 3A).

Next, we investigated the function of ROS production in the induction of senescence by PLA2R. Control and PLA2R-expressing cells were treated with or without the antioxidant *N*-acetyl-cysteine (NAC) just after infection (Catalano *et al*, 2005; Takahashi *et al*, 2006). We performed a colony formation assay to assess proliferation in the presence or absence of NAC. PLA2R-over-expressing cells stopped growing in the absence of the ROS scavenger, but not in its presence (Fig 3B). Accordingly, the senescence-like cell morphology—flattened and enlarged—observed in control PLA2R-overexpressing cells was suppressed by treatment with NAC (Fig 3B). Finally, the NAC treatment strongly decreased SA-β-gal labelling in PLA2R-overexpressing cells (Fig 3C). PLA2R thus seems to induce senescence through the production of ROS. Numerous studies have shown that ROS can induce various cellular stresses; they notably favour a DNA break, inducing a DNA damage response and cellular senescence

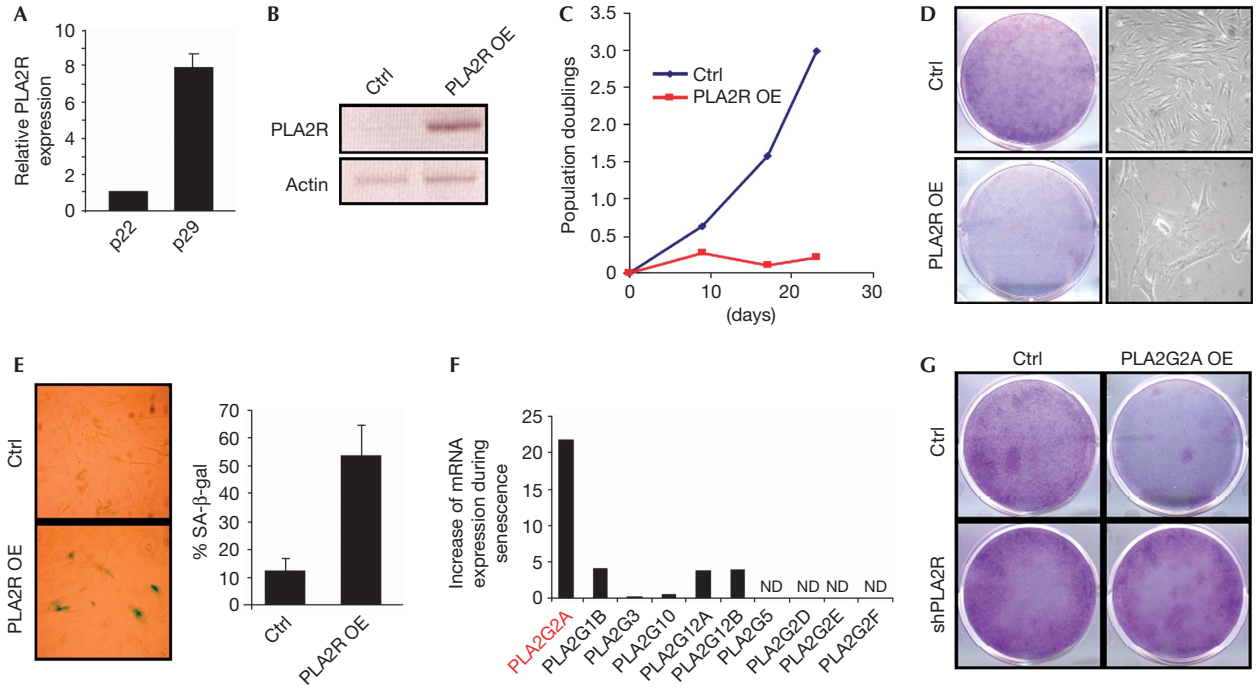


Fig 2 | The ectopic expression of PLA2R triggers premature senescence. (**A**) RNAs from WI38 HDFs at p22 (proliferating) and p29 (senescing) were prepared. After retro-transcription, Q PCR against PLA2R mRNA and GAPDH mRNA were performed. The relative levels of mRNA PLA2R are shown. (**B**) After infection, selection and RNA preparation, ectopic expression of PLA2R was verified by RT–PCR in WI38 cells. (**C**) Growth curve analysis of control versus PLA2R-overexpressing WI38 cells. (**D**) A colony formation assay was performed to illustrate the growth difference of control and PLA2R-overexpressing WI38 cells. (**E**) Control and PLA2R-overexpressing WI38 cells were seeded and SA-β-gal activity was analysed. The number of positive cells was counted and the percentage was calculated for both conditions. (**F**) RNAs were prepared as in (**A**) and the expression of the indicated sPLA2 was analysed at p22 and p29. The results are presented as a fold increase during senescence (ND stands for not detected). (**G**) WI38 cells were first infected with a control or an shPLA2R-encoding vector, and G418 was selected. The cells were subjected to a second round of infection with a control or a PLA2G2A-encoding vector and puromycin was selected. The cells were then seeded at low densities and stained 2 weeks later with crystal violet. Ctrl, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDF, human fibroblasts; mRNA, messenger RNA; OE, overexpression; PLA2R, phospholipase A2 receptor; QRT–PCR, quantitative reverse transcription–PCR; SA-β-gal, senescence-associated β-galactosidase.

(Lee *et al*, 1999; Macip *et al*, 2003). Therefore, we wondered whether PLA2R could activate a DNA damage response.

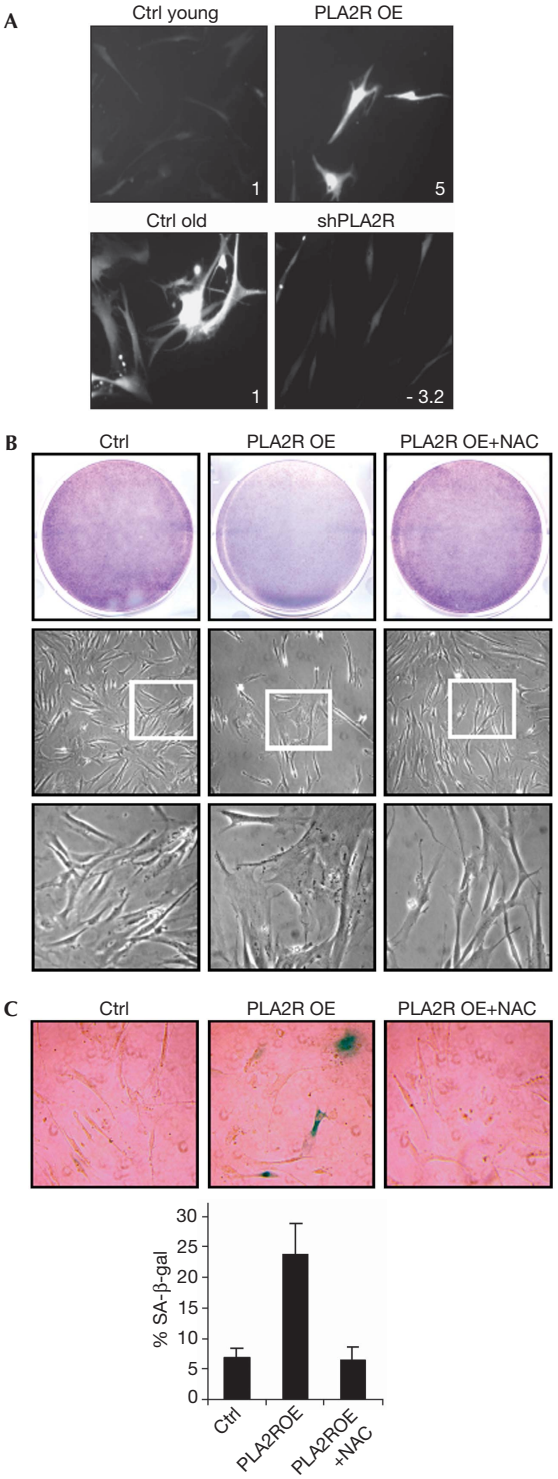
PLA2R regulates senescence in a p53-dependent manner

The presence of DNA strand breaks was assessed by immunofluorescence staining of the phosphorylated histone H2AX (γH2AX), a protein associated with damaged DNA (Rogakou *et al*, 1998). Control and shPLA2R-infected cells were cultured until the control cells entered senescence, after which both populations were immunolabelled. Control senescing cells showed more γH2AX-positive cells (29%) than the shPLA2R-infected cells (13.7%; Fig 4A). A similar experiment was carried out, but this time young HDFs were infected with a control or a PLA2R-expressing vector. About 32% of PLA2R-overexpressing cells showed γH2AX labelling against 14% in control cells, indicating that PLA2R triggers DNA damage (Fig 4B). Interestingly, the ROS scavenger treatment diminished the appearance of the γH2AX labelling in PLA2R-overexpressing cells (Fig 4B), showing the link between ROS production and the accumulation of DNA damage.

We next checked whether the changes observed in the DNA damage level had any impact on the p53 pathway activity. Interestingly, in shPLA2R-infected HDFs, p53, and its targets p21 and human double minute 2 (HDM2), decreased when compared with control cells. Phospho-Rb increased, suggesting that the cells were proliferating (Fig 4C). Conversely, when PLA2R was ectopically expressed, the levels of p53, p21 and HDM2 increased when compared with control senescing cells and phospho-Rb was found to decrease (Fig 4D).

Finally, to confirm functionally that PLA2R regulates senescence through the p53 pathway, we engineered HDFs to express E6 to inhibit p53 (Scheffner *et al*, 1990), E7 to inhibit Rb (Dyson *et al*, 1989), E6E7 to inhibit both. In those HDFs, we ectopically expressed PLA2R or a control green fluorescent protein (GFP). Although E7 did not prevent the growth arrest induced by the expression of PLA2R, E6 was able to efficiently do so according to the colony formation assay (Fig 4E). Nevertheless, the simultaneous inhibition of p53 and Rb reverted completely the growth arrest induced by PLA2R, suggesting that the Rb pathway contributed slightly to the PLA2R effect (Fig 4E). Taken together, these results reveal that PLA2R, through the production of ROS and mainly through the activation of the DNA damage–p53 pathway, regulates the senescence of primary human cells.

Despite the recent discovery of new genes controlling senescence (Acosta *et al*, 2008; Kuilman *et al*, 2008), further



◀ **Fig 3** | PLA2R induces senescence through the production of reactive oxygen species. (**A**) Young WI38 cells were infected with control (Ctrl) or PLA2R-expressing vectors. After 2 weeks, the cells were loaded with H_2DCFDA and the fluorescence was analysed by microscopy and flow cytometry. Pictures and relative mean fluorescence are shown (upper panel). WI38 cells approaching senescence were infected with a control vector or a shPLA2R construct, and when control cells entered senescence, the cells were loaded with H_2DCFDA and the fluorescence was analysed as above (lower panel). (**B**) After infection, 2.5 mM *N*-acetyl-cysteine (NAC) was added or not from day 1 after infection and renewed every 2 days. The cells were stained with crystal violet 2 weeks later or pictures were taken after 10 days. (**C**) WI38 cells were analysed for their SA-β-gal activity, and the percentage of positive cells in each condition was calculated. OE, overexpression; PLA2R, phospholipase A2 receptor; ROS, reactive oxygen species; SA-β-gal, senescence-associated β-galactosidase; sh, short hairpin.

work is still needed to understand in more depth the molecular mechanism underpinning this phenomenon. By performing a genetic screen using an shRNA library, we have identified PLA2R to be a crucial regulator of both replicative- and stress-induced senescence. The downregulation of PLA2R prevents the onset of senescence, whereas its overexpression triggers premature senescence. We found that PLA2R regulates cellular senescence through the production of ROS and the activation of the DNA damage pathway. Interestingly, ROS-induced senescence was overcome by the depletion of PLA2R, suggesting the existence of a feedback loop between them. Such a feedback loop has already been observed between ROS- and telomere-induced senescence (Richter & Proctor, 2007). PLA2R could thus be a crucial factor regulating replicative- (owing to short telomeres) and stress-induced senescence.

To our knowledge, except for the two recently identified chemokine receptors (Acosta *et al*, 2008; Kuilman *et al*, 2008), PLA2R is the only receptor that, when downregulated, allows normal human cells to bypass senescence. Our results indicate that PLA2R generates the production of ROS to affect senescence. Interestingly, the recently identified cytokine receptor CXCR2 is also potentially regulating senescence through ROS production (Acosta *et al*, 2008). CXCR2 is a G-coupled receptor, whereas PLA2R has not been formally described to bind to any signalling proteins. So whether or not the production of ROS results from a common or a different mechanism thus remains an open question. PLA2R could induce the release of arachidonic acid (Fonteh *et al*, 2000) and the activation of the MAPK pathway (Kinoshita *et al*, 1997; Silliman *et al*, 2002). These pathways have been described as activators of ROS production and senescence occurrence (Lee *et al*, 1999; Iwasa *et al*, 2003; Catalano *et al*, 2005). Our preliminary results, however, suggest that PLA2R, although having an impact on the cell cycle (see levels of p53, p21 and cyclin A), does not have a crucial impact on ERK and p38 kinases in our experimental settings (supplementary Fig 2 online). Alternatively, PLA2R could regulate senescence by controlling the production of cytokines, as PLA2R is able to regulate cytokines (Lambeau & Gelb, 2008) and cytokines are involved in senescence outcome (Acosta *et al*, 2008; Kuilman *et al*, 2008). Taken together, our data have identified PLA2R to be a new crucial regulator of senescence in human primary cells.

METHODS

Cell culture and retroviral infection. Normal human diploid fibroblasts WI38, IMR90 (American Type Culture Collection (ATCC), Manassas, VA, USA) and GP293 cells (Clontech, Mountain View, CA, USA) were cultured in DMEM (Invitrogen)

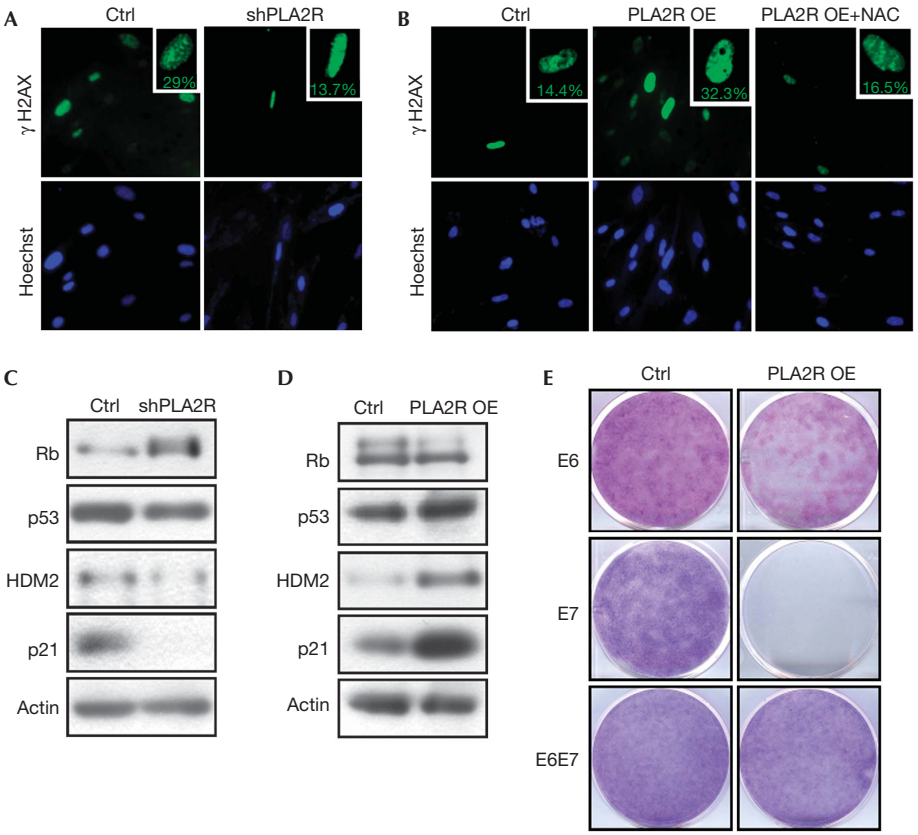


Fig 4 | PLA2R regulates senescence through the p53 pathway. (**A,B**) Old control (Ctrl) and shPLA2R-infected WI38 cells or young control and PLA2R-infected WI38 cells were subjected to γH2AX immunofluorescence, and the percentage of γH2AX-positive cells was determined. (**C**) WI38 cells were cultured until control cells entered senescence. Both control and shPLA2R-infected cells were lysed and western blot analysis was performed. (**D**) When the growth arrest induced by the overexpression of PLA2R was visualized, control and PLA2R-infected WI38 cells were lysed and western blot analysis was performed. (**E**) WI38 cells were first infected with E6- or E7-encoding vectors or both. Cells were then infected with control or PLA2R-encoding vectors. After selection, cells were seeded at low densities and the ability to form colonies was visualized by crystal violet staining. NAC, *N*-acetyl-cysteine; OE, overexpression; PLA2R, phospholipase A2 receptor; sh, short hairpin.

supplemented with 10% fetal bovine serum (Hyclone Perbio, Brackley, UK) in the presence of gentamicin at a final concentration of 80 μg/ml (Invitrogen, Carlsbad, CA, USA). HMECs (Clonetics, Basel, Switzerland) were cultured in mammary epithelial cell growth medium (Promocell, Heidelberg, Germany). Cells were maintained at 37 °C under a 5% CO_2 atmosphere. GP293 packaging cells were used as recommended by the manufacturer (Clontech).

Genetic screening. WI38 cells at p23 (cells are senescing at p30) were infected with the control or pools of the Netherlands Cancer Institute's shRNA library (Berns *et al*, 2004). Each pool is targeting 96 genes, each gene being targeted by three independent shRNAs. An shRNA pool is used to infect 500,000 cells. We set up the infection efficiency at 30% to have, in most cases, one retroviral particle per infected cell. Cells were selected and split every week (one into three) until proliferation stopped. The emerging clones (without clonal selection) were amplified and genomic DNA was purified. Cells were lysed in TNE buffer (Tris–HCl 10 mM pH 8.0, 100 mM NaCl, EDTA 10 mM pH 8.0) with 0.5% SDS and incubated at 37 °C for 2 h in the presence of 50 μg/ml of RNAse A (9707-B; Euromedex, Souffelweyersheim, France). Proteinase K at a concentration of 100 μg/ml (EU0090-B; Euromedex) was then added and the lysate was incubated overnight at 45 °C. A phenol–chloroform–isoamyl alcohol (25/24/1) extraction was performed, followed by an isopropanol precipitation. shRNA inserts were amplified by using nested PCR. The first pair of primers used to amplify the 600-bp sequence was: pRS out forward 5′-CCCTTGAACCTCCTCGTTCGACC-3′ and pRS out reverse 5′-GAGACGTGCTACTTCCATTTGTC-3′. An aliquot of 5 μl of the PCR product was used to perform a second PCR with the primers: pRS in forward 5-ACCTCCTCGTTCGACCC-3′ and pRS in reverse 5′-TGTGAGGGACAGGGGAG-3′. The PCR products were purified using the Jet Quick kit (GENOMED, Lohne, Germany) and cloned into the pGEM-T-easy vector (Promega, Madison, WI, USA). The pRS forward was used to sequence the shRNA insert (GenoScreen, Lille, France).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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SUPPLEMENTARY INFORMATIONS

Methods

Vectors. The shRNA sequence inserted into the pRetroSUPER vector to generate the pRS/PLA2R or pRSneoGFP/PLA2R is: 5'-CGCCGCTCAACTATCTGAA-3', the pRS/PLA2R-6 5'-CGACAAGCCGTTATGAAAG-3' and the pRS PLA2R-9 5'-AGCAGAGGTGGAGTTTCTT -3'. PLA2R cDNA from mouse origin was excised from pCi neo/PLA2R (SalI/ NotI) (Rouault *et al*, 2007) and inserted into the pLPCX (XhoI/ NotI) (Clontech) retroviral vector to generate pLPCX/PLA2R. PLA2G2A was excised from pRC CMVneoPLA2G2A (NotI/XhoI) and inserted into the pPRIPu (NotI/SalI) retroviral vector to generate pPRIPu/hPLA2G2A.

Colony formation assays and growth curves. Colony formation assays were carried out in 6-well plates with 30.000 cells plated per well. Ten to 15 days later, cells were washed with PBS, fixed with 4% PFA and stained with 0.1 % Crystal violet (Sigma-Aldrich). Growth curves were performed in 60 mm dish with 150.000 cells seeded per dish. Cells were counted every 7 days. Population doublings were calculated at each passage using the formula: $\log_2$ (number of cells at time of subculture/number of cells plated).

Immunoblotting and immunofluorescence microscopy. For immunoblot analysis, total cell extracts were separated by gel electrophoresis (SDS-PAGE) and proteins were transferred onto nitrocellulose membrane and detected using ECL (Amersham). The following primary antibodies were used: anti-p53 (DO-1, Santa Cruz Biotechnology), anti-HDM2 (sc-965, Santa Cruz Biotechnology), anti-p21 (sc-397, Santa Cruz Biotechnology), anti-βactin (sc-1616, Santa Cruz Biotechnology), anti-Rb (554136, BD Pharmingen) anti-phospho p38 MAPKinase

(9211, Cell Signalling) and anti-phospho p44/42 MAPKinase (9101, Cell Signalling). Anti-mouse (Amersham), anti-rabbit (Amersham) and anti-donkey (Santa Cruz) secondary antibodies conjugated to horseradish peroxidase were used. Immunofluorescence was performed as described in (Bernard et al., 2001) using the γH2AX Ab (05-636, Upstate).

PCR. Total RNA was isolated from 500.000 cells using TriPure reagent (MRC). For QRTPCR, first-strand cDNA was synthesized from 5 µg of total RNA using 50 U of MMLV reverse transcriptase (#M170A; Promega), 10 U of Rnasin (#M261B; Promega), and 500 ng of oligo dT primers in a final volume of 50 µl. Negative controls without added reverse transcriptase were performed and gave no significant PCR amplification. Quantitative real-time PCR (qPCR) assays were performed with optimized primers as described (Mounier *et al*, 2008). All reactions were performed in a total volume of 16 µl and contained 50 ng of reverse transcribed RNA (based on the initial RNA concentration) and 250 nM of each primer set. The abundance of PLA2R mRNA targets was calculated and compared to the expression of GAPDH mRNA which was used as a reference gene. The comparative Ct method (detailed in the ABI Prism 7700 Sequence Detection System User Bulletin no. 2) was used to determine the relative quantities of each mRNA. For RT-PCR, equal amounts were used to generate cDNAs using the M-MLV reverse transcriptase (Invitrogen) in the presence of random hexamers (fermentas), dNTPs and RNAse Inhibitor (Invitrogen). After reverse transcription, the cDNAs were used for PCR reactions with the following primers: β-actin Fwd 5' - TGACGGGGTCACCCACACTGTGCCCATCTA-3', β-actin Rev 5'- CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' and mPLA2R Fwd 5'- GCCGATACGAAGAAGACGAG -3', mPLA2R Rev 5'- CTCCAGGGTTCCACAGTGAT - 3'.

Senescence analysis. Senescence associated β-galactosidase activity was determined as previously described in (Dimri *et al*, 1995).

Reactive oxygen species analysis. A cell-permeant indicator for reactive oxygen species (H_2DCFDA, Merck) was used to determine the intracellular concentration of ROS. Cells were loaded with 5μg/mL of H_2DCFDA for 5 minutes, washed twice in PBS, and the fluorescence was immediately analyzed by flow cytometry and microscopy.

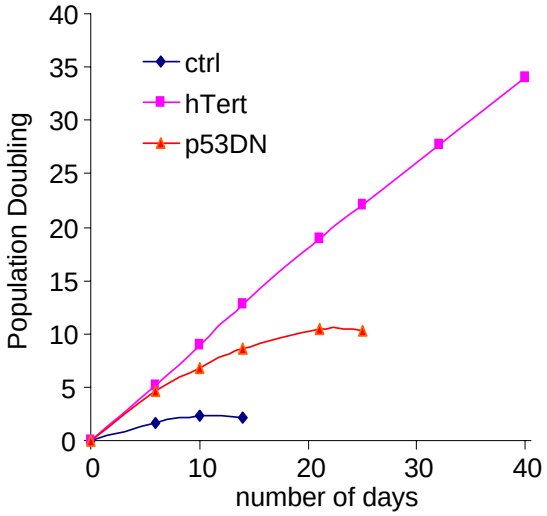
Figure Legends

Fig 1 | hTert expression immortalised WI38 or HMEC cells. (**A**) After infection and selection of HMEC, growth curves were performed and population doublings calculated after each passage. (**B**) After infection and selection of WI38 cells, a colony assay experiment was performed when control-infected cells were approaching senescence.

Fig 2 | Regulation of the MAPK pathway by PLA2R. Old control- and shPLA2R-infected WI38 cells or young control- and PLA2R-infected WI38 cells were lysed and western blot analysis was performed using the indicated antibodies.

Sup Fig. 1

A



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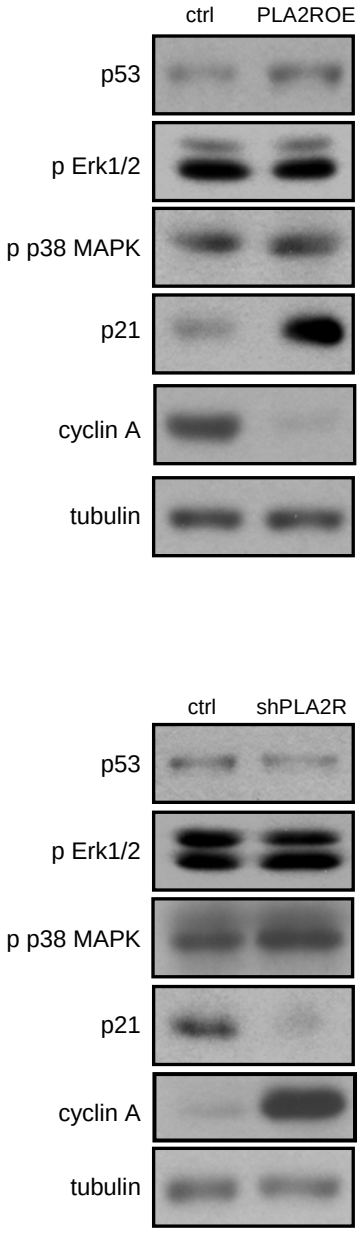
Sup Fig. 2



Many questions arose from these results. We had clearly demonstrated a role of PLA2R1 in regulating cellular senescence. As cellular senescence is a tumour suppressor mechanism and PLA2R1 seems to be a critical regulator of this biological process, we hypothesised that PLA2R1 could exert tumour suppressive actions. It was not an evidence, as cellular senescence regulators can be tumour suppressors in a senescence context and exert oncogenic properties in other contexts [428]. Additionally, ROS are mutagenic and can promote cancer development [429]. We thus setup a few experiments in order to determine whether our hypothesis could be experimentally validated. To this end, we checked PLA2R1 expression in various normal and cancer cells lines and tissues. We also asked what would be the effect of PLA2R1 knockdown on different steps of tumour development (e.g. OIS escape, transformation and tumorigenic potential). Furthermore, we checked what would be the effect of PLA2R1 ectopic expression in cancer cells lines. Finally, alternative questions remained to be answered. Among others, what is the implication of PLA2R1's ligands (sPLA2s) in PLA2R1 mediated effects? What PLA2R1 domains mediate its effects? What pathway/s does PLA2R1 regulate to result in ROS accumulation? How is PLA2R1 regulated? We were hoping that answering some of these questions would shed light on the physiological role of PLA2R1. Figure 26 schematises the various questions we tried to answer.

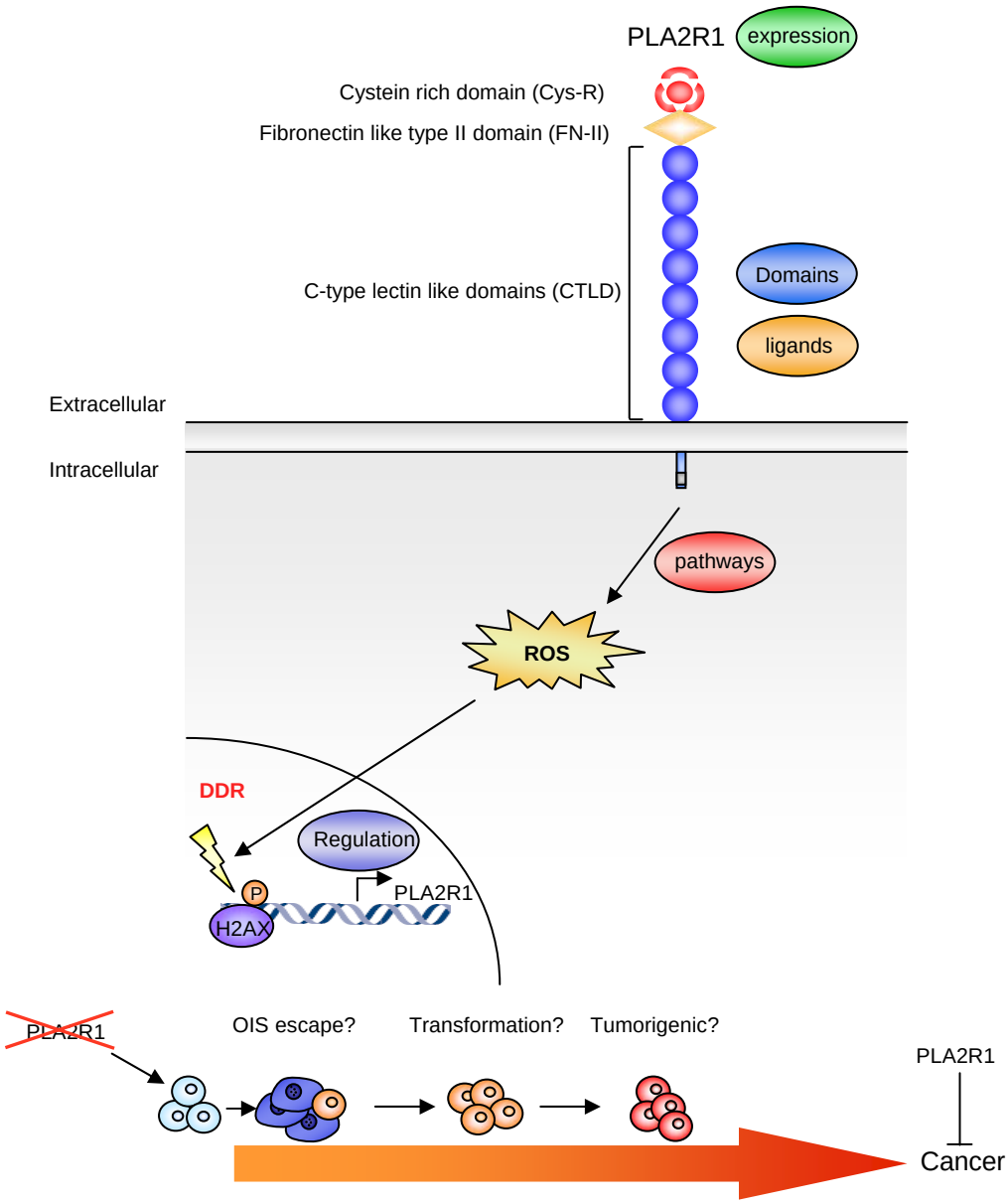


Figure 26: A list of biological questions concerning PLA2R1 physiological role that we were hoping to answer.

I will now move on with the follow up of the first published article, a work that I'm currently finishing in David's laboratory. The article is under preparation for publication. In this work, we tried to answer whether PLA2R1 could have tumour suppressive activities and therefore tackled the questions mentioned earlier.

II.3 Article 2

The mannose family member PLA2R1 displays sPLA2 independent tumour suppressive properties by modulating the mitochondrial redox state

Abstract

The mannose family member PLA2R1 has recently been identified as a critical regulator of "replicative senescence" [424] and a target in idiopathic membranous nephropathy, a renal autoimmune human kidney disease [430-432]. Despite these observations, we have to date very little information concerning the physiological role of this receptor. We hypothesised from our previous work that PLA2R1 could exert tumour suppressive actions. In the present work, we demonstrate that PLA2R1 suppresses tumour progression. PLA2R1 expression is down-regulated in a subset of cancer cell lines and cancer patient samples. This repression is associated with PLA2R1 promoter hypermethylation. In normal cells, PLA2R1 depletion favours an escape from failsafe mechanism and an increased transforming and tumorigenic potential in xenografted mice. In contrast, PLA2R1 expression leads to apoptotic cell death in a myriad of cancer cell lines whereas it preferentially triggers cell cycle arrest and cellular senescence in primary cells. By performing a targeted chemical screen, we uncover a pivotal role of the mitochondria as an effector of PLA2R1 ROS induced apoptosis. Unexpectedly,

PLA2R1 tumour suppressive actions are sPLA2 independent, its only ligands described to date. Taken together our results demonstrate an unpredicted role of PLA2R1 as a sPLA2 independent tumour suppressor candidate with putative therapeutic potential.

Introduction

Cancer is a multistep process leading to the acquisition of several capabilities that govern human tumours [22]. During this process oncogenes and the loss of tumour suppressor genes impact normal cell homeostasis resulting in aberrant proliferation, limitless proliferation, resistance to failsafe mechanism, angiogenesis, metastasis and invasion [433]. Among the failsafe mechanism, cellular senescence represents a crucial barrier against tumorigenesis development. This cellular process can act as a tumour suppressive mechanism during several steps of tumour development. A barrier against immortalisation [181, 182], it also prevents malignant conversion of neoplastic lesions [55, 380] and can be part of the anti-tumoral response generated by clinical drugs [45, 46]. Apoptosis, the other major failsafe mechanism is a genetically controlled program cell death that is often deregulated during cancer development [434]. Identifying new candidate tumor suppressor genes that control failsafe mechanism such as cellular senescence and apoptosis is critical for various reasons. It might reveal new alterations that disrupt normal cell homeostasis favouring tumour development. Additionally, it could lead to the discovery of novel biomarkers and putative anticancer agents. To this end, we have performed a functional loss-of-function genetic screening to identify genes that when down-regulated lead to cellular senescence bypass. Using this strategy, we and others have already isolated and characterized several genes controlling this process [124, 424-426]. In particular, we have described for the first time a putative function of the phospholipase A2 receptor (PLA2R1) in controlling the senescence response in a reactive oxygen species (ROS)

dependent mechanism. Although cellular senescence is a tumour suppressive mechanism, some of its regulators turn out to be context dependent tumour suppressors [428]. Additionally, ROS are mutagenic and can promote cancer development [429, 435, 436]. In contrast, recent evidences suggest that high levels of ROS can preferentially trigger cell death in cells with a cancer genotype that have acquired increased ROS output owing to oncogenic stimulation and very active metabolism [231, 437-440]. Additionally, recent evidences also suggest that oncogenes could activate an antioxidant network to escape from failsafe mechanism [210]. We therefore analysed the role of PLA2R1 during tumour development. PLA2R1 is a membrane receptor of approximately 180-200 kDa made of a large extracellular domain composed of a cysteine rich domain (Cys-R), a fibronectin like domain II (FN-II) and eight c-type lectin like carbohydrate recognition domains (CTLD). It contains a single pass transmembrane domain and a short intracellular cytoplasmic tail [400]. To date, the only PLA2R1 ligands described are the sPLA2. PLA2R1 *in vivo* biological functions and its associated signaling pathways still remain largely unknown [399]. Here we demonstrate that PLA2R1 exerts tumour suppressive functions in a mitochondrial-ROS dependent manner. Additionally we highlight that PLA2R1 acts in a sPLA2 independent manner. Altogether our results identify an unexpected role of PLA2R1 with putative therapeutic potential.

Results

PLA2R1 expression decreases in kidney and breast tumours

In order to determine whether PLA2R1 could be a putative tumour suppressor gene, we first interrogated the public Oncomine database (www.oncomine.org). PLA2R1 expression was analysed by microarrays in 239 unique cases comparing cancer *vs* normal samples. We found that PLA2R1 mRNA decreased in breast and kidney cancers in a myriad of independent studies suggesting that PLA2R1 down-regulation might be a common event in these cancers (Figure 1a/b).

To corroborate this bio-informatics analysis, we determined PLA2R1 mRNA levels by quantitative RT-PCR (qRT-PCR) in cancer versus normal tissues. We analysed PLA2R1 expression in 18 patients' kidney tissues from which we had access to both normal and cancer samples. Strikingly, PLA2R1 mRNA levels strongly decreased in kidney cancer samples versus their normal counterparts (Figure 1c). PLA2R1 expression was also analysed in breast and kidney cell lines. Compared to normal and hTERT immortalised HMEC, 13 out of 16 breast cancer cell lines examined displayed a significant decrease of PLA2R1 mRNA with 6 of them showing no or almost no detectable expression (Figure 1d). Additionally, PLA2R1 down-regulation was also observed in a subset of kidney cell lines (Figure S1a).

Promoter hypermethylation plays a major role in cancer progression through the transcriptional silencing of bona fide tumour suppressor genes [441-443]. Using the CpG island predictor software (CpG ProD), a potential CpG-rich island of ~ 1500bp was found in the promoter and first intron region of PLA2R1. Interestingly, whereas HMEC and immortalized HMEC did not display any methylation, breast cancer cells showed promoter

hypermethylation (Figure 1e). In accordance, treatment of these cells with 5'-aza-2' deoxycytidine (an inhibitor of the DNA methyltransferases) restored PLA2R1 expression whereas it had no effect on HMEC and immortalized HMEC (Figure 1f). Collectively, these results suggest that PLA2R1 is found down-regulated in tumour tissue samples and its down-regulation is associated with promoter hypermethylation.

a

PLA2R1 expression in breast tumours vs normal

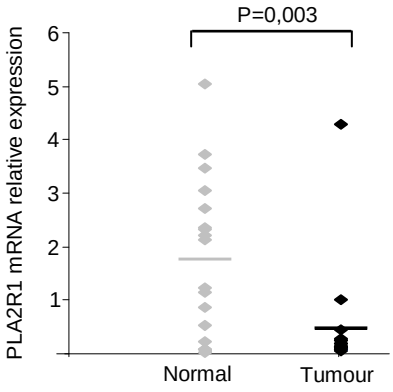
Study	Cancer Tissues vs Normal	Fold changes	P value	Gene Rank
Zhao Breast	Invasive Ductal Breast Carcinoma	-2,52	1.30E-15	in top 1%
Richardson Breast 2	Ductal Breast Carcinoma	-3.684	2.13E-8	in top 3%
Sorlie Breast	Lobular Breast Carcinoma	-1.757	0.036	in top 7%
	Ductal Breast Carcinoma	-2.484	0.009	in top 10%
Sorlie Breast 2	Lobular Breast Carcinoma	-1.789	0.026	in top 9%
	Ductal Breast Carcinoma	-2.498	0.008	in top 11%
Perou Breast	Ductal Breast Carcinoma	-2.411	0.043	in top 15%
Turashvili Breast	Invasive Ductal Breast Carcinoma	-2.701	0.019	in top 6%

b

PLA2R1 expression in kidney tumours vs normal

Study	Cancer Tissues vs Normal	Fold changes	P value	Gene Rank
Yusenko Renal	Chromophobe Renal Cell Carcinoma	-76.573	4.14E-5	in top 1%
	Papillary Renal Cell Carcinoma	-2.111	2.94E-8	in top 1%
	Clear Cell Renal Cell Carcinoma	-13.649	2.46E-8	in top 1%
Higgins Renal	Granular Renal Cell Carcinoma	-2.714	0.031	in top 6%
	Chromophobe Renal Cell	-3.081	0.031	in top 9%
	Papillary Renal Cell Carcinoma	-3.202	0.028	in top 13%
	Clear Cell Renal Cell Carcinoma	-2.612	0.042	in top 19%
Cutcliffe Renal	Renal Wilms Tumor I	-4.639	4.11E-4	in top 4%
	Clear Cell Sarcoma of the Kidney	-6.910	1.16E-4	in top 4%
Lenburg Renal	Clear Cell Renal Cell Carcinoma	-2.032	1.83E-4	in top 4%
Beroukhim Renal	NH Clear Cell Renal Cell Carcinoma	-1.540	4.38E-4	in top 13%
	H Clear Cell Renal Cell Carcinoma	-1.576	2.96E-4	in top 20%
Gumz Renal	Clear Cell Renal Cell Carcinoma	-3.745	0.006	in top 19%

c



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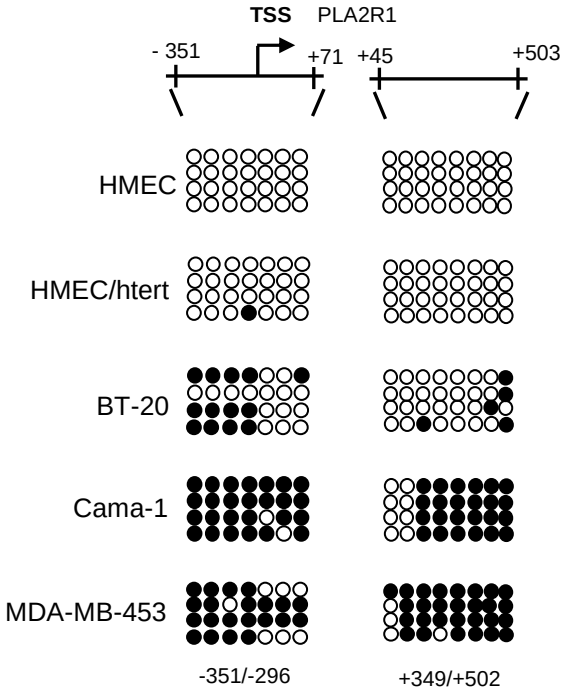

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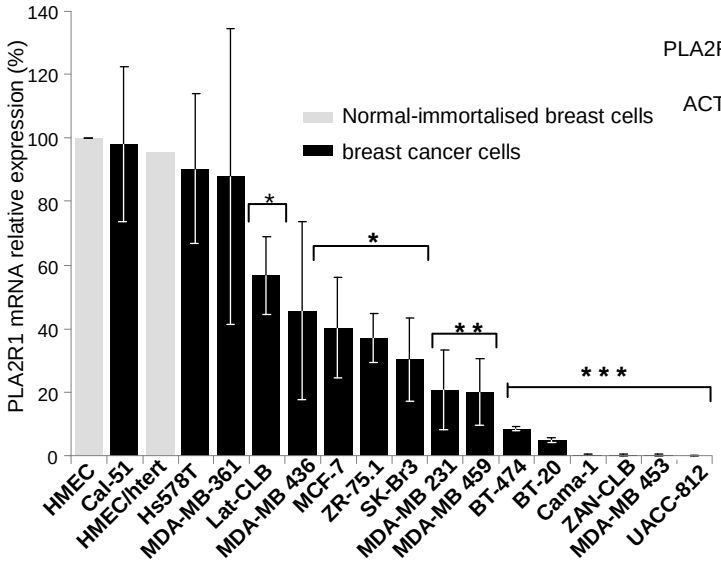

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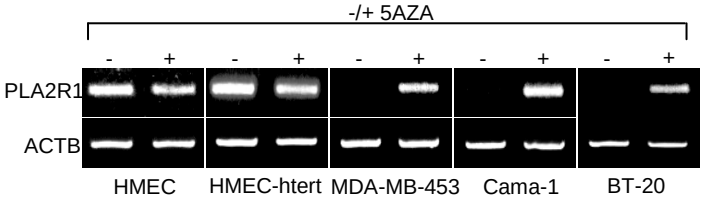


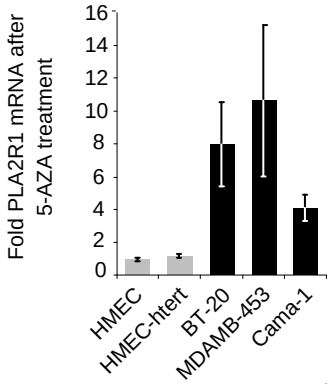


Fig.1

Figure 1: PLA2R1 expression decreases in kidney and breast tumours.

(a) PLA2R1 expression in kidney cancer tissues versus normal tissues. Expression profiles were obtained from the Oncomine database. Six out of eight studies displayed significant ($p<0.05$) down-regulation of PLA2R1 in breast tumours.(b) PLA2R1 expression in kidney cancer tissues versus normal tissues. Expression profiles were obtained from the Oncomine database. Six out of six studies displayed significant ($p<0.05$) down-regulation of PLA2R1 in kidney tumours.(c) RNAs of both normal and cancer tissue from the same patients were extracted and PLA2R1 expression was analysed by qRT-PCR (n=36). p value=0.003.(d) qRT-PCR analysis of PLA2R1 expression in 16 breast cancer cell lines versus normal and immortalised HMEC. (* P<0,05; ** P<0,01; *** P<0,001).(e) Bisulfite sequence analysis of the PLA2R1 promoter. Each row represents a single clone (n=4). Each circle represents a CpG dinucleotide. Black (filled) circles correspond to methylated CpG sites. Open (white) circles are unmethylated CpG sites. The two zones studied are (-351;+71) and (+45;+503) in respect to the transcription starting site (TSS). (f) qRT-PCR analysis following treatment with the DNA methyltransferase inhibitor 5-AZA (5µM; 72hrs).

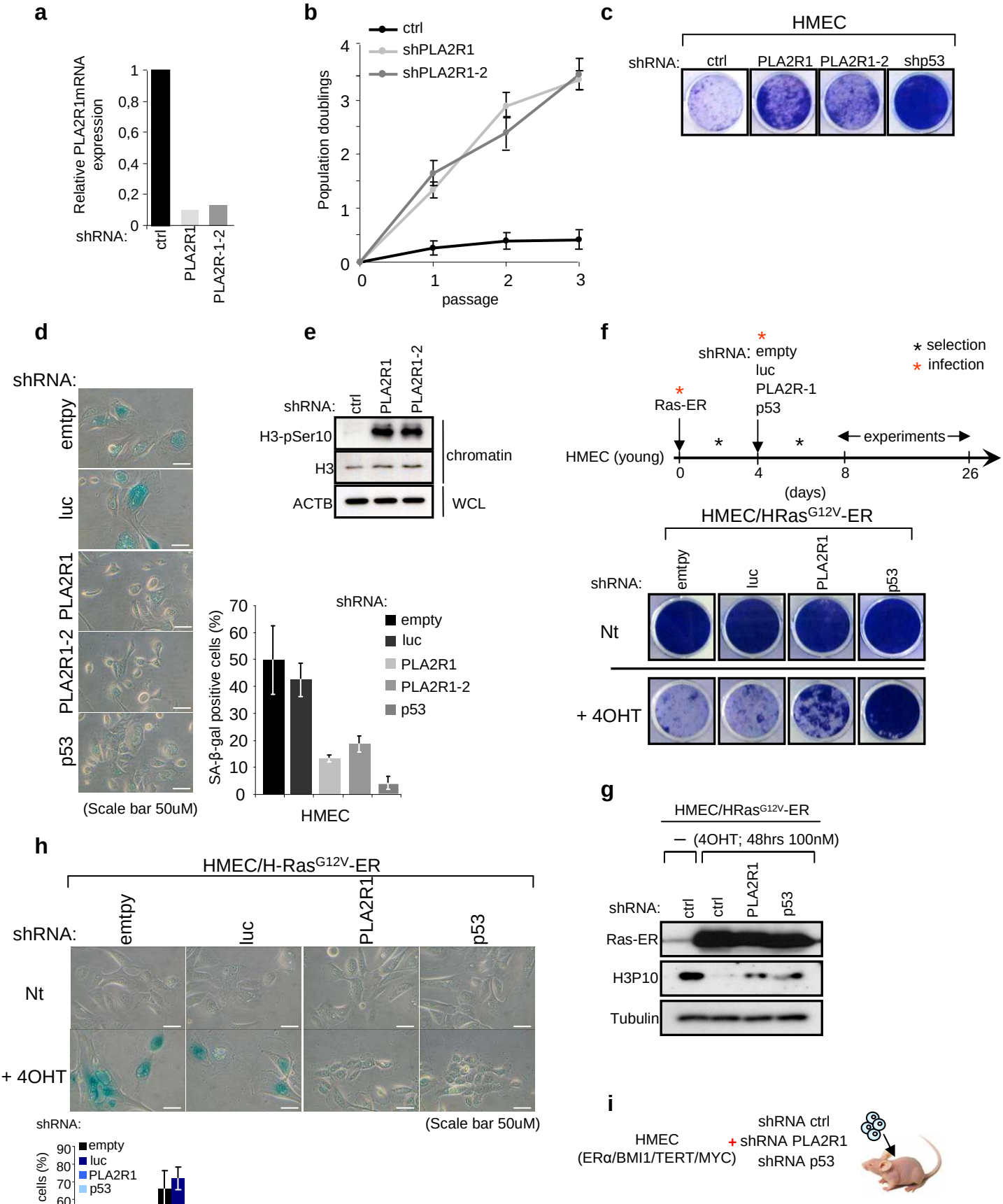
PLA2R1 depletion favours failsafe programs escape and tumorigenesis

As PLA2R1 decreased expression might be associated with tumor development we next examined whether PLA2R1 depletion could favour an escape from early stages failsafe programs. Failsafe programs, such as senescence, are activated in normal cells in response to aberrant signals and lead to cell growth blockage to avoid the potential appearance of abnormally proliferating cells [47]. Our previous results have highlighted a role of PLA2R1 in replicative senescence of normal human fibroblasts [424]. We examined its putative role as a cellular senescence regulator of normal human epithelial cells. HMEC were infected with PLA2R1 shRNA encoding vectors, and efficiency of the knock-down was checked by qRT-PCR (Figure 2a). PLA2R1 shRNA encoding vectors were also validated by co-transfection experiments in 293GP cells (Figure S2a) and at the endogenous level in HMEC cells (Figure S2b). As expected, the knock-down of PLA2R1 extends HMEC lifespan as judged by the growth curve analysis and the colony assay with p53 depletion used as a positive control

(Figure 2b-c). PLA2R1 depleted cells are less SA-βgal positive (Figure 2d) and express the mitotic marker (H3-pSer10) whereas control cells do not (Figure 2e).

Next, we examined what could be the effect of PLA2R1 depletion on oncogene induced senescence (OIS), a key step in the malignant conversion of benign lesions [55, 265]. Young HMEC cells were engineered to express the inducible form (ER coupled) of a constitutively active form of Ras (H-Ras^{G12V}). HMEC/Ras^{G12V}-ER cells were infected with control shRNAs or shRNA targeting PLA2R1 or p53. As expected, oncogenic stress induced by Ras activation blocked cell growth (Figure 2f), led to a decrease of the (H3-pSer10) mitotic marker (Figure 2g) and an increase SA-βgal activity (Figure 2h). By contrast, emerging clones were observed in PLA2R1 or p53 knock-down cells (Figure 2f). Additionally, the cells sustained (H3-pSer10) expression (Figure 2g) and displayed a decreased SA-βgal activity (Figure 2h). Interestingly, PLA2R1 depletion also favoured an escape from OIS induced with Mek or Her-2 oncogenes suggesting that this effect was not limited to oncogenic Ras (Figure S2c-f). We investigated how PLA2R1 depletion could favour this escape. We had previously demonstrated that PLA2R1 knock-down resulted in decreased ROS levels in normal fibroblast primary cells [424]. Moreover, several publications have functionally linked ROS to the regulation of both replicative and oncogene induced senescence [30]. Interestingly, OIS in HMEC was associated with ROS accumulation that was partially reverted in PLA2R depleted cells (Figure S3a-d). Altogether, it suggests that PLA2R1 depletion can desensitize normal human epithelial cells to replicative or oncogenic stresses through ROS mediated mechanism and may thus be a step required to favour failsafe mechanism bypass.

We next analysed whether PLA2R1 depletion could favour tumorigenesis. To test our hypothesis, we focused our interest on rather high PLA2R1 expressing cancer cells according to the qRT-PCR analysis (Figure 1d, S1a). We first generated control and shPLA2R1 expressing MDA-MB-436, Hs578T and ACHN cells and the subsequent decrease in PLA2R1 mRNA was confirmed by qRT-PCR in these cancer cell lines (Figure S4 a-d). PLA2R1 knock-down resulted in increased transforming potential as judged by the number of colonies and/or the colonies size (Figure S4a-d). Finally, the tumorigenic potential following PLA2R1 knock-down was assessed in HMEC $^{(ER\alpha\text{-}Bmi1\text{-}hTERT\text{-}Myc)}$ cells which have been specifically designed as an oestrogen breast cancer dependent model. These cells form tumours when injected in immunodeficient mice [444]. We used this model to assess whether the depletion of PLA2R1 could increase the tumorigenic potential of these cells. We decided to assess tumour growth and sacrifice mice as soon as tumours from a condition were visible. Interestingly, PLA2R1 depleted HMEC $^{(ER\alpha\text{-}Bmi1\text{-}hTERT\text{-}Myc)}$ cells formed tumours with an incidence of 5/5 in xenografted mice. The same results where obtained with p53 depleted cells (Figure 2i). However in control HMEC $^{(ER\alpha\text{-}Bmi1\text{-}hTERT\text{-}Myc)}$ cells, no tumours were observed when mice were sacrificed (Figure 2i). Taken together, our results highlight that PLA2R1 depletion favours an escape from failsafe mechanisms and leads to increased transforming and tumorigenic capabilities.



condition	Tumour incidence
HMEC/ ERα/BMI1/TERT/MYC + shRNA control	0/5
HMEC/ ERα/BMI1/TERT/MYC + shRNA PLA2R	5/5
HMEC/ ERα/BMI1/TERT/MYC + shRNA p53	5/5

Fig.2

Figure 2: PLA2R1 depletion favours failsafe programs escape and tumorigenesis.

(a) qRT-PCR analysis of control or PLA2R1 depleted HMEC (shRNA PLA2R1 or PLA2R1-2).(b) Growth curve analysis of control and PLA2R1 depleted HMEC (shRNA PLA2R1 or shRNA PLA2R1-2). (c) Colony assay formation of control, PLA2R1 and p53 depleted HMEC. p53 depleted cells were used as positive controls. (d) SA-βgal analysis of control (empty and luciferase shRNAs) versus PLA2R1 and p53 HMEC. p53 depleted cells were used as positive controls. Scale bar represents 50μM.(e) Western blot analysis of the mitotic marker (H3-pSer10) in control or PLA2R1 depleted cells.(f) HMEC/H-RAS^{G12V}ER cells were infected with control (empty or luciferase), PLA2R1 or p53 specific shRNAs. The cells were then counted and plated for the different experiments. Treatment with 4 hydroxytamoxifen (4OHT) was performed at 100nM for 48 hrs. Colony formation assay was assessed 15 days later using crystal violet. A shRNA targeting p53 was used as a positive control.(g) Western blot analysis of cells described in (f). Cells were assessed 10 days post 4OHT treatment.(h) SA-βgal analysis of the cells described in (f). Cells were assessed 15 days after 4OHT treatment. Scale bar represents 50μM.(i) HMEC $^{(ERα\text{-}Bmi1\text{-}hTERT\text{-}Myc)}$ cells infected with a shRNA control, a shRNA PLA2R1 or a shRNA p53 were injected subcutaneously into the flanks of nude mice. The shRNAs contained a GFP cassette allowing bioluminescence imaging of the tumours. The shRNA targeting p53 was used as a positive control.

PLA2R1 ectopic expression specifically triggers apoptotic cell death in cancer cells while triggering cell cycle arrest and senescence in primary cells

A down-regulation of PLA2R1 results in senescence escape and favours transforming and tumorigenic potential. Accordingly, its expression is repressed in cancer cells and cancer patient tissues. We next investigated the effect of re-introducing PLA2R1 in non-expressing cancer cells such as BT-20, Cama-1 and MDAMB-453 (Figure 1d). PLA2R1 ectopic expression completely blocked cancer cell growth according to growth curves and colony assays analyses (Figure 3a-d). PLA2R1 expression altered cancer cells membrane integrity, as measured by robust increase in trypan blue positive cells suggesting the induction of strong cell death (Figure 3e). PLA2R1 dying cancer cells displayed a strong increase in activated caspase 3 and in the cleavage of PARP-1, one of its targets (Figure 3f). Additionally, PLA2R1 expressing cells were TUNEL positive (Figure 3g) highly validating the apoptotic traits

previously described and demonstrating that PLA2R1 was preferentially inducing apoptotic cell death.

Most importantly, whereas the constitutive PLA2R1 expression induced apoptotic cell death in cancer cells its expression in normal primary cells (WI-38 and HMEC) did not result in apoptosis (Figure 3h) indicating that PLA2R1 selectively killed cells with a cancer genotype. This was not due to PLA2R1 expression levels as normal cells expressed similar or higher PLA2R1 amount (Figure 3h). In contrast PLA2R1 induced cell cycle arrest with cellular senescence features in normal cells. Indeed, HMEC ectopically expressing PLA2R1 entered a growth arrest (Figure 3i-j) and were SA-βgal positive (Figure 3k). PLA2R1 ectopic expression in WI38 cells also led to cell cycle arrest (Figure 3i) with senescence features (Figure S5b-c) and no apparent sign of apoptosis (Figure S5a). Taken together, our results demonstrate that PLA2R1 is a strong inducer of apoptotic cell death in cancer cells whereas it preferentially induces cell cycle arrest and cellular senescence in primary cells.

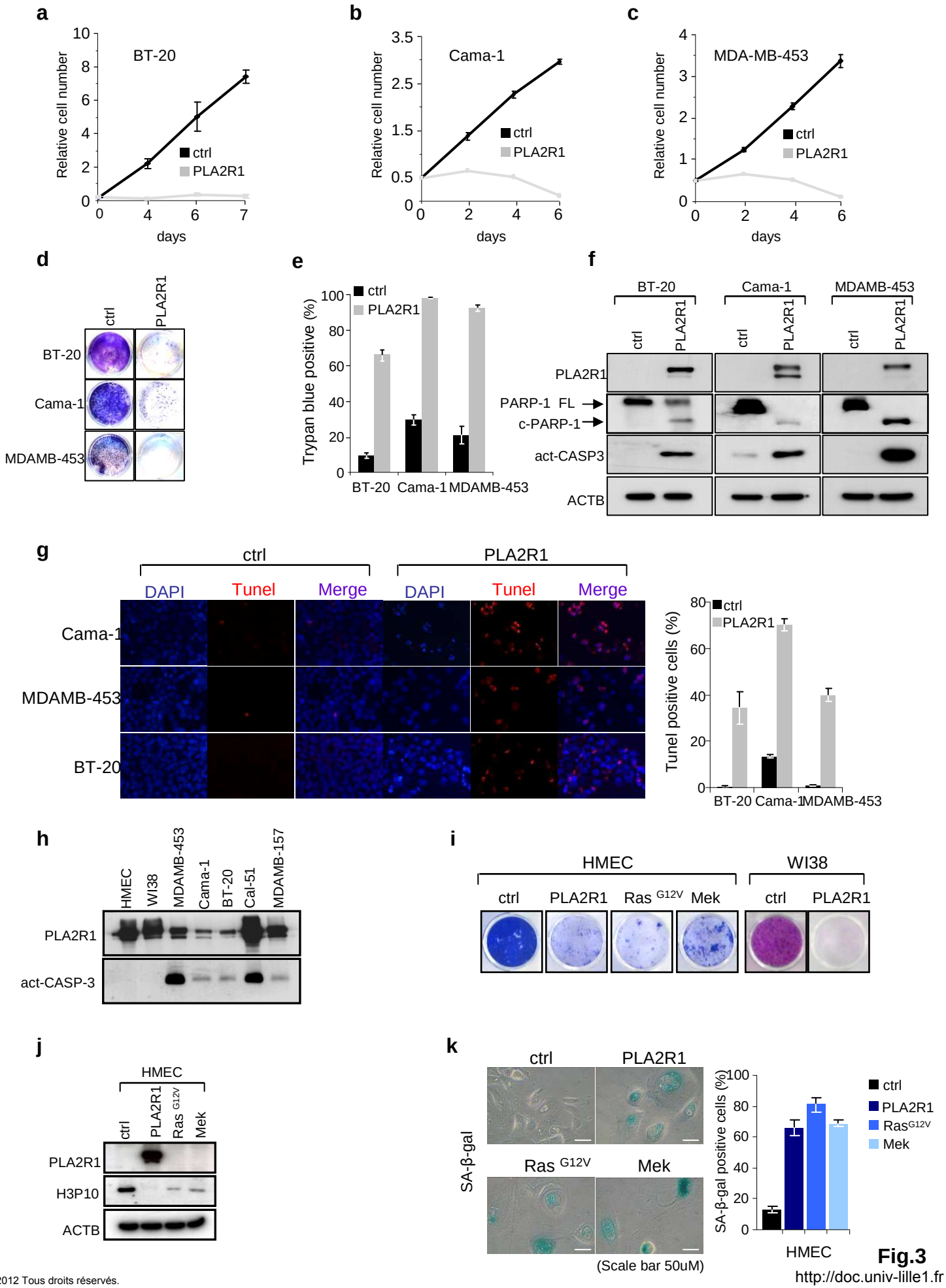


Fig.3

Figure 3: PLA2R1 ectopic expression specifically triggers apoptotic cell death in cancer cells while triggering cell cycle arrest and senescence in primary cells.

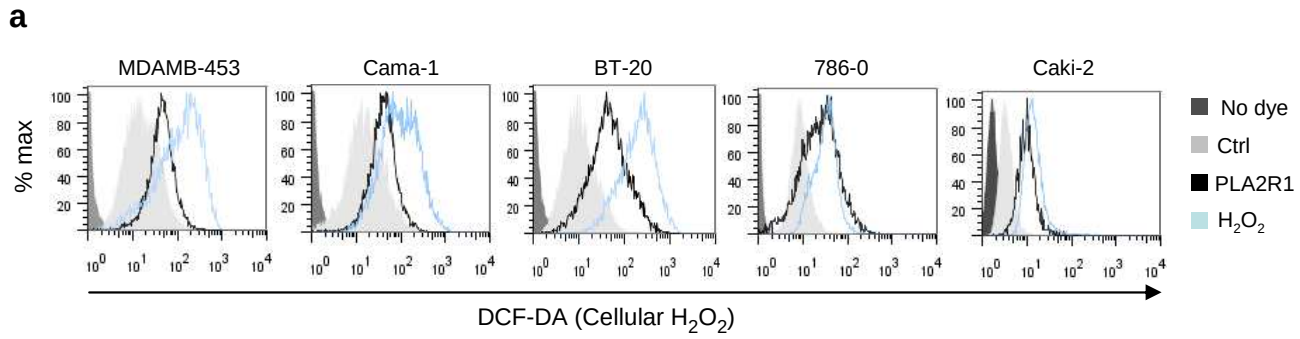
(a) Growth curve analysis of control and PLA2R1 expressing BT-20 cells.(b) Growth curve analysis of control and PLA2R1 expressing Cama-1 cells.(c) Growth curve analysis of control and PLA2R1 expressing MDAMB-453 cells.(d) Colony formation assay of control and PLA2R1 expressing BT-20, Cama-1 and MDAMB-453 cells.(e) Control and PLA2R1 expressing BT-20, Cama-1 and MDAMB-453 cells were analysed for trypan blue exclusion test.(f) Western blot analysis of control and PLA2R1 expressing BT-20, Cama-1 and MDAMB-453 cells. Cells were probed for PLA2R1 expression, and apoptotic markers (activated caspases 3 and cleaved PARP-1). ACTB was used as a control.(g) TUNEL analysis of control and PLA2R1 expressing cells BT-20, Cama-1, and MDAMB-453 cells.(h) Primary and cancer cells expressing PLA2R1 were assessed for activated caspase 3.(i) Colony formation assay of HMEC and WI38 expressing a vector alone or PLA2R1. H-Ras^{G12V} and MEK were used as positive controls.(j) Western blot analysis of control, PLA2R1, H-Ras^{G12V} and MEK expressing cells. H3-pSer10 was used as a proliferation marker. H-Ras^{G12V} and MEK were used as positive controls.(k) The same cells as in (j) were assessed for SA-βgal. Scale bar represents 50μM.

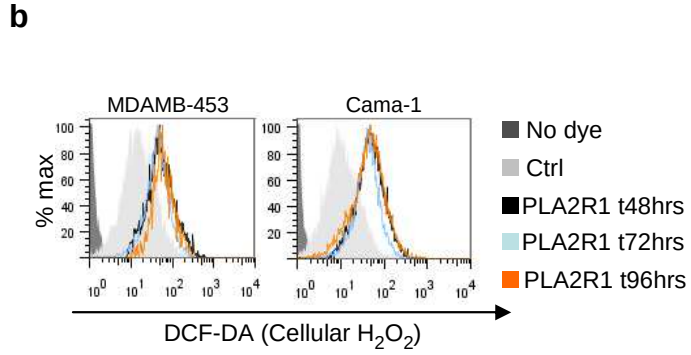
PLA2R1 expression induces cancer cell apoptosis through ROS mediated mechanism

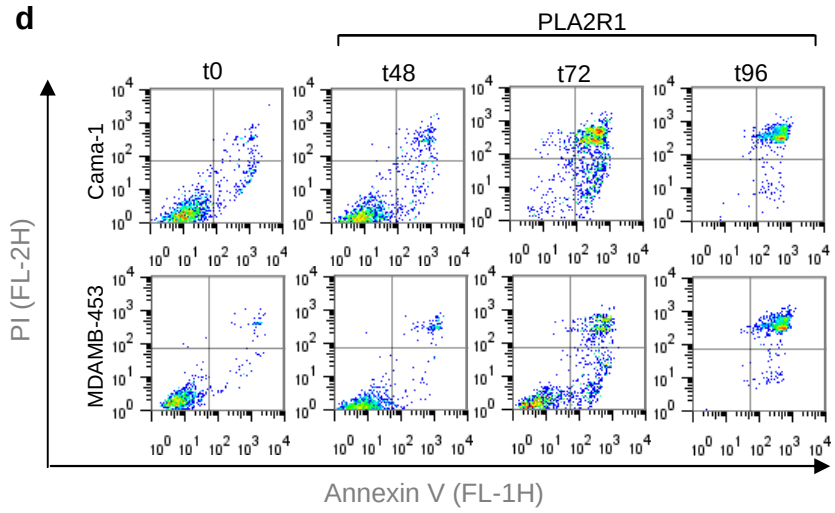
We have previously demonstrated that PLA2R1 regulates senescence in normal human fibroblasts by modulating ROS levels [424]. Moreover, PLA2R1 depletion leads to decreased intracellular ROS levels and this is associated with an escape from OIS (Figure 2f-g, S2, S3c/d). For these reasons, ROS appear as good candidates to mediate PLA2R1 induced cell death in cancer cells. Interestingly, PLA2R1 ectopic expression led to ROS accumulation in most of cancer cell lines (Figure 4a). To uncover whether ROS accumulation preceded apoptotic cell death, we performed kinetic experiments. Interestingly, 48 hrs post infection, ROS accumulation was already observed in the cell lines ectopically expressing PLA2R1 (Figure 4b) whereas the same kinetic performed to analyse apoptotic markers (activated caspase 3 and PARP-1 cleavage) revealed that apoptosis was observed 72 hrs post infection (Figure 4c). As these markers are late stage apoptotic markers, we also performed an AnnexinV/PI FACS analysis. Forty eight hrs post infection, no increase in the number of

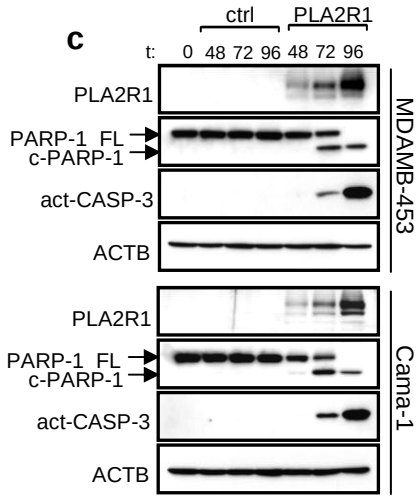
AnnexinV positive cells was observed in PLA2R1 expressing cells (Figure 4d). Indeed, the percentage of AnnexinV positive cells strongly increased 72 hrs post infection and peaked at 96 hrs post infection (Figure 4d). Most importantly, control infected cells did not show any sign of ROS accumulation or apoptotic markers (Figure S6a-S7a). These results suggest that ROS production is not a consequence of apoptotic cell death but might rather be a cause. In accordance, SKBR-3 cells, the only cancer cell line resistant to PLA2R1 (Figure S8b-c), does not show any sign of ROS accumulation after PLA2R1 expression (Figure S8a).

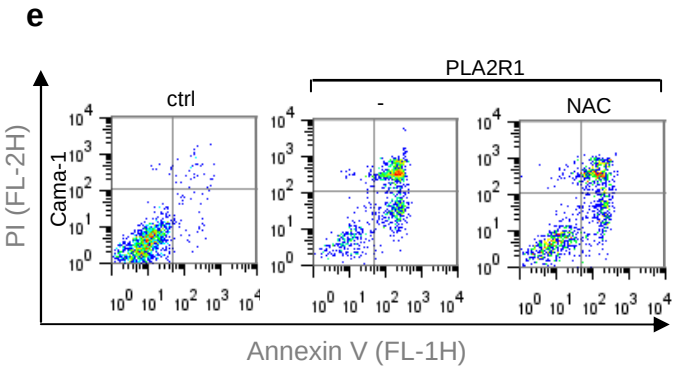
To functionally demonstrate the involvement of ROS in PLA2R1 induced apoptotic cell death, we investigated the effect of the well described anti-oxidant N-acetyl-cysteine (NAC) [445]. Incubation of PLA2R1 infected cancer cells with NAC reverted partially PLA2R1 induced apoptosis (Figure 4e-g). These results pointed out a major functional role of ROS in PLA2R1 mediated cancer cell death. High ROS levels have been demonstrated as potent cancer cells killers with an ability to induce cell death in numerous cell lines [437]. In accordance, PLA2R1 ectopic expression induced cell death in countless cancer cell lines from various origins (Figure S9a-f). Additionally, as cells are cultured under normoxic conditions (21% O_2 levels) we tested what would be the effect of PLA2R1 ectopic expression under physiological oxygen conditions. Interestingly, PLA2R1 was still capable of triggering apoptosis under these conditions (Figure S9g).



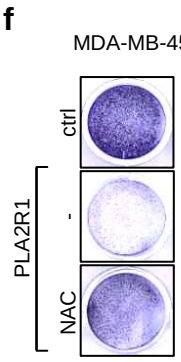








Viable cells: 93,12% 18.47% 43.05%



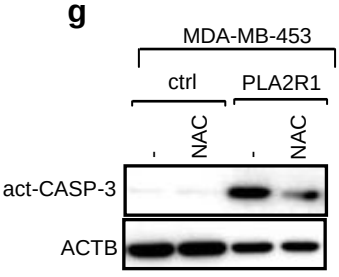


Fig.4

Figure 4: PLA2R1 expression induces cancer cell apoptosis through ROS mediated mechanisms.

(a) The indicated cancer cells lines expressing PLA2R1 or not were incubated with H_2DCF-DA (1hrs; 3µM) and their ROS contents analysed by FACS. H_2O_2 was used as a positive control. Unstained cells were used as negative control.(b) Kinetic analysis of ROS levels following PLA2R1 expression. H_2O_2 was used as a positive control. Unstained cells were used as negative control.(c) Kinetic western blot analysis of apoptotic markers in control and PLA2R1 expressing cells (Cama-1; MDAMB-453). The apoptotic markers used are activated caspases 3 and cleaved PARP-1.(d) AnnexinV/PI analysis of control and PLA2R1 expressing Cama-1 and MDAMB-453 were performed at the same time as in (b) and (c).(e). AnnexinV/PI analysis of control, PLA2R1 expressing cells (Cama-1) incubated or not with N-acetyl cysteine (NAC; 3µM).(f) Colony assay of the cells MDAMB-453 control or PLA2R1 treated or not with NAC (0.5µM). (g) Western blot analysis of cells in (f).

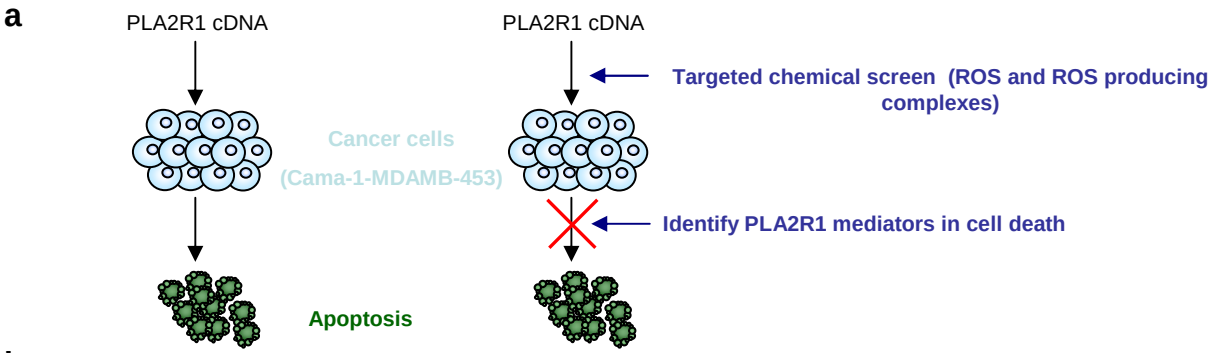
PLA2R1 modulates the mitochondrial respiratory chain activity to exert its tumour suppressive role

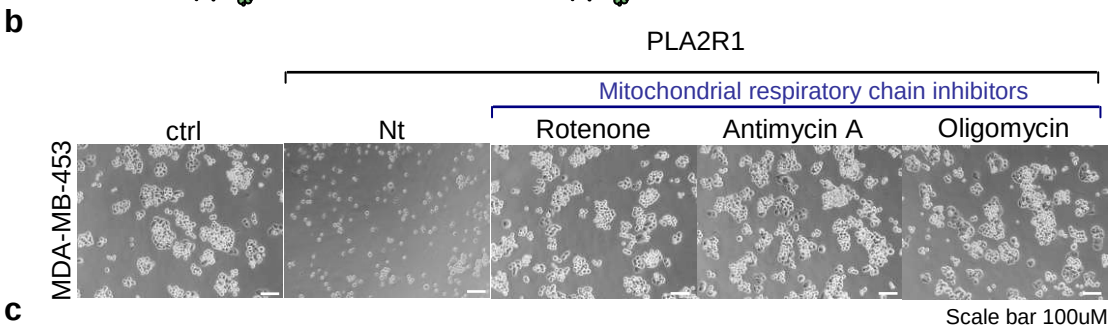
In order to identify ROS generating complexes or ROS that contribute to PLA2R1 induced cell death, we performed a targeted chemical screen (Figure 5a, S10). The chemical screen was performed in two cancer cell lines in order to avoid identifying cell type specific mechanism. Although the reversion of PLA2R1 induced cell death was partial with the anti-oxidant NAC, we hypothesised that if PLA2R1 modulated specific ROS generating complexes or ROS, targeting it could be sufficient to revert potently PLA2R1 phenotype and hence identify a specific route leading to PLA2R1 cell death. Twenty three inhibitory molecules including global antioxidants, inhibitors of inducible nitric oxide synthetase (iNOS), nitric oxide (NO), peroxinitrite but also ROS generating complexes such as NAPDH oxidase (NOX) complexes and mitochondria were tested (Figure S10a). Strikingly, we found that altering the mitochondria respiratory chain was efficient in reverting PLA2R1 induced cell death. More specifically, targeting the complex I with rotenone and III with antimycin A1 which are the main sites of electron leakage and ROS production [446], leads to a strong

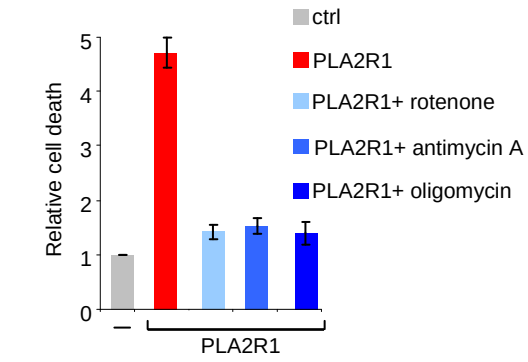
apoptosis reversion (Figure 5b-c). Conversely in control infected cells, the blockade of complex I and III led to ROS increase and a slight increase in apoptosis in accordance with previous studies (data not shown) [447, 448]. The simple fact that these chemical inhibitors were able to reverse PLA2R1 induced cell death and ROS increase (Figure 5b-d) suggests that PLA2R1 boosts the mitochondrial respiratory chain activity. Additionally, PLA2R1 induced cell death was also reversed with oligomycin (Figure 5b-c), an inhibitor of the F0 proton channel of the ATP synthetase and indirectly of the burst of oxygen consumption [449]. Oligomycin was not only an apoptosis inhibitor as it was able to reverse PLA2R1 induced ROS accumulation prior apoptosis induction (Figure 5d).

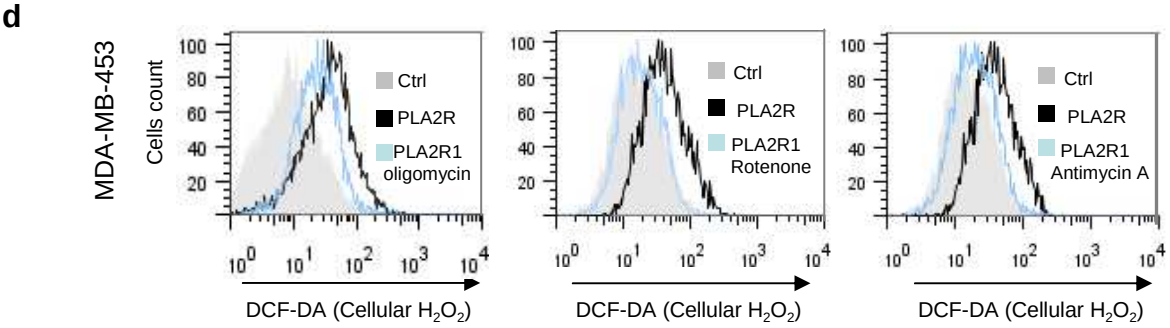
Next, we determined whether PLA2R1 could induce mitochondrial oxidative damage. We analysed ROS mediated mitochondrial lipid peroxidation. As a good indicator of lipid peroxidation, we checked with nonyl acryl orange (NAO) the oxidative damage of cardiolipin, a mitochondrial membrane component. Loss of NAO fluorescence corresponds to an oxidation of cardiolipin. We performed a kinetic of PLA2R1 expressing cells and analysed lipid peroxidation by FACS. NAO was plotted against ethidium bromide (EB) to monitor cell death. PLA2R1 ectopic expression led to cardiolipin oxidation 72 hrs post infection (Figure 5e). Interestingly, ethidium bromide negative cells displayed cardiolipin oxidation. This suggests that mitochondrial oxidative damage did not result from PLA2R1 induced apoptosis but was a result of PLA2R1 induced ROS accumulation. Finally, inhibiting the mitochondrial complex I, III and V partially protected cells from PLA2R1 induced mitochondrial oxidative damage (Figure 5f). Additionally, PLA2R1 expression not only resulted in mitochondrial oxidative damages but also led to mitochondrial transmembrane potential loss (Figure S10b). Altogether these results suggest that the mitochondria respiratory chain is an essential mediator of PLA2R1 tumour suppressor properties as inhibiting ROS generating complexes I

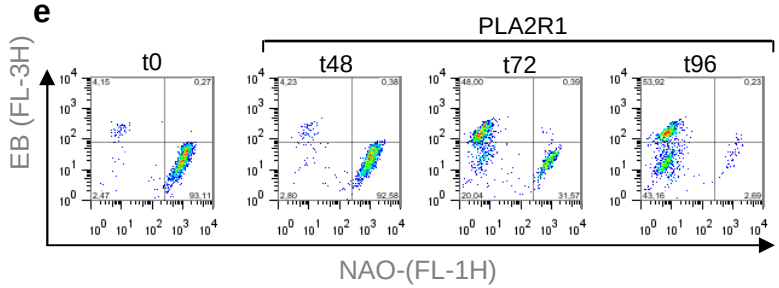
and III or shutting down oxidative phosphorylation and mitochondrial oxygen consumption with oligomycin impeded PLA2R1 induced ROS and subsequently apoptosis.











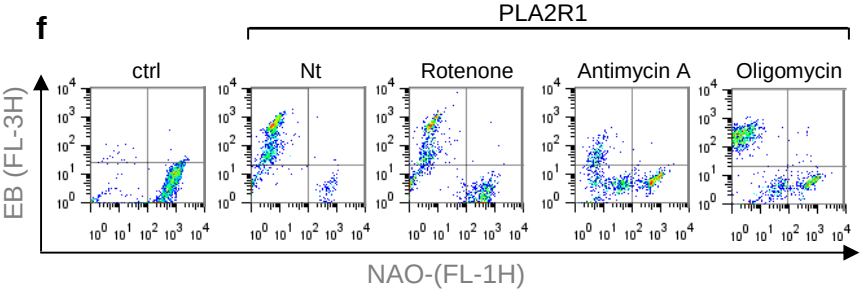


Fig.5

Figure 5: PLA2R1 modulates the mitochondrial respiratory chain activity to exert its tumour suppressive role.

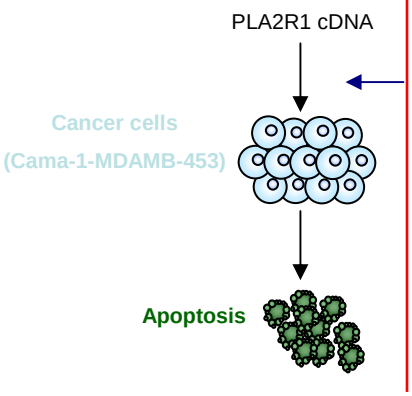
(a) Schematic representation of the targeted chemical screen performed in MDAMB-453 and Cama-1 cells.(b) Cellular morphology of control or PLA2R1 expressing MDAMB-453 treated or not with mitochondrial respiratory chain inhibitors (rotenone 1µM, Antimycin A 10µM, Oligomycin 10µM) was analysed and representative photos were taken. Scale bar represents 100µM.(c) AnnexinV/PI analysis of control or PLA2R1 expressing MDAMB-453 treated or not with mitochondrial respiratory chain inhibitors (rotenone 1µM, Antimycin A 10µM, Oligomycin 10µM).(d) The cells in (c) were analysed for their ROS contents.(e) A kinetic of PLA2R1 expressing cells was performed to analyse cardiolipin oxidation (NAO) and cell death (EB) by FACS.(f) Cardiolipin oxidation (NAO) and cell death (EB) of control or PLA2R1 expressing cells treated or not with mitochondrial respiratory chain inhibitors (rotenone 1µM, Antimycin A 10µM, Oligomycin 10µM) were analysed by FACS.

PLA2R1 exert its tumour suppressive properties in a sPLA2 independent manner

PLA2R1 was identified as a binding site for two neurotoxic monochains phospholipase A2 (OS1) and (OS2) isolated from the venom of Taipan snake [390]. To date, the only biological responses mediated by PLA2R1 implicate sPLA2, its only ligands described to date [399]. We therefore checked whether sPLA2 or sPLA2 activated pathways could be involved in PLA2R1 induced ROS increase and apoptosis. We performed a targeted chemical screen inhibiting sPLA2, cPLA2 (cytosolic PLA2) and iPLA2 (a calcium independent phospholipase A2). We also inhibited sPLA2 key mediators such as lipooxygenase 5/12 (5-LO and 12-LO) and cyclooxygenase 1 and 2 (COX1 and 2) (Figure 6a). To our surprise, none of the tested inhibitors yielded positive results (Figure 6a). Inhibitors were used at various concentrations previously determined to be sufficient to inhibit their target (G.Lambeau, personal communication). To investigate this surprising result, we generated PLA2R1 mutants. Sequential truncations starting from the N-terminal cystein rich domain (Cys-R) of PLA2R1 were performed (Figure 6b). Interestingly, successively deleting domains in the N-terminal part of PLA2R1 partially reverted PLA2R1 induced apoptosis (Figure 6b-c).

PLA2R1 induced cell death was greatly impeded with a PLA2R1 mutant (CTLD2-Cter) deleted in the cystein rich (Cys-R), the fibronectin like type II (FN-II) domains and the first C-type lectin like carbohydrate recognition domain (CTLD1). This observation strongly suggested that the CysR-FN-II-CTLD1 domains play a crucial role in PLA2R1 mediated effects (Figure 6b-c). This was unexpected but in accordance with the targeted chemical screen suggesting a PLA2R1 sPLA2 independent role. Indeed, the sPLA2 binding is mediated by the CTLD4-5-6 with an important role of CTLD5 [406]. Altogether, these results suggest that PLA2R1 mediates biological responses associated with its tumour suppressive activities in a sPLA2 independent fashion at least in this specific context.

a



PLA2 and PLA2 related pathways inhibitors tested for the chemical screen

Chemicals	Doses	target	Phenotype reversion
HELSS	1-10uM	i PLA2	None
Wyeth	1-10uM	cPLA2	None
Pyrrolidine-2	1-10uM	cPLA2	None
AZ-1	1-10uM	cPLA2	None
LY329722	1-10uM	sPLA2 (IIA, X)	None
RO 05097A	1-10uM	sPLA2 PAN	None
RO 032007A	1-10uM	sPLA2 PAN	None
Indomethacin	1-10uM	COX1/COX2	None
Rofecoxib	1-10uM	COX2	None
Baicalein	1-10uM	12-LO	None
MK886	1-10uM	5-LO	None

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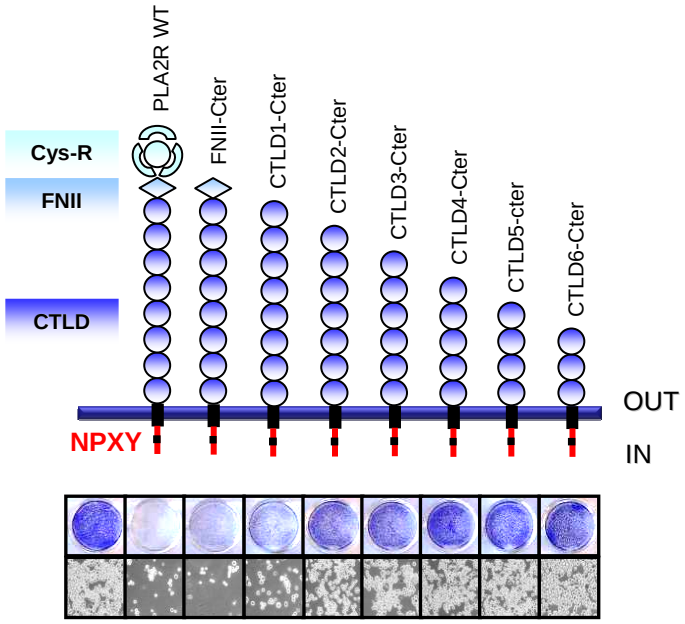

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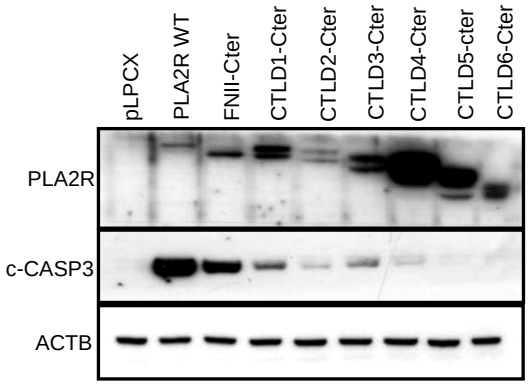


Fig.6

Figure 6: PLA2R1 exerts its tumour suppressive properties in a sPLA2 independent manner.

(a) Targeted chemical screen inhibiting sPLA2/cPLA2/iPLA2 and the downstream pathways they modulate.(b) Colony assay of PLA2R1 mutants generated (see materiel and methods). Cells were fixed and stained with crystal violet 10 days post the start of the experiment. (c) Western blot analysis of PLA2R1 mutants (c). The activated caspase 3 was used as an apoptotic marker. PLA2R1's mutants expression was probed with a PLA2R1 antibody.

Discussion

To date, little is known about PLA2R1 physiological biological functions especially in the cancer field. We reported its ability to regulate replicative senescence in normal human fibroblasts [424] and others recently identified PLA2R1 as a target in idiopathic membranous nephropathy, a renal autoimmune human kidney disease [430-432]. In the present study, we demonstrate that PLA2R1 displays strong tumor suppressive properties and thus deserves to be studied in depth in that context. According to our results, the expression of PLA2R1 decreases in breast and kidney cancers. Even if the Oncomine database analysis oriented us to these forms of cancer, it is probable that PLA2R1 may be deregulated in other cancer types. Interestingly, PLA2R1 has been described as a major antigen for the "idiopathic membranous nephropathy" auto-immune kidney disease. PLA2R1 may thus be of particular importance in regulating kidney biology [430-432].

To decipher how PLA2R1 can decrease in tumor cells we analysed its expression at both genetic and epigenetic levels. We examined more than 40 kidney samples with comparative genomic hybridation (normal vs tumoral) and no significant changes in PLA2R1 gDNA content were observed (data not shown). Beside genetic alterations, epigenetic alterations such as promoter hypermethylation play a major role in cancer progression through the transcriptional silencing of bona fide tumour suppressor genes [441-443]. We indeed found

hypermethylation of CpG residues in PLA2R1 promoter. As expected, in cancer cell lines displaying a PLA2R1 promoter hypermethylated, chemical inhibition of DNMT resulted in PLA2R1 re-expression whereas it had no effect in PLA2R1 expressing cells. Thus, the epigenetic regulation of PLA2R1 seems to be an important event in PLA2R1 repression observed in cancers.

PLA2R1 regulates replicative senescence of normal human fibroblasts [424]. Here we extend these observations by showing that a down-regulation of PLA2R1 extends the replicative potential of normal human epithelial cells (HMEC) and favours a bypass from various oncogenic stress, in particular from HER-2, an important player of breast tumorigenesis [450]. Additionally, the loss of PLA2R1 favours transforming and tumorigenic potential of various cell lines. One critical question that comes to mind is how does PLA2R1 depletion lead to such a phenotype? Interestingly, we found that PLA2R1 depleted cells were more resistant to stress induced ROS accumulation and it has recently been proposed that oncogenes can activate an antioxidant program to favour ROS detoxification and tumorigenesis [210]. Additionally, ROS play a crucial role in replicative and oncogene induced senescence and ROS modulation has been functionally involved in replicative and oncogene induced senescence [124, 261, 283, 287, 288, 291, 292, 297, 298, 424, 451]. Altogether these data show that altering PLA2R1 levels can have potent effects on normal cell homeostasis favouring escape from failsafe mechanism and tumorigenic properties through ROS mediated mechanism.

In contrast, re-introducing PLA2R1 expression resulted in apoptotic cell death in a ROS dependent manner in a myriad of cells with cancer genotypes. Interestingly, PLA2R1 expression preferentially triggered cell cycle arrest and cellular senescence in primary cells. A chemical screen identified the mitochondria respiratory chain as the main site for PLA2R1

induced cell death. Chemically inhibiting specific ROS generating complexes or the oxidative phosphorylation led to insensibility to PLA2R1 induced cell death and partially reversed PLA2R1 mediated oxidative mitochondrial damage. This suggests a critical role of the mitochondrial respiratory chain in PLA2R1 mediated effects. Our results suggest that PLA2R1 might increase mitochondrial oxygen consumption. Indeed, inhibiting the mitochondrial complex in control cells led to ROS production and slight apoptosis. It therefore suggests that mitochondrial respiratory chain inhibitors, to counteract PLA2R1 ROS increase and apoptosis, must maintain normal mitochondrial oxygen consumption in PLA2R1 expressing cells. This suggests that PLA2R1 boosts this activity that that normal activity is restored in presence of mitochondrial inhibitors. Nonetheless, it has to be confirmed experimentally. An alternative explanation, that might synergise or be non exclusive, could be formulated. PLA2R1 might have an impact on mitochondrial biogenesis and therefore increase the mass of the numbers of mitochondrion per cell.

Finally, we uncovered an unexpected sPLA2 independent role of PLA2R1. This finding could have major implications in term of future research perspectives. One might postulate that PLA2R1 could bind alternative ligands that are yet to be discovered. Alternatively, PLA2R1 could interact or be involved in the dimerization with receptors mediating signaling pathways. In line with this hypothesis, Endo180, which belongs to the same family as PLA2R1, interacts with urokinase-type plasminogen activator receptor (uPAR) [452]. Taken together, we identify PLA2R1 as a new and powerful regulator of breast and kidney tumorigenesis through the modulation of mitochondrial ROS dependent mechanism. Moreover, our results breach exciting new research perspectives on the role of this protein.

Experimental procedures

Cell Culture

Human cancer cell lines (ATCC) were cultured in the following media: DMEM (Invitrogen) for MDA-MB 453, MDA-MB-157, MDA-MB-231, MDA-MB-361, MDA-MB-436, Hs-578T, MCF-7, Cal-51, Caki-2, 786-O, RPMI (Invitrogen) for RCC4, RCC10, MEM (Invitrogen) for Cama-1, BT-20, ACHN and Mc Coy's (Invitrogen) for SK-Br3 cells. All medium were supplemented with 10 % FBS (Lonza), 1% penicillin/streptomycin (Invitrogen), 0, 36 % gentamycin (Invitrogen). For Cama-1, BT-20 and ACHN cells lines, 1% Non essential Amino-Acid (Invitrogen) was added. Virus producing GP293 cells (Clontech) were cultured in DMEM media (Invitrogen) supplemented with 10 % FBS (Lonza), 1% penicillin/streptomycin (Invitrogen) and 0, 36 % gentamycin (Invitrogen). Normal human diploid fibroblasts WI38 (American Type Culture Collection (ATCC)) were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Lonza), 1% Non essential Amino-Acid (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 0, 36 % gentamycin (Invitrogen). HMECs (Clonetics) were cultured in mammary epithelial cell growth medium (Promocell) in the presence of 1% penicillin/streptomycin (Invitrogen) and 0, 36 % gentamycin (Invitrogen).

Vectors construction

Wild-type membrane-bound (GenBank NM 007366) deletion mutants of human PLA2R1 were generated by PCR from the pSupF human PLA2R1 vector (unpublished). Each construct was first ligated into the pGEMTeasy vector (Promega), fully sequenced and subsequently subcloned in the pLPCX retroviral vector (Clontech) using XhoI/NotI restriction

sites. PCR conditions were 98°C for 1 min, followed by 30 cycles of 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 1-5 mins. This was followed by a final extension of 72°C for 10 mins. Primer sequences are available upon request.

Transfection and infection

GP293 cells were transfected using PEI reagents according to manufacturer's recommendations (Euromedex). Two days after transfection, viral supernatant mixed with fresh media (1/2) and polybrene (final concentration at 8ug/ml) was used to infect target cells. Cells were infected for a period of 12-24 hrs depending on the cell type. Importantly, infection protocols were designed so that virtually all cells were infected as judged by GFP control infection condition. One day post infection, cells were selected using puromycin at the final concentration of 0,75-1,5µg/ml depending on the cell type.

Antibodies and immunoblot

Cell lysates were prepared in ice cold Giordano buffer (50 mM Tris·HCl, pH 7.4, 250 mM NaCl, 0.2% Triton X-100, 5 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche). Lysates were clarified by centrifugation at 14,000 rpm for 30 min at 4°C. Protein concentrations were measured using Bradford protein assay (Biorad #500-0006). For immunoblot analysis, cell extracts were separated by SDS-PAGE gel electrophoresis under non reducing conditions (specifically for PLA2R1 detection) or reducing conditions and proteins were transferred onto nitrocellulose membrane. The following primary antibodies were used: anti-PLA2R1 (HPA012657, Atlas), anti-active caspase3 (ab32042, Abcam), anti-tubulin (T6199, Sigma), anti-actin (A5316, Sigma), anti H3-pSer10 (#9701, Cell Signalling)

and anti cyclin A (sc-751, H432; Santacruz). After overnight incubation at 4°C with primary antibodies, blots were washed with PBS tween 0.05%, incubated with a secondary antibody coupled to peroxidase, washed again PBS tween 0.05% and antigen-antibody complexes were detected using ECL (Amersham).

RNA extraction, retro-transcription and quantitative PCR

Normal and tumoral human kidney tissues were provided by the tumorothèque HEH. Total RNA extraction was performed using a phenol–chloroform method using TriReagent (Sigma-Aldrich). PhaseLockGel tubes (Eppendorf) were used for phase separation. The cDNA synthesis was performed from 3ug of total RNA using the First-Strand cDNA Synthesis Kit (GE Healthcare). The RT reaction was diluted 1/60 and used as cDNA template for qRT-PCR analysis. TaqMan quantitative PCR analysis was carried out on a LightCycler 2.0 System (Roche Applied Science). PCR mixtures contained LightCycler TaqMan mix, 200 nM primers and 1.67µl of cDNA template in a 6.67µl reaction volume. Housekeeping genes (*ACTB*, *PGK1*), used for normalization of target mRNA expression in each sample type, were selected by systematic geNorm analysis as previously described [453]. Real-time PCR intron-spanning assays were designed using the ProbeFinder software (Roche Applied Science).

Colony formation assays

Colony formation assays were carried out in 6 or 12 well plates. Five to ten days later, cells were washed with PBS, fixed with 4% paraformaldehyde and stained with 0.05% Crystal violet (Sigma-Aldrich).

Bioinformatics analysis

The analysis was performed by interrogating the oncomine public database (www.oncomine.org).

Methylation study

Genomic DNA was extracted following the manufacturer instructions (Qiagen). Genomic DNA (1µg) with carrier DNA (3µg) was placed at 65°C for 10 mins. 12µl of NaOH (5N) was added as well as H_20 to reach a final volume of 100µl. The mixture was incubated 30 mins at 37°C. For the bisulfite modification, 350µl of sodium bisulfite (Merck) and 50µl of freshly prepared hydroquinone was added to the mixture and incubated for 5 to 16 hrs at 50°C. Bisulfited DNA was desalted using the Wizard DNA clean up system (Promega) according to the manufacturer recommendations. 3µl of NaOH (5N) was added to the desalted DNA and incubated 15 mins at room temperature. DNA precipitation was performed with the PCR purification kit (Qiagen) according to the manufacturer recommendations. Two regions (nt positions −351 to +71, and nt positions +45 to +503 from the *PLA2R1* transcription start site) were analyzed. PCR amplifications were performed in 100µl using the HotStart *Taq* DNA polymerase Kit (Qiagen) and 0.4µM of primers after 10 mins at 95°C for *Taq* polymerase activation and 40 cycles (30 seconds denaturation at 94°C, 1 min annealing at 56°C, and 1.5 mins extension at 72°C). To determine the proportion of methylated CpG, PCR products were cloned into a pGEM-T vector (Promega) as recommended by the manufacturer and 5 clones from each sample were analyzed by sequencing. Primers sequences are available upon request. For 5-aza-2'-deoxycytidine (5-aza) treatments were performed at 5µM for 72 hrs.

Soft-agar colony formation assay

To measure anchorage-independent growth, cells were detached with trypsin and re-suspended in growth medium. Base agar was prepared with 0.75% low-melting agarose (Lonza) in growth medium. The top agar contained the suspension of cells in 0.45% low-melting agarose (1.5-3.0 x 10^4 cells/well in 6 well plates depending on the cell type). Plates were incubated for 3 weeks at 37°C and colonies were counted under a bright light microscope. The mean surface was quantified using the Image J software.

Tunel assay

Supernatants of various conditions were collected and added to cells harvested by trypsination. After centrifugation cells were re-suspended in FBS (100%) and 1.10^5 cells were cytospined on microscopic slides. After fixation with PFA 3.7%, cells were washed three times in PBS 1X and permeabilised 15 mins in PBS 0.2% triton X100. Cells were serially washed in PBS 1X and then with TdT buffer (Tris 30mM, Sodium cacodylate 150mM, Ph 7.5) to which was added up extemporaneously CoCl2 (1mM). dUTP addition at the 3'-OH DNA termini was performed by incubating cells 1 hr at 37°C in a solution of TdT/CoCl2 buffer in the presence of biotin-dUTP (Roche; 6µM final) and the Terminal deoxynucleotidyl Transferase (TdT) enzyme (Roche; 2,4U/µl final). Reaction was stopped by washing with TB buffer (NaCl 300mM, Sodium Citrate 34,1mM) during 15 mins at room temperature. Cells were then washed in PBS 1X, blocked 10 mins in a solution of PBS 1X containing 2% BSA and incubated 1 hr with Cy3-coupled-streptavidine in PBS 1X. Nuclei were stained with Hoechst for 10 mins, washed three times in PBS 1X and mounted using Fluoromount-G (SouthernBiotech). Cells were visualised using a Carl Zeiss Axiophot2 fluorescence

microscope with an objective (X 40). Pictures were taken with an Axiocam MRC camera. Results are representative of two independent experiments.

SA-βgal analysis

SA-βgal analyses were performed as in [130]. Briefly, subconfluent cells were washed twice with PBS (1X). Cells were fixed with a fixation solution (with glutaraldehyde (0.2%) and formaldehyde (2%) in PBS 1X) for 5 mins and washed twice with PBS 1X. The staining solution (40 mM citric acid/Na phosphate buffer, 150 mM sodium chloride, 5 mM K4(Fe(CN)6) 3H2O, 5 mM K3(Fe(CN)6), 2 mM magnesium chloride and 1 mg/ml X-gal in distilled water) was then added for 8-16 hrs at 37°C. The next day, cells were washed with PBS 1X and visualised with phase contrast microscopy. At least 5 different fields were counted for each condition representing at least 500 events.

SAHF analysis

SAHF analysis was performed as in [138]. The cells were washed twice with PBS 1X, fixed 15 mins with PFA 4% and stained with 0.13µg/ml DAPI for 2 mins at room temperature. The DNA foci were visualised and at least 200 events were recorded.

ROS quantification

Cellular ROS contents were measured by incubating cells with 3µM H_2DCF-DA probe for 60 mins. Cells were washed in PBS 1X buffer, trypsinised and re-suspended in 300µl of PBS 1X buffer. Cells were counterstained with popidium iodide (PI) in order to exclude dead

cells from the analysis. ROS levels were analysed by flow cytometry using FACS Calibur with 10.000 events recorded. The final data was analysed using Flow Jo 7.6 software. Results are representative of three independent experiments.

Cardiolipin oxidation assessment

The structure and the integrity of mitochondria was indirectly assessed analysing cardiolipin oxidation with 10-N-nonyl acridine orange (NAO) as in [454]. Briefly, cells from different conditions were quickly trypsinized and transferred with "floating cells" in falcon tubes. Cells were centrifuged at 1000g for 5 mins at 4°C. 500µl of NAO solution (25ng/ml in PBS 1X-BSA (0.1%)) was added to the cells and the mixture NAO-cells was incubated at 37°C for 10 mins. At the end of the incubation, cells were placed on ice and 4ml of PBS 1X-BSA (0.1%) was added. Cells were centrifuged at 1000g for 5 mins at 4°C. Finally, the cells were re-suspended in 300µl of ethidium bromide (EB) solution and analysed by flow cytometry. At least 15.000 events were recorded. Data analysis was performed using Flow Jo 7.6 software. Results are representative of three independent experiments.

Mitochondrial transmembrane potential ($\Delta\Psi_m$) assessment

The mitochondrial transmembrane potential ($\Delta\Psi_m$) measured with the Rhodamine 123 (Rh-123) probe was performed as in [454] and is the same procedure as for cardiolipin oxidation assessment with NAO [454]. The working solution is 1mg/mlof Rh-123 in PBS 1X-BSA (0.1%) buffer. The final data was analysed using Flow Jo 7.6 software. Results are representative of three independent experiments.

Chemical screen

During the targeted chemical screen, at least 4 different doses were used for each inhibitor. Additionally, control cells were also treated with the inhibitors to determine any kind of drug cytotoxicity.

Annexin V/PI analysis

Cells collected by centrifugation were counted then incubated with annexin V-FITC in binding buffer 1X (10mM Hepes/NaOH ph7, 140 mM NaCl, 2.5 mM CaCl2) for 10 mins in the dark according to the manufacturer protocol (Abcyss). DNA staining was then performed by incubating cells in propidum iodide (0.6µg/ml) for 10 additional mins. For each condition 10 000 events were recording by FACS (Facscalibur) and results were analysed with Flow Jo 7.6 software. Results are representative of three independent experiments.

Trypan blue analysis

Cells collected by centrifugation were re-suspended in complete medium then incubated 5 mins in trypan blue (Gibco) at a final concentration of 0.066 %. At least 300 cells were counted for each condition. Results are representative of three independent experiments.

Figure legends

Figure S1: PLA2R1 decreases in kidney cell lines.

(a) qRT-PCR analysis of PLA2R1 expression in kidney cancer cell lines versus normal kidney tissue.

Figure S2: PLA2R1 depletion favours an escape from oncogene-induced-senescence (OIS).

(a) Co-transfection of control or PLA2R1 in the presence or not of shRNAs targeting PLA2R1 (PLA2R and PLA2R1-2). A GFP vector was used as a loading control.

(b) Western blot analysis of endogenous PLA2R1 in HMEC cells targeted or not with shRNAs. ACTB was used as a loading control.

(c) Colony assay of HMEC/MEK-ER control or PLA2R1 depleted cells treated or not with 4OHT (100nM; 48hrs). p53 depleted cells were used as positive controls.

(d) Western blot of cells in (c).

(e) Colony assay of control or PLA2R1 depleted cells following the over-expression of Her-2.

(f) Western blot analysis of cells in (e).

Figure S3: PLA2R1 depleted cells are more resistant to ROS induced cellular senescence.

(a) HMEC/HRAS^{G12V}-ER cells were treated with 4OHT (100nM; 48hrs) and ROS (H_2DCF-DA (1hrs; 3µM)) contents were analysed by FACS at the indicated times.

(b) HMEC/MEK-ER cells were treated with 4OHT (100nM; 48hrs) and ROS (H_2DCF-DA (1hrs; 3µM)) contents were analysed by FACS at the indicated times.

(c) HMEC/HRAS^{G12V}-ER control and PLA2R1 depleted cells were treated with 4OHT (100nM; 48hrs) and ROS (H_2DCF-DA (1hrs; 3µM)) contents were analysed by FACS. p53 depleted cells were used as positive controls.

(d) HMEC/MEK-ER control and PLA2R1 depleted cells were treated with 4OHT (100nM; 48hrs) and ROS (H_2DCF-DA (1hrs; 3µM)) contents were analysed by FACS.

Figure S4: PLA2R1 depletion favours cellular transformation.

(a) Hs578T control and PLA2R1 depleted cells were assessed for transformation potential. Cells were seeded on agar plates at 30.000 cells per 6 well plates. The colonies numbers were counted and their size analysed. qRT-PCR was performed to validate the shRNA efficiency.

(b) ACHN control and PLA2R1 depleted cells were assessed for transformation potential. Cells were seeded on agar plates at 15.000 cells per 6 well plates. The colonies numbers were counted and their size analysed. qRT-PCR was performed to validate the shRNA efficiency.

(c-d) MDAMB-436 control and PLA2R1 depleted cells were assessed for transformation potential in both 10% and 1% FBS. In the 1% FBS conditions, cells were seeded on agar plates at 30.000 cells per 6 well plates. The colonies numbers were counted and their size analysed (10% FBS). qRT-PCR was performed to validate the shRNA efficiency.

Figure S5: PLA2R1 ectopic expression lead to premature senescence in WI38 cells.

(a) Western blot analysis of control and PLA2R1 expressing WI38 cells. RAS^{G12V} was used as a positive control for cellular senescence. Adriamycin treatment (1µM; 24hrs) was used as a positive control for apoptosis.

(b) SA-βgal analysis of cells in (a).

(c) SAHF analysis of cells in (a).

Figure S6: Cells expressing control vector alone do not show an increase in ROS contents.

(a) Cama-1 control cells were incubated with H_2DCF-DA (3µM; 1hrs) and analysed by FACS at the indicated time. H_2O_2 was used as a positive control.

(b) MDAMB-453 control cells were incubated with H_2DCF-DA (3µM; 1hrs) and analysed by FACS at the indicated time. H_2O_2 was used as a positive control.

(c) BT-20 control cells were incubated with H_2DCF-DA (3µM; 1hrs) and analysed by FACS at the indicated time. H_2O_2 was used as a positive control.

Figure S7: Cells expressing control vector alone do not show an increase in AnnexinV/PI staining.

(a) Cama-1 and MDAMB-453 control cells were AnnexinV/PI analysed by FACS at this indicated time.

Figure S8: The SKBR-3 cancer cell line is resistant to PLA2R1 induce ROS accumulation and apoptosis.

(a) Control and PLA2R1 expressing SKBR-3 were analysed for their ROS contents (H_2DCF-DA; 3µM-1hrs). H_2O_2 was used as a positive control. Unstained cells were used as negative controls.

(b) Western blot analysis of cells in (a).

(c) Colony assay of cells in (a).

Figure S9: PLA2R1 induces apoptosis in a myriad of cancer cell lines.

(a) Colony assay of control and PLA2R1 expressing breast cancer cells.

(b) Colony assay of control and PLA2R1 expressing kidney cancer cells.

(c) Colony assay of control and PLA2R1 expressing pancreatic cancer cells.

(d) Colony assay of control and PLA2R1 expressing melanoma cancer cells.

(e) Colony assay of control and PLA2R1 expressing colon cancer cells.

(f) Western blot analysis of control and PLA2R1 in a subset of cancer cell lines. Active caspases 3 was used as an apoptotic marker. PLA2R1 expression was also analysed. ACTB was used as a loading control.

(g) Colony assay formation of control and PLA2R1 expressing cells under different conditions of oxygen levels (20%, 5%, 2%).

Figure S10: PLA2R1 regulates the mitochondria redox state.

(a) The list of the targeted ROS producing complex and ROS. Additional information can be found in the materiel and methods section. The table contains the chemical used, their targets and the phenotype reversion potential. (-) no effects; (+) partial effects; (++) strong effects; (+++) very potent effects.

(b) The mitochondrial transmembrane potential ($\Delta\Psi_m$) of PLA2R1 expressing cells was measured by FACS analysis, at the indicated time, with the Rhodamine 123.

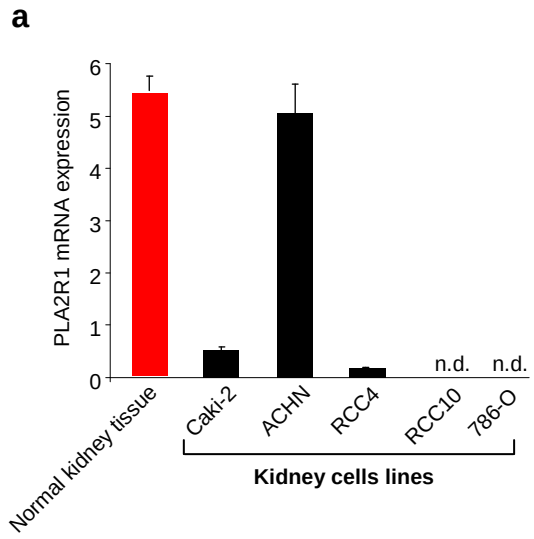
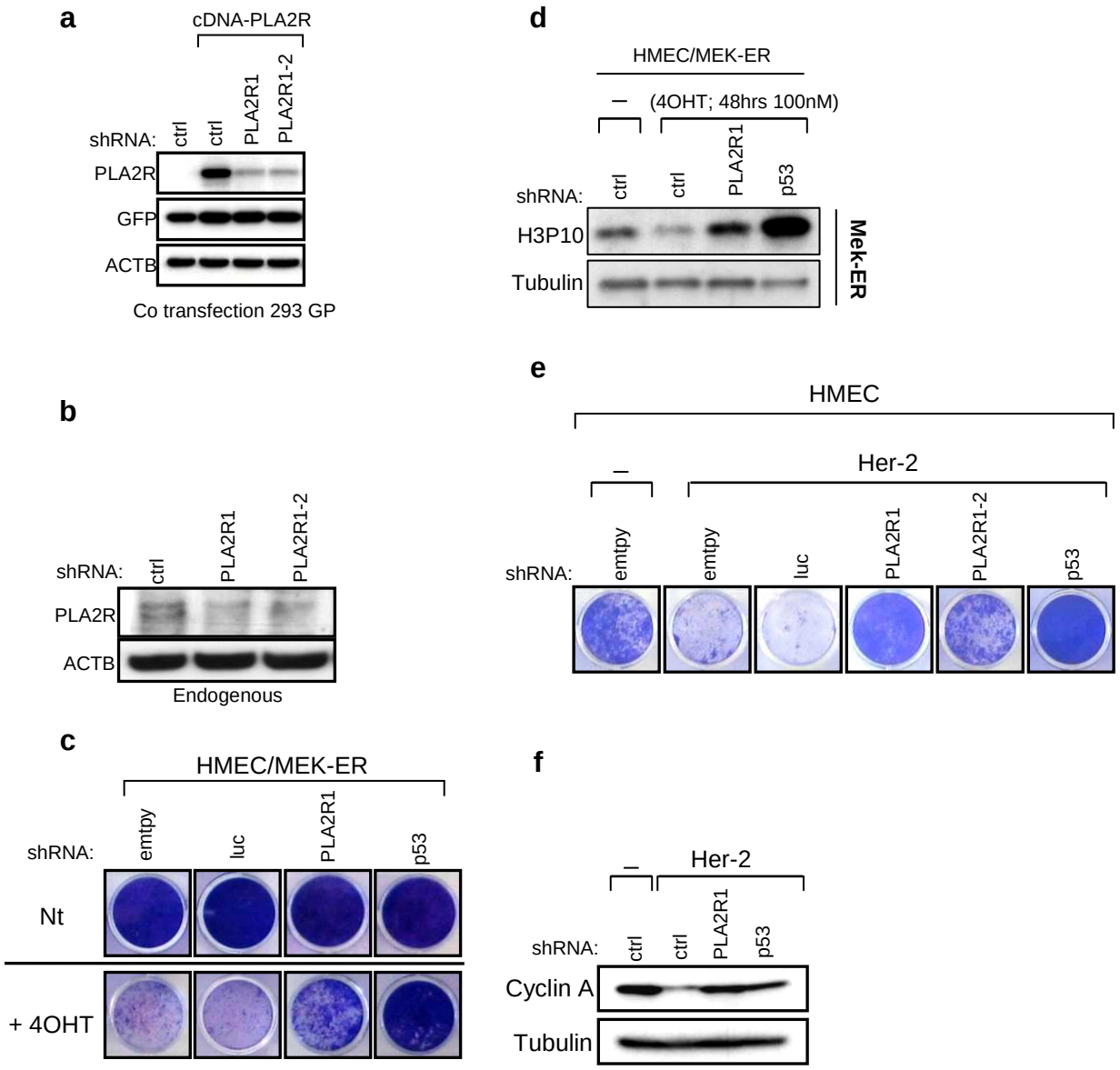


Fig S1



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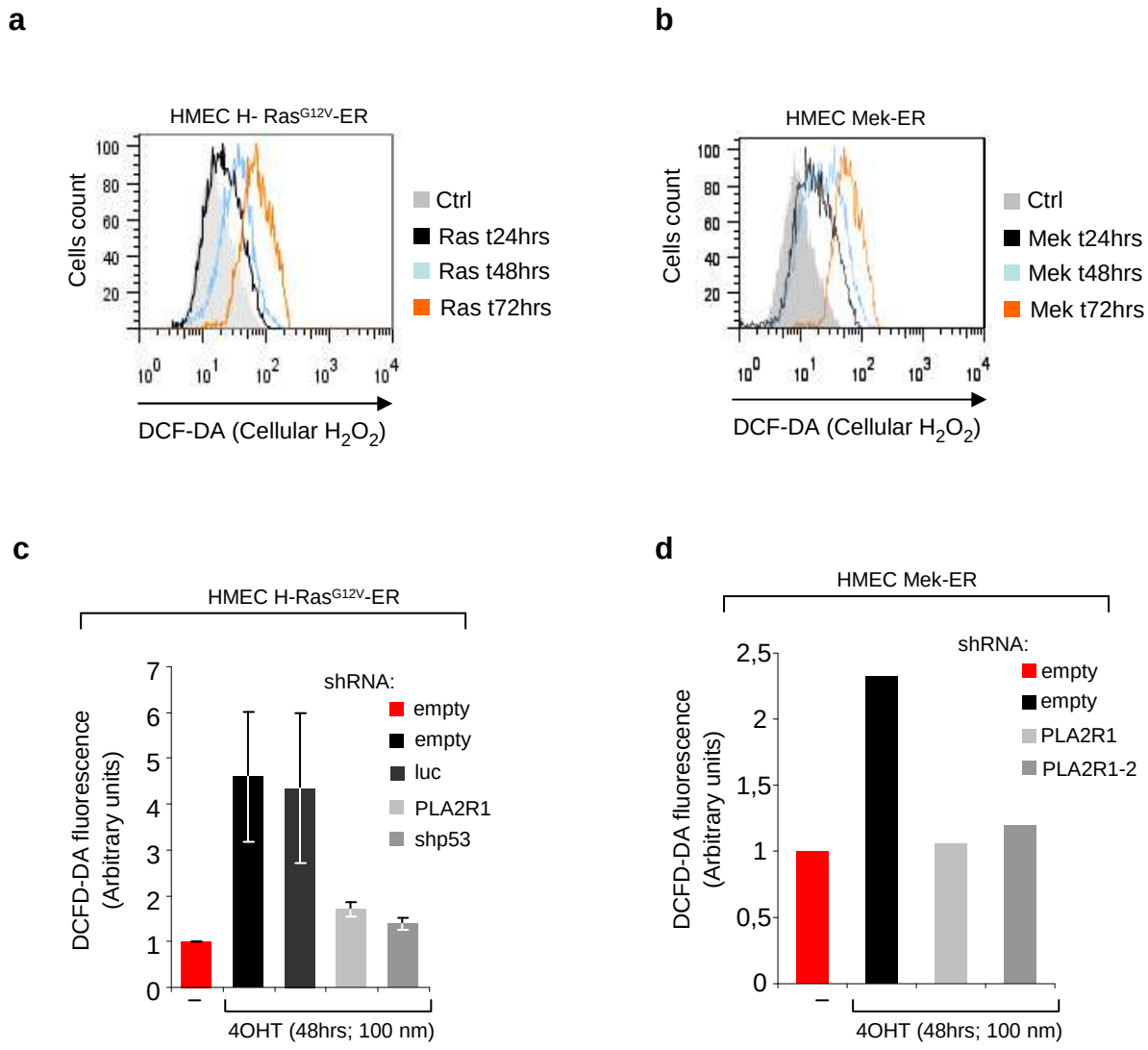

Fig S3

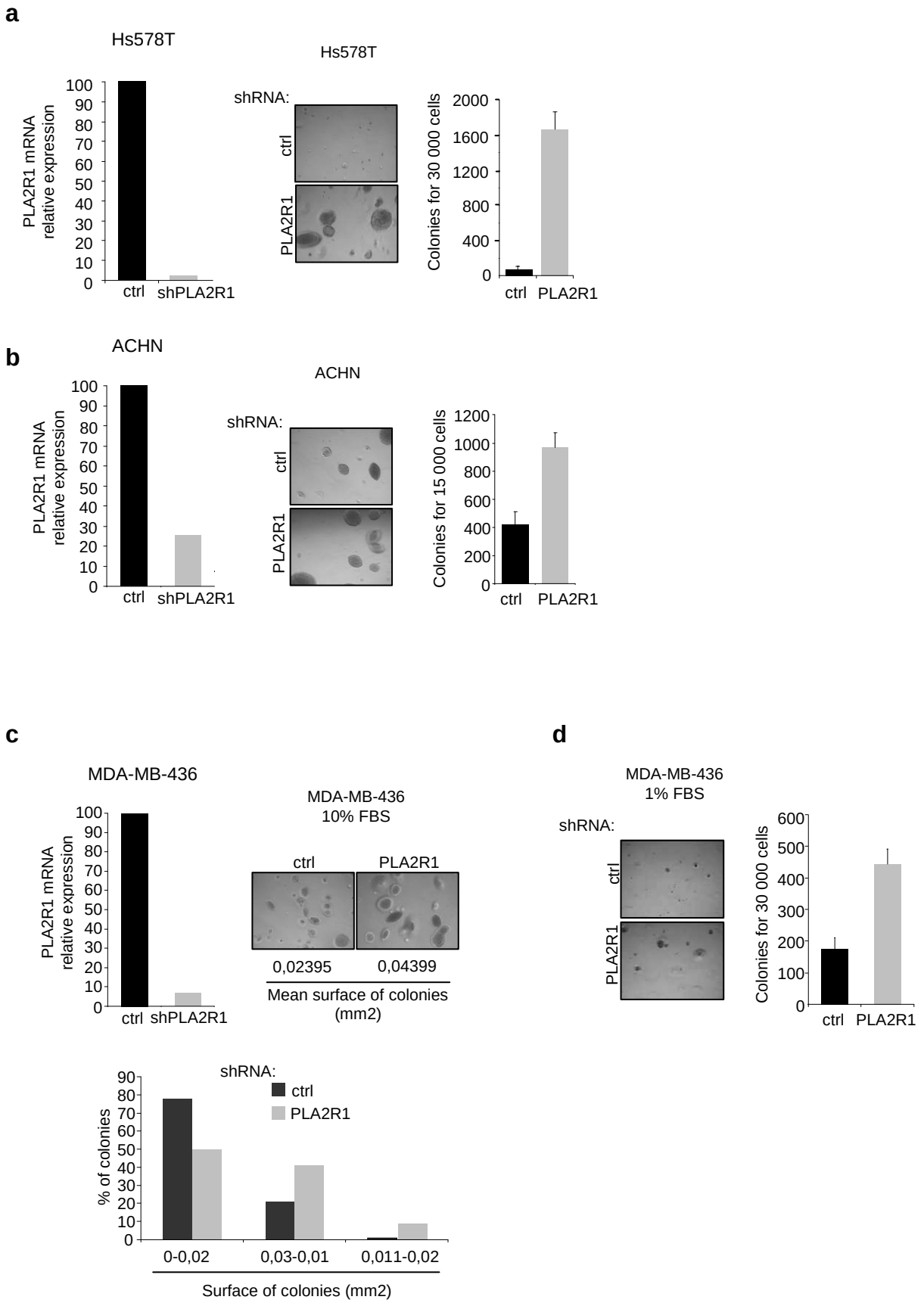

Fig S4

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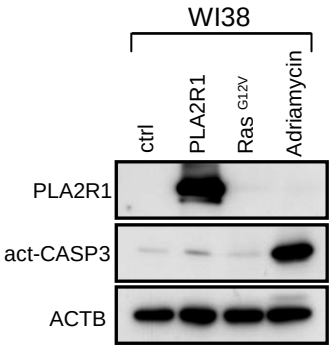
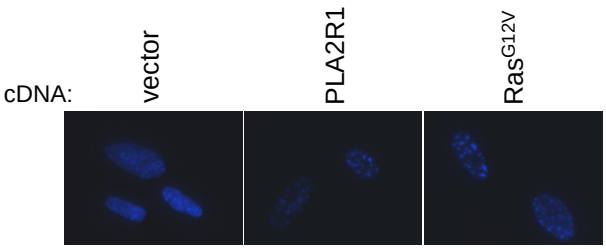


b

(Scale bar 50uM)

SA-βgal(%): 7% (+/-2) 50% (+/-7) 70% (+/-10)

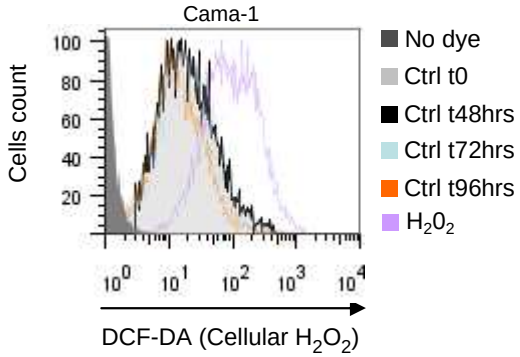
c



SAHF(%): 7% (+/-2) 35% (+/-4) 65% (+/-3)

Fig S5

a



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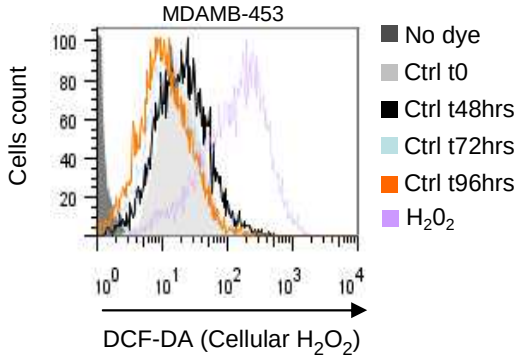

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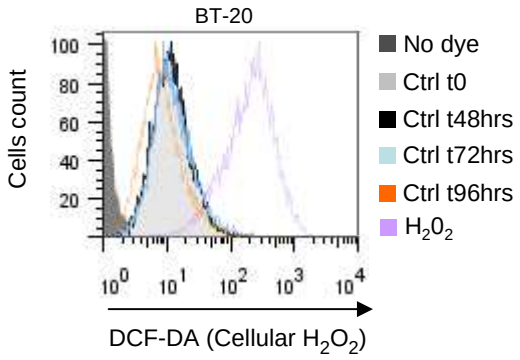

Fig S6

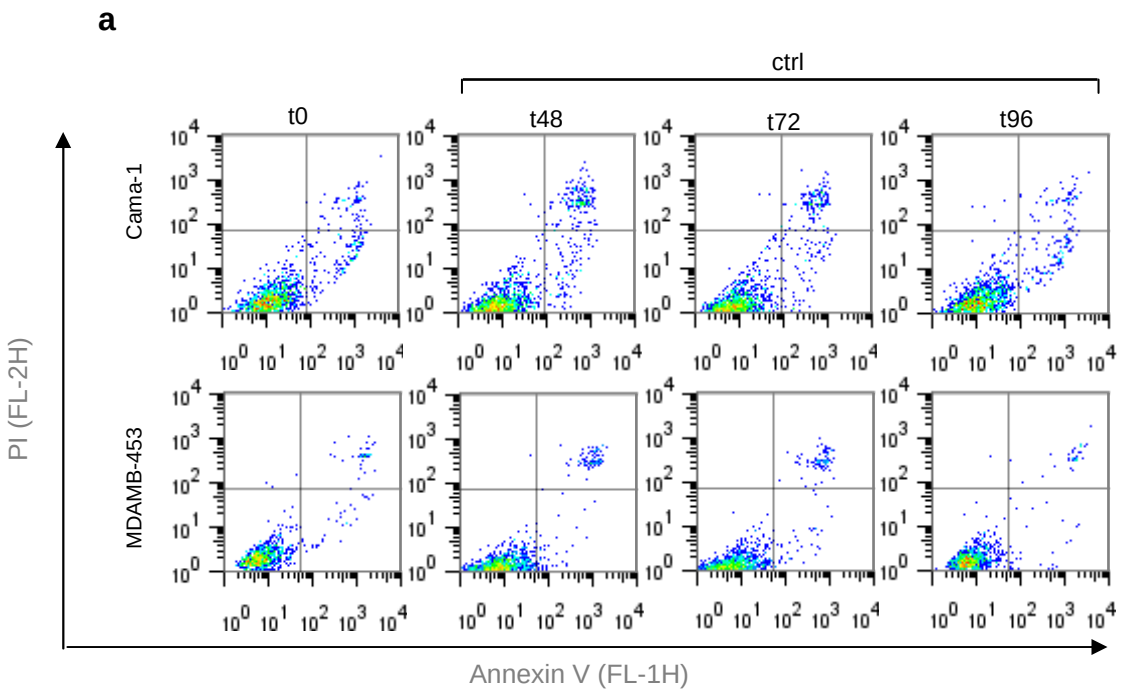

Fig S7

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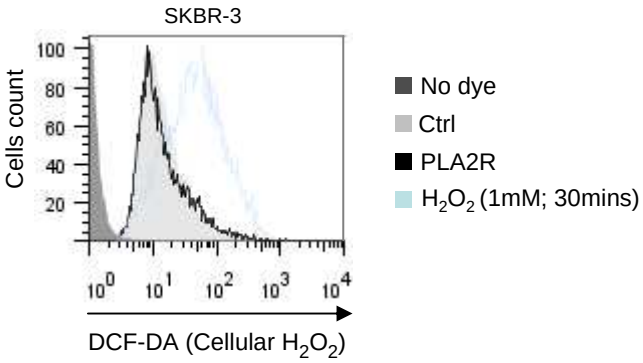

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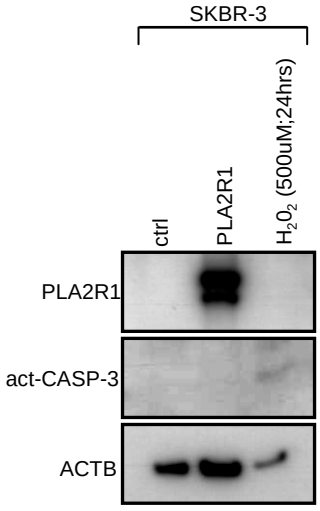

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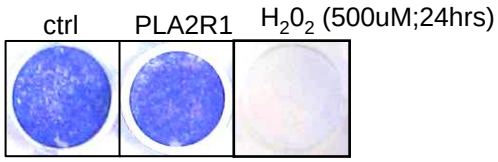

Fig S8

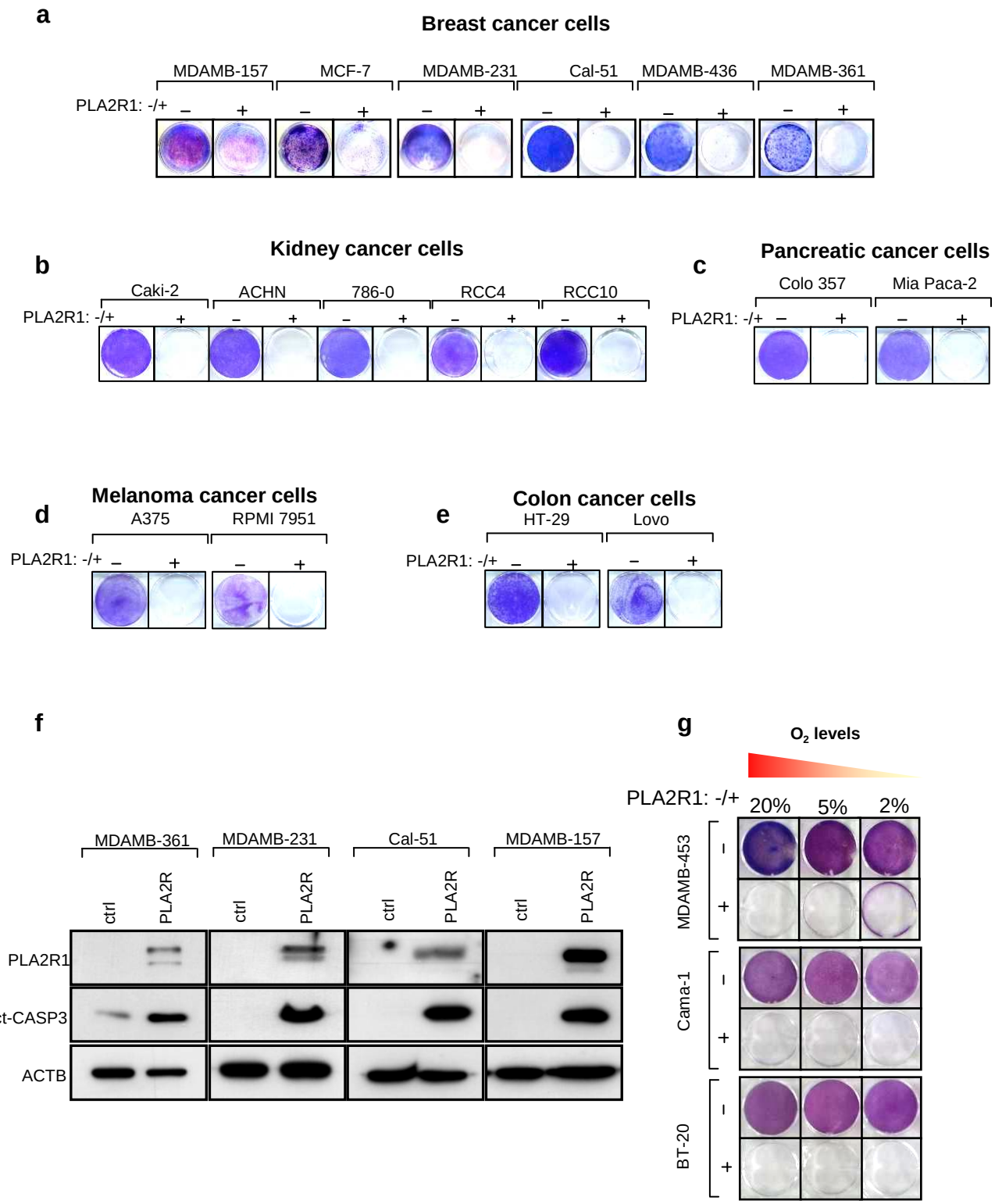

Fig S9

a

Targeted chemical screen (ROS- ROS producing complexes)

	Chemicals	target	Phenotype reversion
	Malate	Antioxidant	-
	Na-pyruvate	Antioxidant	-
	NADPH	Antioxidant	-
	Carboxy-PTIO	Nitric oxide	-
	Catalase	Antioxidant	-
	Tempol	Antixoidant	-
	Tiron	Antioxidant	-
	L-NMMA	iNOS	-
	L-NAME	iNOS	-
	L-NNA	iNOS	-
	Aminoguanidine hemisulfate	iNOS	-
	Ebselen	peroxinitrite	-
	FeTMPyP	Peroxinitrite	-
	MnTBAP	Peroxinitrite-O_2-	-
	NSC23766	Rac (NOX)	-
	Apocycin	NOX	-
Mitochondria	DPI	Mitochondria + NOX	+ +
Mitochondria	NAC	Antioxidant	+
Mitochondria	Rotenone	Mitochondria (Cplx I)	+ +
Mitochondria	Antimycin A	Mitochondria (Cplx III)	+ +
Mitochondria	Oligomycin	ATP synthetase –oxidative phoshphorlation	+ + +
	TFFA	Mitochondria (Cplx II)	-
	KCN	Mitochondria (Cplx IV)	-

b

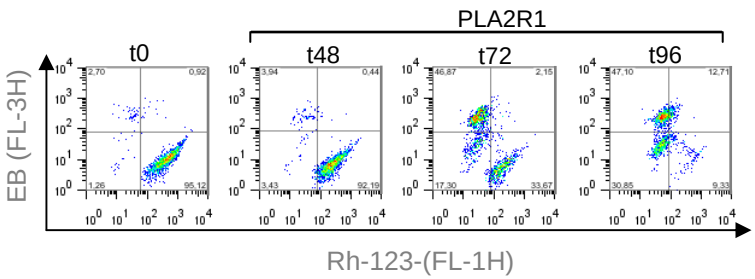

Fig.S10

CONCLUSIONS AND PERSPECTIVES

III/ CONCLUSIONS AND PERSPECTIVES

shRNA library screens and identification of several new cellular senescence regulators

The genetic screen enabled us to identify 11 new putative cellular senescence regulators. This technology, when applied to an adequate cellular model and used in a feasible manner (e.g for example to uncover regulators of a phenotype that can be induced by one single genetic event), is a power tool to decipher new functional genetic components of biological pathways [381, 455]. Indeed, such genetic screenings have been successfully performed in various contexts leading to the identification of new regulators/networks underpinning biological processes [381].

However there are also limitations to shRNA library screens. For example, as shRNAs can lead to "off target" effects, it is important to validate each "hit" by using at least two independent shRNAs [427]. During the "hits" validation process, identifying other shRNAs inhibiting the gene of interest was sometimes a major challenge. To confirm the effect observed with a 2nd shRNA, we opted for commercially available pSHAG MAGIC vectors (pSM2c) which are shRNAs containing retroviral vectors. Due to technical difficulties or poor knock-down efficiency, most of the vectors tested did not result in significant targeted gene down-regulation and cellular senescence bypass. Finally, compared to the NKI library that I used (theoretically targeting 8000 humans genes), it is also possible to target a greater number of genes as whole genome shRNA libraries are available [456].

Our validation method led us to select genes that determined cellular senescence fate in opposite ways depending on their expression (premature senescence was induced during

ectopic expression whereas senescence bypass was observed during gene depletion). This method did not preclude that other "hits" could also be cellular senescence regulators. For example, FBXO32 (an F-Box protein) which constitutes one of the subunits of the ubiquitin protein ligase complex called SCFs (SKP1 cullin-F-box) did not meet our validation criteria. Nonetheless, FBXO32 was found to be negatively regulated by polycomb proteins [457] that are known cellular senescence repressors. Moreover, its promoter was found hypermethylated in cancer cells and restored FBXO32 expression led to cancer cell apoptosis suggesting tumour suppressor activities [458]. Among the other "hits", MED12 (mediator complex subunit 12) was found to be in a positive co-regulator complex of p53 target genes [459] suggesting that it could also control cellular senescence fate. Altogether, even if we have excluded some genes based on our initial validation method, it is possible that other "hits" could be true cellular senescence regulators.

Regulation of PLA2R1 expression

We have provided compelling evidence that PLA2R1 is a critical regulator of cellular senescence. However major questions persist. Among others, how is PLA2R1 expression regulated? We have shown that PLA2R1 mRNA levels increase during "replicative senescence" suggesting the involvement of a transcriptional regulation.

Identifying components of PLA2R1 transcriptional regulation could shed light on the regulatory process governing PLA2R1 activities not only in cellular senescence but also in various biological responses mediated by PLA2R1. Putative targets include polycomb proteins. Polycomb members, as previously mentioned, are involved in the repression of a subset of cellular senescence regulators and decrease during cellular senescence [127, 270, 271].

Promoter sequence analysis predicted binding sites for NF-Kβ, Rel A and C/EBPβ which are crucial regulators of the pro-inflammatory network associated with cellular senescence [98, 124]. Interestingly, PLA2R1 has been linked to cytokines production [398]. It is therefore possible that during cellular senescence NF-Kβ family members and/or C/EBPβ activate PLA2R1 transcription resulting in pro-inflammatory cytokines production which could in turn be involved in cellular senescence induction.

Considering the data we have accumulated so far on PLA2R1, promoter sequence analysis also highlighted the potential binding of 2 very interesting transcriptional factors (Nrf2 and FOXM1). Both transcription factors can be activated in response to constitutive oncogenes (e.g Ras^{G12V}) to activate an antioxidant program associated with cellular senescence bypass and tumorigenesis [210, 451]. One could hypothesize that these antioxidant promoting transcription factors regulate ROS by activating antioxidant mediators (as it has been demonstrated) but also by repressing pro-oxidant genes such as PLA2R1. In accordance, FOXM1 has been associated with transcriptional repression [460]. However the transcriptional repression associated with Nrf2, if any, has not been demonstrated so far.

The transcriptional regulation of PLA2R1 might also play important role during later stages of tumour development. We have recently shown that PLA2R1 promoter was hypermethylated in cancer cell lines and was associated with PLA2R1 inhibition. We are currently trying to determine whether PLA2R1 is also found hypermethylated in cancer tissues which is an important step to establish whether PLA2R1 promoter hypermethylation is a physiological process acquired during tumorigenesis. If such is the case, it could be interesting to identify the mechanism underlining this methylation.

DNA methyltransferases (DNMTs) are major enzymes involved in DNA methylation [461]. We have demonstrated that chemically inhibiting DNMTs with 5-aza-2'-deoxycytidine (5-aza) in cell lines where PLA2R1 promoter is methylated results in PLA2R1 re-expression. It would therefore be interesting to determine which DNMTs are involved in the methylation of PLA2R1 promoter. Specific genetic inhibition of DNMT-1 (the key DNA methyltransferase involved in maintenance and *de novo* methyltransferase) but also the specific inhibition of *de novo* methyltransferase DNMT3a and 3b should shed light on the mechanism involved in the methylation of PLA2R1 promoter. In the last years, it has been demonstrated that the DNMT machinery can be directly recruited by transcription factors [462]. It will thus be interesting to identify the mechanism involved in DNMTs recruitment.

Alternatively and/or additionally, large scale projects could lead to the discoveries of PLA2R1 unexpected regulators. For example, a RNAi screen could be performed in cancer cells lines that posses a hypermethylated PLA2R1 promoter (e.g MDAMB-453) and PLA2R1 expression could be monitored. In this context, the knock-down of PLA2R1 epigenetic repressors would lead to PLA2R1 re-expression. In contrast to PLA2R1 hypermethylated cells, both forward (cDNA) and reverse (siRNA) genetic screenings in normal cells, could lead to the identification of positive and negative PLA2R1 transcriptional regulators. As our results suggest that the transcriptional and epigenetic regulation of PLA2R1 might be important in the regulation of PLA2R1 functions, it will be crucial to identify its components.

PLA2R1/sPLA2 relationship and new alternatives mechanisms

We have demonstrated that several sPLA2s are found up-regulated during cellular senescence [424]. Additionally, the ectopic expression of sPLA2-IIA (PLA2G2A) resulted in

premature senescence [424]. In accordance with this result, Kim and colleagues demonstrated later during the year that sPLA2-IIA ectopic expression also resulted in cellular senescence of their model of interest [463]. They also demonstrated that cellular senescence was not limited to sPLA2-IIA but could also be induced by a recombinant sPLA2-IB protein [463]. These results were in accordance with ours suggesting a role of sPLA2s during cellular senescence. Although Kim and colleagues had previously demonstrated, in conformity with our work, that PLA2R1 is up-regulated during cellular senescence [123], they did not investigate the role of the receptor in sPLA2 mediated cellular senescence [463]. Additionally, they did not demonstrate whether it was the sPLA2s enzymatic activity or their binding properties that was responsible for their pro-senescent effect.

We found that PLA2R1 depleted cells were resistant to sPLA2-IIA pro-senescent effects [424]. This was rather surprising because sPLA2-IIA, which is a ligand of mouse PLA2R1, does not seem to bind human PLA2R1 [464]. These observations suggest that an indirect interplay might exist between sPLA2-IIA and PLA2R1. However to date, we were unable to identify the missing link. Kim and colleagues demonstrated that sPLA2-IB induced ROS to trigger cellular senescence [463]. It is therefore plausible that sPLA2-IIA acts through the same pathway. In conclusion, it is possible that PLA2R1 and sPLA2s activate the same pathways to generate synergic effect.

The effect of sPLA2s during cellular senescence might not be limited to extracellular interaction with a receptor. sPLA2s could also exert intracellular actions. Indeed, sPLA2-IB was found to be internalised and transported to the nucleus [422]. Additionally, unlike Kim and colleagues, we were unable to induce premature senescence with the purified sPLA2s (sPLA2-IB, sPLA2-IIA) protein recombinant tested (data not shown). In our hands, we were

only able to detect an effect when sPLA2s were ectopically expressed. This could be due to the fact that we did not use the right doses of recombinant proteins and/or did not repeat the treatment as often as we should have. Nonetheless, it also suggests that sPLA2s may also have actions in the intracellular component of the cell.

The PLA2R1/sPLA2 relationship is quite complex as mentioned above. Our recent results bring additional complexities to this relationship. We were surprised by the fact that inhibitors of sPLA2/cPLA2/iPLA2 and their related pathways had no effects on PLA2R1 induced cell death. PLA2R1 mutants experiments later confirmed that this receptor seem to exert its tumour suppressive function in a sPLA2s independent fashion. Indeed, PLA2R1 CTLD4-5-6 domains have been identified as the sPLA2 binding sites with a crucial role of CTLD5 [406]. PLA2R1 domain deletion experiments revealed that these domains were not required for PLA2R1 mediated cell death. Altogether it suggests that PLA2R1, can at least in some contexts, exert biological functions independently of sPLA2s.

Alternative ligands of the receptor might also involve sugars. Indeed, it has been demonstrated that PLA2R1 can bind sugars such as mannose and galactose [395, 406]. This could limit sugar availability which in turn might lead to cellular stress and ROS production. However sugar binding has been mapped on the CTLD5-6-7-8 at least for the rabbit PLA2R1 [395, 406].

The fact that PLA2R1 domains required for cancer cell death involves alternative domains than the ones implicated in ligand recognition so far raises a multitude of questions. For example, can PLA2R1 bind other ligands in its N terminal domains to mediate its effects? Does PLA2R1 bind with other receptors or transmembrane proteins? PLA2R1 also exists as a

soluble form. Therefore, can soluble PLA2R1 exert tumour suppressive actions just like the membranous form? If so, can it act as a ligand itself?

While waiting for these results, we have tried to answer some of the questions ourselves by using alternative methods. We have performed additional PLA2R1 mutants. Interestingly, soluble PLA2R1 was still able to induce cell death (data not shown). Moreover, a soluble form of PLA2R1 containing the N terminal part (Cys-R/FN-II/CTLD1-2-3) which should not bind to sPLA2s, was still able to induce cell death (data not shown). Altogether, these results suggest that PLA2R1 could potentially also act as a ligand. This is an appealing possibility and the production and the use of a recombinant protein, corresponding to the shortest soluble PLA2R1 mutant that still has an effect, could provide interesting insights.

Finally, we are currently testing the effect of the three other mannose receptor family members (MR, Endo180, DEC-205) on ROS production and apoptosis and are also testing additional PLA2R1 mutants in order to identify, if possible, the shortest domain(s) responsible for its tumour suppressive actions. Altogether, these various approaches should lead to the identification of the mechanism underlining PLA2R1 functions.

PLA2R1-ROS relationship

We analysed candidate signaling pathways that could mediate ROS production by PLA2R1. Recent observations suggest that Akt can induce ROS accumulation [287]. We investigated this pathway through the use of LY294002, an inhibitor of the PI3K/Akt pathway. LY294002 did not inhibit PLA2R1 mediated response suggesting that this pathway was not involved in the phenotype observed (data not shown). Moreover inhibition of Rac

with NSC23766, a PI3K activated protein that in turns leads to ROS production [43], had no effect on PLA2R1 mediated responses. Finally, as the MAPK pathway activation can also lead to ROS accumulation [283, 435] we checked if this pathway was involved in PLA2R1 mediated effects. Pharmacological inhibition of MEK by U0126 had no effect. Taken together, it highlights that signalling pathways triggered by PLA2R1 leading to ROS generation are yet to be determined. Lipidomic and metabolomic might be interesting approaches to unravel new pathways regulated by PLA2R1 and identify missing links between PLA2R1 and ROS.

As we have demonstrated a critical role of mitochondria in PLA2R1 induced ROS accumulation, analyzing the mitochondrial activity in PLA2R1 over-expressing cells could give interesting insights. For example, PLA2R1 ectopic expression could lead to mitochondrial oxygen consumption. To test this hypothesis, we are currently collaborating with a team that will analyse oxygen consumption from isolated mitochondria. PLA2R1 induced ROS accumulation might not be limited to mitochondria oxygen consumption but could also results from alternative regulations. For example, PLA2R1 could have an impact on mitochondrial biogenesis. Taken together, our results indicate that the mechanisms by which the mitochondria are activated to produce ROS in response to PLA2R1 are still unclear. Additionally, although mitochondria's role in PLA2R1 modulation of ROS levels seems undeniable, we cannot exclude at this stage that ROS increase may also be the result of a decrease in antioxidant enzymes or redox modulating enzymes (e.g glutathione (GSH), glutathione peroxidase (GPX), catalase). Measuring the levels/activity of antioxidant enzymes should give interesting insights.

As previously mentioned, ROS can cause lethal cellular alterations including oxidative damage to proteins, lipids, and DNA [465]. We have demonstrated that PLA2R1 resulted in mitochondrial cardiolipin oxidation. Alternatively, we could also analyse DNA oxidation by measuring the level of 7,8-dihydro-8-oxo-29-deoxyguanosine (8-oxo-dGuo), one of the major products of DNA oxidation [429]. We took a first step trying measure DNA oxidation by looking at the DNA damage sensor protein H2AX. We found that activated H2AX (γH2AX) levels increase following PLA2R1 expression, however its increase is cell type specific suggesting that some cells might die of alterative oxidative damage. However, as this analysis is quite indirect, it would be more informative to analyse directly the oxidative DNA damages (e.g 8-oxo-dGuo). Finally, as proteins are major targets of oxidative damages [466], it will be interesting to analyse proteins oxidative damages in a context of PLA2R1 expression. Western blot, mass spectrometry and high pressure liquid chromatography (HPLC) could be used to identify the oxidised proteins.

We wanted to analyse the pathways activated by ROS in a PLA2R1 context. In primary cells, the DNA damage/p53 pathway was shown to be a critical regulator of PLA2R1-ROS induced cellular senescence [424]. Interestingly, in cancer cells lines, PLA2R1 does not require a functional p53 pathway to trigger cell death. Indeed, most of the cell lines tested had a non functional p53 pathway [467]. This is not surprising because high amount of ROS can cause lethal cellular alterations including oxidative damage to proteins, lipids, and DNA [465]. Such cellular damage might not be controlled by a single pathway. In accordance, ROS can trigger cell death independently of the p53 pathway [437, 468].

Other signaling pathways mediating ROS induced cell death implicate the stress kinases. Pharmacologically inhibiting stress kinases such as the c-Jun N-terminal kinase

(JNK) with SP600125 and p38MAPK with SB239063 or SB203580, which are known as ROS induced cell death mediators, had no effect. Additionally, inhibiting caspases activation with Z-VAD-FMK only partially reverses PLA2R1 induced cell death (data not shown). These results suggest that if a cell death program is blocked in response to PLA2R1-ROS induced damages, cells can die by alternatives cell deaths. Taken together, this highlights that once ROS are produced in high amount, the signalling pathways that mediate their effect might be numerous, non exclusive and hard to counteract.

PLA2R1 mediated response Cellular senescence vs Apoptosis

We have observed that PLA2R1 preferentially trigger cellular senescence in primary cells whereas it is associated with massive cell death in cancer cells. Various hypotheses can be formulated to explain such difference. First it is known that cancer cells display higher ROS levels [231]. Indeed, oncogenic activation has been associated with increase ROS levels [435, 440]. PLA2R1 ROS accumulation in cancer cells could therefore lead to a higher ROS output than in normal cells [231]. We have observed that PLA2R1 ectopic expression in normal cells leads to ROS accumulation but to a far lesser extend than in cancer cells (data not shown). As for now, we do not have an explanation for this. The fact that PLA2R1 induces cancer cell death can also be explained because cancer cells usually proliferate faster than normal cells. ROS induced damages could lead to more drastic phenotype in highly proliferative cells. Finally, it is also possible that normal cells have better “reparation systems” to cope with ROS induced damages.

In contrast to normal cells in which we have demonstrated a critical role of the DNA damage/p53 pathway in PLA2R1 induced cellular senescence, we have not analysed the

molecular pathways following PLA2R1 induced apoptosis in cancer cell lines. Apoptosis can be activated by two main pathways which include the intrinsic and/or extrinsic pathway [434]. Western blot analysis of PLA2R1 expression kinetic suggests that the caspases of both intrinsic and extrinsic pathways are activated (data not shown). Moreover, the analysis of the cytochrome C release from the mitochondria and levels of pro and anti apoptotic members (which determine cytochrome C release) has not been performed yet but would be informative. Indeed, it has been proposed that ROS can have an impact on the levels of pro and anti apoptotic family members [469, 470]. In line with this, we are currently testing if anti apoptotic proteins can reverse PLA2R1 induced cell death.

PLA2R1 physiological role as a tumour suppressor

Our results obtained *in vitro* and *in vivo* suggest a role of PLA2R1 as a tumour suppressor gene. However functional studies *in vivo* are yet to be performed. In order to analyse this functional role, we will study the phenotypes associated with PLA2R1$^{-/-}$ knockout mice. In 1997 Hanasaki and colleagues analysed basic phenotypes associated with PLA2R1$^{-/-}$ mice. They found that they were viable, sterile and necropsy and microscopic examination of major tissues revealed no evident histopathological abnormalities [416]. However the PLA2R1$^{-/-}$ knockout mice were not analysed in a context of tumour development. It will therefore be interesting to study PLA2R1$^{-/-}$ knockout mice in that context. Various experiments could be carried out such as the crossbreed of these mice with cancer prone mice models. Indeed, it will be interesting to know whether PLA2R1$^{-/-}$ knockout mice in a context of oncogene driven tumorigenesis (e.g Her-2 in mammary tumours) can lead to a quicker escape from cellular senescence and favour/increase tumour development.

Additionally, the susceptibility of PLA2R1$^{-/-}$ mice to develop tumours in chemically induced carcinogenesis experiments will bring additional insights into the tumour suppressive actions of PLA2R1 [471]. Skin treatment with 7,12-dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13- acetate (DMBA/TPA) leads to a constitute active form of H-Ras and cellular senescence [295]. Cellular senescence is eventually bypassed leading the tumour progression. It will therefore be interesting to analyse in that context whether PLA2R1$^{-/-}$ mice develop tumours quicker than control cells.

PLA2R1 physiological role in other biological responses

It has been proposed that PLA2R1 could have a role in the pathogenesis of idiopathic membranous nephropathy [430-432]. As the functional role of PLA2R1 in that disease has not been studied, adequate mouse models could be used. However PLA2R1$^{-/-}$ mice could also be used to study other biological responses such as wound healing and ageing as both biological processes have been associated with cellular senescence [262, 378]. PLA2R1$^{-/-}$ mice could be treated with chemical agents and/or physical agents to induce liver injury or cutaneous injury [43, 44]. It would be interesting to test whether PLA2R1$^{-/-}$ mice could have defects in wound healing. It is unclear whether or not Hanasaki and colleagues analysed ageing phenotype of PLA2R$^{-/-}$ mice [416]. It will also be interesting to analyse whether PLA2R1 insufficiency could cause the early onset of ageing in mice.

PLA2R1 as a putative anti-tumoral compound and a cancer biomarker

We recently filled a patent for PLA2R1 potential use as a cancer biomarker and an anti-tumoral compound (N° 11305885.3 in Europe). To date, we have only been able to detect

PLA2R1 at the mRNA levels which limits its use as a biomarker. However, with new available sources of antibodies [430], we are hoping we will be able to detect PLA2R1 at the protein level. Immunohistochemistry detection of PLA2R1 is currently being performed.

PLA2R1 could also have putative anti-tumoral effects. Indeed, we have recently demonstrated that it preferentially triggers massive cell death in cancer cells whereas it tends to induce a cell cycle arrest in normal cells. Several strategies could be adopted. In a foreseeable future, gene therapy with retroviral vectors expressing PLA2R1 could be used. However to date, the therapeutic use of this strategy is limited and major progress still have to be made. Nonetheless, the first clinical trails with such approaches have already begun to take place [472].

An alternative strategy could be to use a PLA2R1 recombinant protein. Such approach is appealing but before being launched, we are trying to identify the shortest PLA2R1 domain responsible for its tumour suppressive activities. The generation and testing of new mutants is currently underway and should help us identify this domain. In our hands and on the cell lines tested, we have found that PLA2R1 is a more potent inducer of cell death than the master tumour suppressor p53 (data not shown). It has recently been proposed that p53 reactivation in early stages tumours can lead tumour regression [164, 349] but in later stages is not sufficient to counteract tumour progression [473]. The fact that PLA2R1 seems to target cancer cell lines with a cancer genotype rather than an oncogene addiction suggests that its spectrum of action could be larger than classical tumour suppressors.

Taken together, the work performed during my thesis has enabled us to identify PLA2R1 as an unexpected cellular senescence regulator and a putative tumour suppressor

gene with therapeutic potential. As to date, many gaps remain to be filled to understand PLA2R1 biological role.

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IV/ REFERENCES

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ANNEXES

V/ ANNEXES

Priority Report

A Genetic Screen Identifies Topoisomerase 1 as a Regulator of Senescence

Nicolas Humbert,[1] Sébastien Martien,[1] Arnaud Augert,[1] Marco Da Costa,[2] Sébastien Mauen,[3] Corinne Abbadie,[1] Yvan de Launoit,[1] Jesús Gil,[2] and David Bernard[1]

[1]UMR8161, Institut de Biologie de Lille, Centre National de la Recherche Scientifique/Universités de Lille 1-2/Institut Pasteur de Lille, IFR142, Lille, France; [2]Cell Proliferation Group, Medical Research Council Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Campus, London, United Kingdom; and [3]Moores Cancer Center, University of California San Diego, La Jolla, California

Abstract

Normal cell growth can be permanently blocked when cells enter a state known as senescence. This phenomenon can be triggered by various stresses, such as replicative exhaustion, oncogenic stimulation, or oxidative stress. Senescence prevents transmission of aberrant signals to daughter cells and thus prevents irreversible damage that could favor cancer development. To identify new genetic events controlling senescence, we have performed a loss-of-function genetic screen on normal human cells. We report that knockdown of topoisomerase I (Top1) results in an increased replicative potential associated with a decrease in senescence markers and a diminished DNA damage response. In addition, Top1 depletion also favors a bypass of oncogene-induced senescence. Conversely, Top1 constitutive expression induces growth arrest, the appearance of a senescence marker, and an activation of the DNA damage response. Altogether, these results reveal an unanticipated function of Top1 in regulating senescence. [Cancer Res 2009;69(10):4101–6]

Introduction

Cancer cells derive from normal cells that accumulate genetic and epigenetic alterations. During this process and among other steps, normal cells acquire immortality and enhanced stress resistance (1). Cellular senescence was originally described as a replicative potential limit of normal cells. It has been redefined recently as a cellular stress response program occurring in normal cells and occasionally in cancer cells, depending on the context. It is now widely accepted that the genetic events triggering a bypass of cellular senescence favor tumorigenesis by extending the replicative potential, rendering cells more resistant to cellular stresses, or both (2).

The p53 pathway is a major regulator of senescence and tumorigenesis. During tumorigenesis, multiple cooperating genetic events can result in inactivation of the p53-tumor suppressor pathway (2). One major activator of the p53 pathway is induction of DNA damage. Interestingly, the DNA damage response pathway is activated during senescence and its loss allows senescence escape and is an early step required for tumor development (3–5).

To identify new loss-of-function genetic events involved in the control of cellular senescence, we have performed a genetic screen on normal human diploid fibroblasts (HDF), using an shRNA library. We have thus identified topoisomerase 1 (Top1) as a regulator of senescence, revealing a new function for an old target of cancer treatment.

Materials and Methods

Cell culture and vectors. WI38 and IMR-90 cells (American Type Culture Collection) and packaging GP 293 cells (Clontech) were cultured in DMEM (Invitrogen) supplemented with 10% FCS (Invitrogen) and gentamicin (Invitrogen). The pRS retroviral library was prepared from the pRS library (6). The shRNA sequence inserted into the pRS to generate pRS/Top1.2 was 5′-GGTTCCATTAAATACATCA-3′. The 2nd shRNA used was purchased and used as recommended (V2HS_171464; Open Biosystems). The pMC-GFP-Top1 vector was kindly provided by Dr. M.O. Christensen (Institute of Clinical Chemistry and Laboratory Diagnostics, Dusseldorf, Germany; ref. 7).

Tansfection, infection, and genetic screening. GP 293 cells were transfected using PEI reagents (Euromedex). Three days after transfection, viral supernatant mixed with fresh media (1/2) and polybrene (8 μg/mL) was used to infected target cells. In the genetic screening experiments, WI38 cells close to senescence were infected with the pRS control retroviral vector or with the pRS library (6) and infected cells were selected by puromycin treatment at 500 ng/mL. The selection pressure was maintained at 200 ng/mL. WI38 cells were split every week for 3 wk, and the gDNA of proliferating cells was prepared. Nested PCRs were performed with the following primer pairs: PCR1 reverse 5′-GAGACGTGCTACTTCCATTTGTC-3′ and forward 5′-CCCTTGAACCTCCTCGTTCGACC-3′, PCR2 reverse 5′-TGTGAGGGACAGGGGAG-3′ and forward 5′-ACCTCCTCGTTCGACCC-3′. A PCR (20 cycles) was performed with primer pair PCR1, and 1% of this PCR reaction product was then subjected to a 20-cycle PCR with primer pair PCR2. The PCR product was cloned with the help of the TOPO TA cloning kit (Invitrogen) and subjected to sequence analysis.

Colony formation assays. In the WI38 or IMR-90 colony formation assays, 90,000 cells were seeded and left to grow for between 1 and 2 wk. Then the cells were fixed with 4% formaldehyde and stained with crystal violet. In the case of U2OS cells, 200,000 cells were seeded into 10-cm dishes 1 d after transfection and selection was done with 500 ng/mL puromycin for 2 d. The cells were fixed and stained 10 d after seeding. Experiments were at least performed independently twice.

Senescence analysis. When cells acquired typical morphology of senescent cells, analysis of senescence markers was performed. Senescence-associated β-galactosidase activity analysis was performed as described by Dimri and colleagues. (8). For senescence-associated heterochromatin foci analysis (9), cells were fixed by formaldehyde 4%, wash with PBS and stained with Hoechst (Sigma). DNA staining was examined under a Zeiss fluorescence microscope.

Immunofluorescence, immunoblotting, staining, and antibodies. For immunofluorescence, cells were fixed with 4% paraformaldehyde in

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: David Bernard, Centre National de la Recherche Scientifique UMR 8161, Institut de Biologie de Lille, 1 rue du Pr Calmette BP 447, 59021 Lille Cedex, France. Phone: 33-3-20-87-11-27; Fax: 33-3-20-87-11-11; E-mail: david.bernard@ibl.fr.

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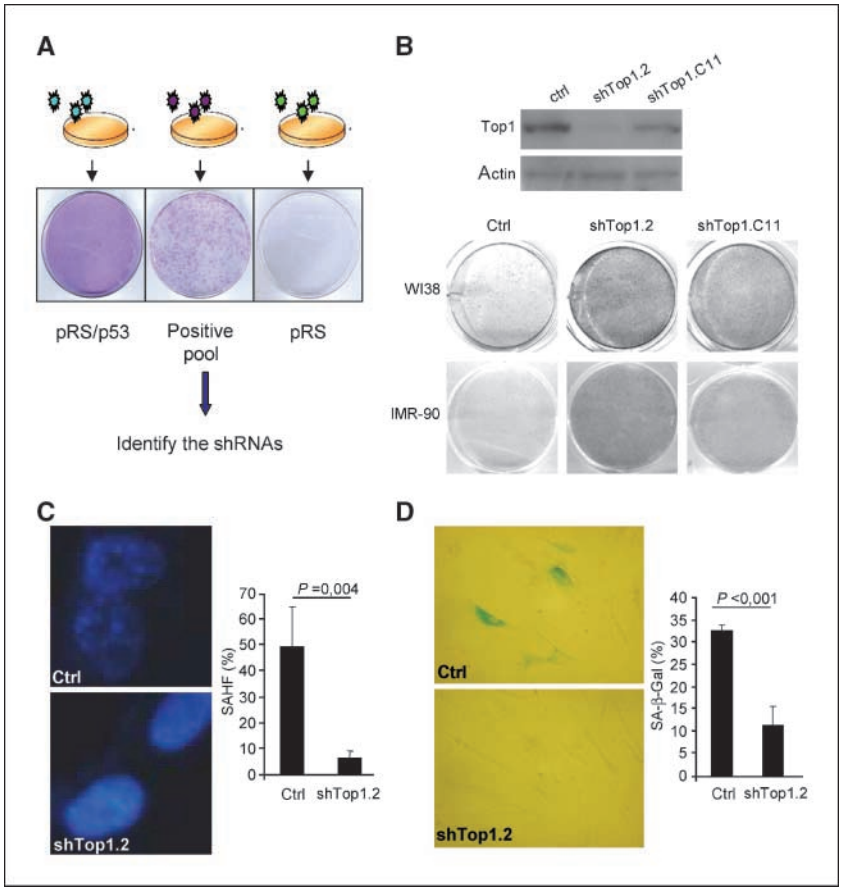

Figure 1. Top1 down-regulation extends the cellular life span. *A,* summary of the genetic screen. *B,* WI38 cells were infected with retroviral vectors encoding an shRNA directed against Top1 or a control vector. After selection, cell extracts were prepared and resolved. The Top1 protein level was revealed using a Top1 antibody and an actin antibody was used as a loading control (*ctrl*). Alternatively after selection, WI38 and IMR-90 were seeded at low density. After 2 wk, the cells were crystal violet stained. *C,* WI38 cells were infected with the indicated retroviral vector and puromycin selected. When cell growth was starting to slow down, senescence-associated heterochromatin foci (*SAHF*) marker was analyzed. *D,* analysis of senescence-associated β-galactosidase marker (*SA-β-Gal*).

PBS, permeabilized with 0.2% Triton X-100 and blocked in PBS/serum 10%. Briefly, cells were incubated with a primary antibody followed by a secondary antibody coupled to Rhodamin (Amersham). Nuclei were stained with Hoechst 33258 at 1 mg/mL for 3 min.

For immunoblotting, cell extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The primary antibodies used were anti-Top1 (556597; BD Biosciences), anti–phospho-p53 (9284; Cell Signaling), anti–phospho-ATM (4526; Cell Signaling), anti-p53 (sc-126; Santa Cruz Biotechnology), anti–phosphoSer10-H3 (ab14955; Abcam), anti-p21 (sc-397; Santa Cruz Biotechnology), and antiactin (A5316; Sigma-Aldrich). The corresponding peroxidase-labeled secondary antibody was detected with Western Lighting Chemiluminescence Reagent Plus (NEL103; Perkin-Elmer).

Comet assay. For each condition, 10,000 cells were embedded in 80 μL of 0.5% low-melting-point agarose at 37°C, and the suspension was immediately pipetted onto a TREVIGEN, Inc., cometslide. Agarose was allowed to solidify at 4°C for at least 30 min. The slides were immersed in prechilled lysis solution [2.5M NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, and 1% Triton (pH 10)] and left at 4°C for 90 min in the dark. The cometslides were placed in a horizontal electrophoresis unit, and equilibrated in electrophoresis buffer for 30 min at 4°C in the dark. For assessing DNA strand breaks, the migration step was performed at pH of >13 in alkaline solution (300 mmol/L NaOH, 1 mmol/L EDTA) for 20 min at 1 V/cm, after which the migration slides were neutralized with 0.4 mol/L Tris (pH 7.5). The slides were stained with propidium iodide (2.5 μg/mL). Comet assays were performed twice.

Statistics. Microsoft Excel software was used to perform a student test. *P* values are directly indicated in the figures.

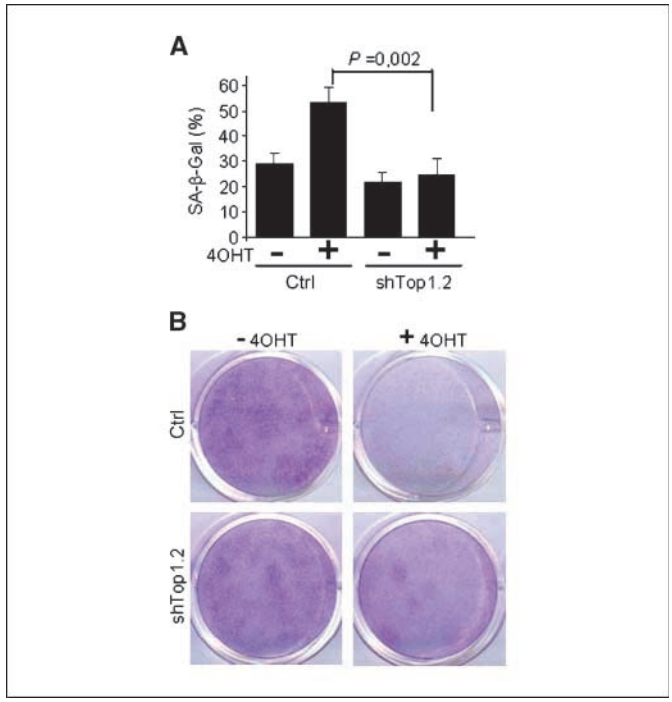

Figure 2. Top1–down-regulated cells bypass oncogene-induced senescence. WI38 cells were coinfected with a MEK/ER vector and with a control or a shTop1 vector. Cells were selected and seeded at the same density. After 2 d, 4OHTamoxifen was added once at 100 nmol/L. Senescence-associated β-galactosidase analysis was performed after 3 d (*A*) and growth analysis after 3 wk (*B*).

Results

A decreased Top1 level prevents senescence. In recent years, senescence escape has emerged as a key step in tumor

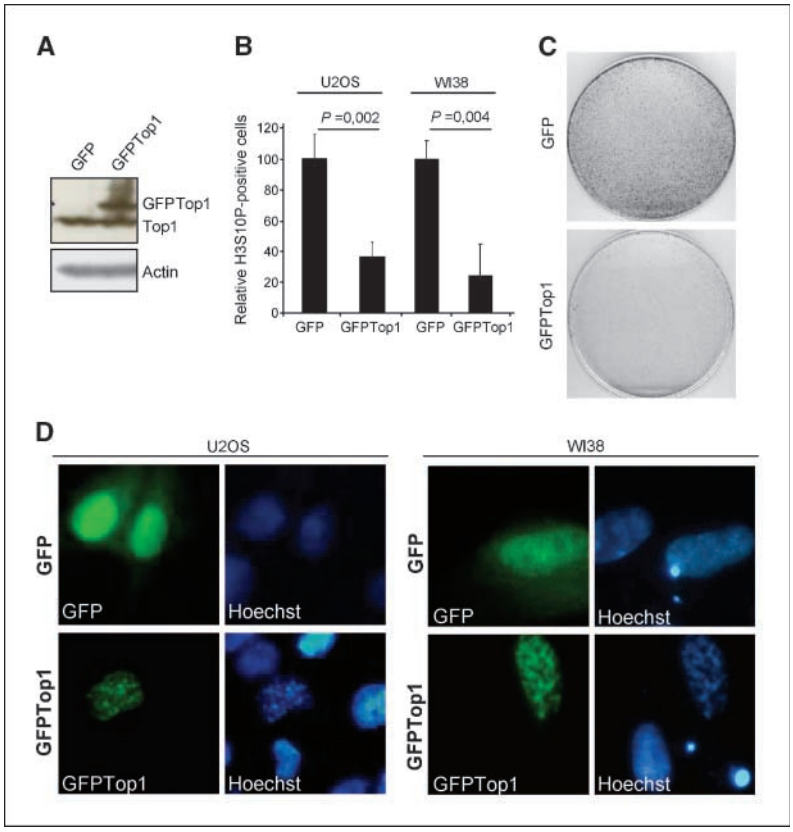

Figure 3. Constitutive Top1 expression blocks cell growth. *A,* U2OS cells were transfected with a GFP- or a GFPTop1-encoding vector. Two days after transfection, cell extracts were prepared and immunoblotting was performed. *B,* 2 d after transfection of U2OS or WI38 cells, immunofluorescence staining was performed using an antibody against phosphoS10-H3 to label mitotic cells. Percentages of H3S10P-positive cells among GFP- and GFPTop1-transfected cells were calculated. *C,* 1 d after transfection, U2OS cells were seeded at low density and puromycin selected for 2 d. After 10 d, the cells were fixed and stained with crystal violet. *D,* WI38 or U2OS cells were transfected with the GFP- or the GFPTop1-encoding vector, fixed, and their chromatin stained with Hoechst.

development (10–12). It is thus necessary to understand in more depth the signals involved in senescence and its regulation. Functional genetic screening is a powerful approach to deciphering new key genetic events involved in senescence control (13). To isolate new genes involved in the senescence program, WI38 normal HDFs approaching senescence were infected with the shRNA library. HDFs infected with a pRS-negative control, a pRS/p53-positive control, or the shRNA library were selected and split every week. After the control-infected HDFs entered senescence, genomic DNA was isolated from the emerging library-infected HDFs and the shRNA was identified (Fig. 1*A*). Using this strategy, we identified an shRNA directed against Top1 along with four other hits. Three are still under investigation, whereas work on PLA2R was described elsewhere (14).

Vectors encoding the shRNA-isolated (shTop1.2) and a second Top1-targeting shRNA (shTop1.C11) were then generated and used to infect HDFs. After selection, levels of Top1 protein were checked by immunoblotting. ShTop1.2 was found to decrease the Top1 protein level strongly, more so than shTop1.C11 (Fig. 1*B, top*). Both shRNAs allowed colony formation in two models of HDF (WI38 and IMR-90), but colony formation was more efficient in the case of the stronger inhibition (Fig. 1*B, bottom*). Colony formation by Top1-depleted HDFs correlated with a loss of senescence markers: the formation of senescence-associated heterochromatin foci and the appearance of senescence-associated β-galactosidase activity (Fig. 1*C* and *D*). Top1 knockdown extended the life span of WI38 cells to ~10 more population doublings (Supplementary Fig. S1) but did not affect telomere length (Supplementary Fig. S2), whereas hTert expression immortalised (data not shown) and sustained telomere length (Supplementary Fig. S2). These results suggest that Top1 affects senescence downstream of the telomeres length status.

We next wondered whether Top1 knockdown could favor bypass from oncogene-induced senescence. WI38 cells stably expressing MAP/ERK kinase (MEK)/ER with a control or an shTop1 were seeded at the same density. Three days after 4OHTamoxifen treatment, 50% of control cells were senescence-associated β-galactosidase–positive, whereas no increase was observed in shTop1-expressing cells (Fig. 2*A*). Three weeks after treatment, cells were stained by crystal violet. Top1-depleted cells treated with 4OHTamoxifen formed colonies, whereas control cells did not (Fig. 2*B*). Taken together, these results reveal that down-regulation of Top1 expression induces a bypass of senescence.

Top1 constitutive expression blocks cell growth. Because Top1 affects senescence, we next sought to determine whether Top1 expression might influence the growth of normal and cancer cells. For this, we transiently expressed a GFPTop1 fusion protein in U2OS cancer cells and checked its overexpression by immunoblot (Fig. 3*A, top band*). Proliferation of U2OS and WI38 cells was assayed by measuring the ability of transfectants to enter mitosis. Growth of U2OS or WI38 cells constitutively expressing GFPTop1 was much slower than that of control-transfected U2OS cells (Fig. 3*B*). This was confirmed by the inability of U2OS cells expressing GFPTop1, after brief selection, to form colonies after 10 days of culture when seeded at low density (Fig. 3*C*). Interestingly, DNA labeling revealed that Top1 expression in U2OS cells as well as in WI38 cells induced appearance of senescence-associated heterochromatin foci (Fig. 3*D*). Thus, Top1 constitutive expression induces a growth arrest and the appearance of a senescent marker in WI38 and U2OS cells.

Top1 modulates the DNA damage–p53 response. Because the DNA damage pathway is involved in regulation of senescence (3–5), we next investigated whether the DNA damage pathway might

be altered by Top1. We first observed the effect of stable Top1 down-regulation on the DNA damage pathway. To this end, we first performed a comet assay that consists in visualizing DNA strand breaks by migrating denatured DNA of individual cells (Fig. 4*A*). The quantification of these results showed a difference, ~6-fold, between the numbers of cells with DNA breaks in the control senescent HDFs versus the Top1-depleted HDFs (Fig. 4*B*). This was further confirmed by performing immunoblot analysis. Indeed, Top1-depleted cells displayed lower levels of phospho-ATM, phospho-p53, p53, and its target p21 at protein levels and RNA level for p21 (Fig. 4*C* and *D*).

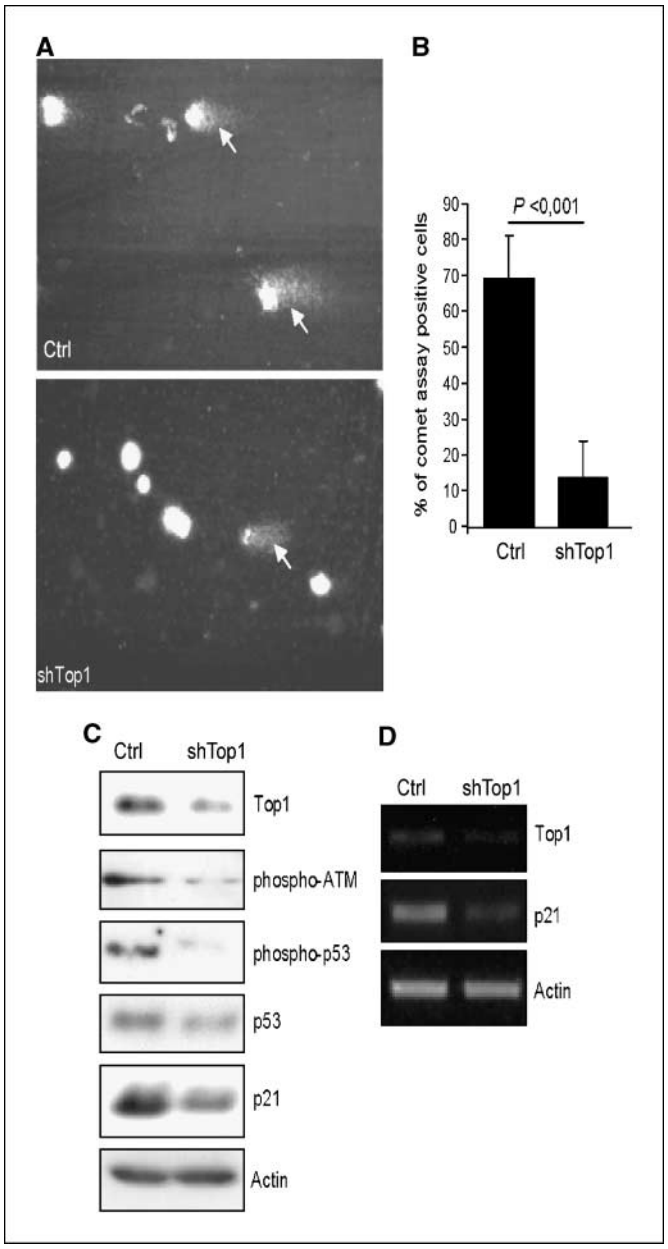

Figure 4. Top1 knockdown decreases the DNA damage response during senescence. WI38 cells were infected and selected. *A* and *B,* comet assays were performed when control cells were starting to senesce. Representative fields are shown in *A*. Quantification in *B* was performed by counting 10 independent fields. *C,* after infection and selection, cell extracts were prepared and immunoblotting was performed with the indicated antibodies. *D,* after infection and selection, RNAs were prepared and reverse transcription-PCR was performed to reveal changes in the level of mRNA corresponding to the p53 target p21.

We next used appropriate antibodies (anti–phospho-ATM, anti–phospho-p53, and anti-p53) to examine the expression of key players of the pathway in cells expressing GFPTop1 or GFP. Interestingly, U2OS cells expressing the GFP-encoding vector displayed weak to no antibody labeling (Fig. 5*A, top*), whereas expression of the GFPTop1 protein resulted in accumulation of all three DNA damage markers (Fig. 5*A, bottom*). In WI38 cells, a majority of GFPTop1-transfected cells were positive for phospho-ATM labeling, whereas ~20% of GFP-transfected cells were positive (Fig. 5*B*). Furthermore, measurement of p53 activity in the presence of an increasing amount of Top1 with a constant amount of a p53 activity reporter revealed dose-dependent activation of p53 by Top1 (Fig. 5*C*). To further confirm the involvement of the p53 pathway, we examined the effect of p53 inhibition by a dominant negative form of p53 (p53DN) over the proliferation arrest induced by GFPTop1. Interestingly, p53 pathway inhibition reverted efficiently the proliferation arrest induced by GFPTop1 (Fig. 5*D*). Altogether, these results support that Top1 acts through the DNA damage-p53 pathway.

Discussion

In summary, we here describe a loss-of-function genetic screen, performed on HDFs that enabled us to identify Top1 as a regulator of senescence. We show that Top1 down-regulation is delay senescence of normal cells, and that its overexpression arrests proliferation of both normal and cancer cells.

Top1 is known to attack the DNA and forms a covalent Top1-DNA intermediate required for DNA relaxation during cellular processes such as replication and transcription. Compounds such as camptothecin are used in clinic to provoke death of dividing cells by stabilizing the Top1-DNA intermediate and inducing DNA damage (15). Interestingly, camptothecin also induces senescence in HDFs (16). Analysis on p16 reveals no change in its expression in control or in Top1-depleted cells (data not shown). Herein, we show that endogenous Top1 regulates senescence through the DNA damage-p53 pathway.

We checked whether Top1 expression increases during senescence. Even, if Top1 expression increased during oncogene-induced senescence (Supplementary Fig. S3*B*), we did not detect any increase during replicative senescence (Supplementary Fig. S3*A*). However, we cannot discard that Top1 might be regulated, at least during replicative senescence, at different levels than its expression such as by phosphorylation (17) and/or by activity of corepressor or coactivator (18) or /and by acting in synergy with other pathways.

We did not detect change in telomere length between shTop1-expressing cells and control cells, suggesting that Top1 is acting downstream of telomere length. This is supported by the fact that Top1 knockdown favors the oncogene-induced senescence escape, a senescence independent of telomere length. Interestingly, one of the factors contributing to replicative or oncogene-induced senescence is the accumulation of DNA lesions at telomere site or at replicative foci, respectively (3–5). As endogenous DNA lesions can recruit Top1 at the damaged sites (19), Top1 manipulation affects to the response to DNA damage.

Top1 down-regulation enables HDFs to keep growing whereas control cells enter senescence, Top1 might thus be expected to exert an antioncogenic function. Yet Top1 expression is generally

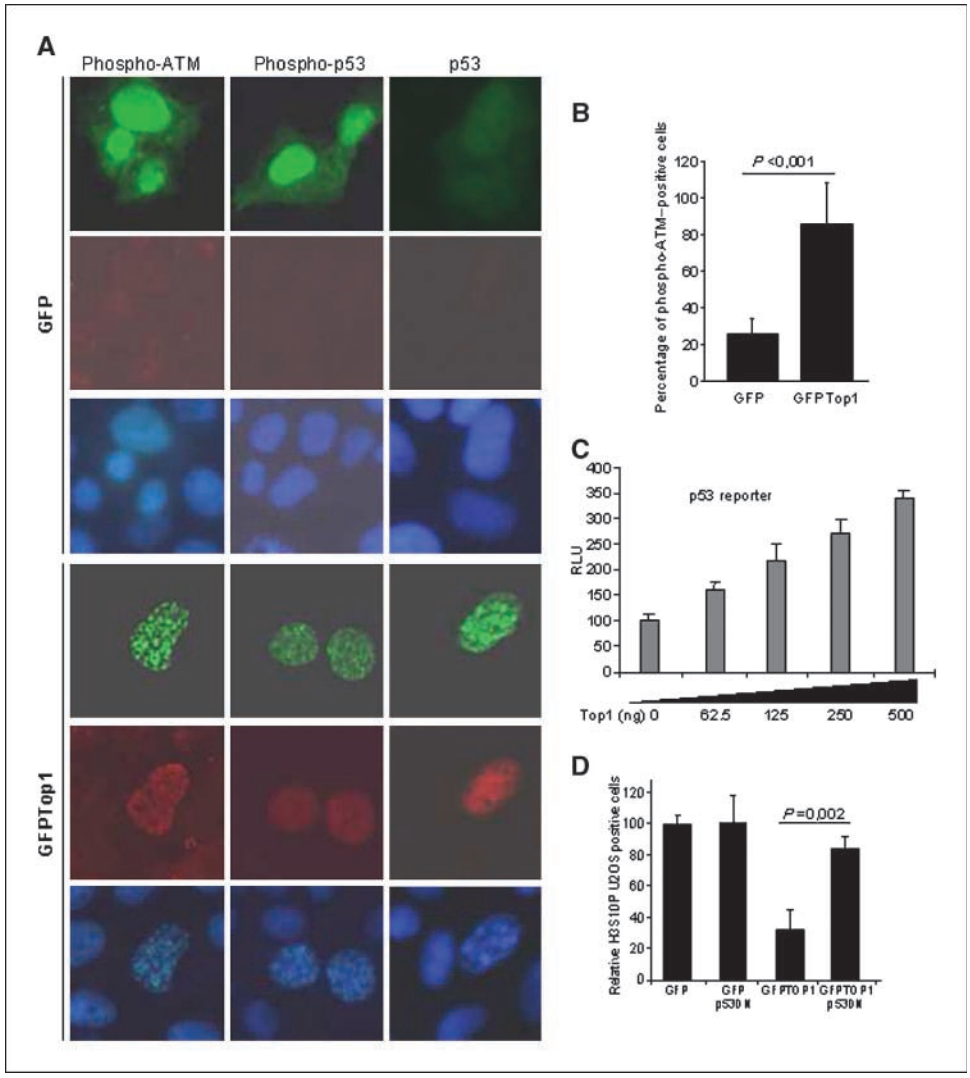

Figure 5. Top1 expression activates the DNA damage-p53 pathway. *A,* U2OS cells were transfected with a GFP- or a GFPTop1-encoding vector. Two days after transfection, immunofluorescence against phospho-ATM, phospho-p53, or p53 was performed. The nuclei were stained with Hoechst dye. *B,* young WI38 cells were transfected with a GFP- or a GFPTop1-encoding vectors. Immunofluoresence directed against phospho-ATM was performed 2 d after transfection and percentage of phospho-ATM–positive cells among the transfected cells calculated. *C,* U2OS cells were cotransfected with a p53 reporter-Luc vector, a normalizing β-galactosidase–encoding vector, and increasing amounts of Top1-encoding vector. Luciferase activity was normalized with respect to β-galactosidase activity, and the relative luciferase activity (*RLU*) is shown. *D,* U2OS cells were cotransfected with a control- or a p53DN-encoding vector and with a GFP- or a GFPTop1-encoding vector. Two days after transfection, an immunofluorescence against phosphoS10-H3 was performed. Relative percentages of labeled cells in GFP- or GFPTop1-cells were displayed.

increased in cancer samples (Oncomine database; ref. 20), excepted, eventually, in Top1-inhibitors unresponsive tumors (21). It should be stressed, however, that in most cancers the DNA damage-p53 pathway is disabled (1, 22, 23). We thus propose that the effect of Top1 on senescence can be largely circumvented if the DNA damage-p53 pathway is attenuated or inactivated, as is generally the case in cancer cells. As an illustration, HDFs immortalized by hTert displayed a decrease in Top1. When large T (a p53 inhibitor) is added, Top1 increases. When an oncogenic Ras is further added, Top1 strongly increases (24). Accordingly, we observed that oncogenic Ras results in Top1 increase (Supplementary Fig. S3*B*). Ras or other oncogenes are able to induce senescence in normal cells but not in DNA damage response-p53 inhibited cells (3, 5), a response in which Top1 could be involved according to our results. In conclusion, our data reveal a complex role of Top1 during immortalization and transformation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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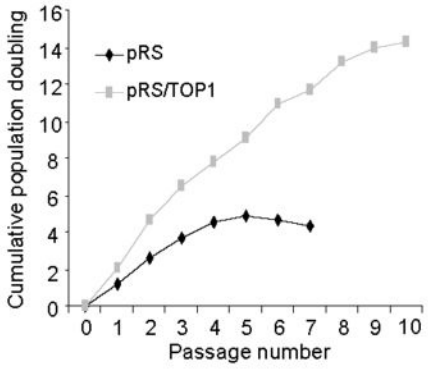
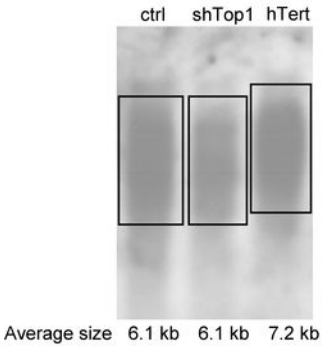
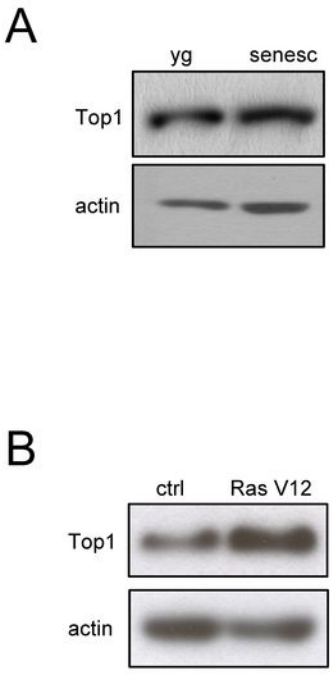


Sup Figure 1



Sup Figure 2



Sup Figure 3

SUPPLEMENTARY INFORMATION

Supplementary Figure Legends

Supplemental Figure 1. WI38 cells were infected with retroviral vectors encoding an shRNA directed against Top1 or a control retroviral and puromycin selected. WI38 cells were seeded at the same density, split every week and counted. The population doublings was calculated at each passage.

Supplemental Figure 2. After infection and selection, WI38 cells were maintained for 5 passages. At this stage, gDNA were prepared from control infected cells (senescing), from shTop1 or hTert expressing cells (growing). Telomere length assay was performed using TeloTAGGG Telomere length assay (Roche) according to the manufacturer's recommendations.

Supplemental Figure 3. Top1 expression during senescence. *A*, cell extracts from young and senescing normal WI38 fibroblasts were prepared and resolved by SDS-PAGE. The Top1 protein level was revealed with an antibody directed against Top1, and an antibody directed against actin was used as a loading control. *B*, WI38 cells were infected with a control or a RasV12 encoding vector. Four days after infection, cell extract were prepared and the level of Top1 was analysed as described above.

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Regulation of ploidy and senescence by the AMPK-related kinase NUAK1

Nicolas Humbert[1,7], Naveenan Navaratnam[2,7], Arnaud Augert[1,3], Marco Da Costa[4], Sébastien Martien[1], Jing Wang[5], Dolores Martinez[6], Corinne Abbadie[1], David Carling[2], Yvan de Launoit[1], Jesus Gil[4] and David Bernard[1,3,*]

[1]UMR8161, Institut de Biologie de Lille, CNRS/Universités de Lille 1 et 2/Institut Pasteur de Lille, Lille, France, [2]Cellular Stress Group, MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Campus, London, UK, [3]UMR5238, Apoptose, Cancer et Développement, CNRS/Université de Lyon/Centre Léon Bérard, Lyon, France, [4]Cell Proliferation Group, MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Campus, London, UK, [5]University of Nebraska Medical Center, Eppley Institute for Research in Cancer and Allied Diseases, Nebraska Medical Center, Omaha, NE, USA and [6]London Research Institute, Cancer Research UK, London, UK

Senescence is an irreversible cell-cycle arrest that is elicited by a wide range of factors, including replicative exhaustion. Emerging evidences suggest that cellular senescence contributes to ageing and acts as a tumour suppressor mechanism. To identify novel genes regulating senescence, we performed a loss-of-function screen on normal human diploid fibroblasts. We show that downregulation of the AMPK-related protein kinase 5 (ARK5 or NUAK1) results in extension of the cellular replicative lifespan. Interestingly, the levels of NUAK1 are upregulated during senescence whereas its ectopic expression triggers a premature senescence. Cells that constitutively express NUAK1 suffer gross aneuploidies and show diminished expression of the genomic stability regulator LATS1, whereas depletion of NUAK1 with shRNA exerts opposite effects. Interestingly, a dominant-negative form of LATS1 phenocopies NUAK1 effects. Moreover, we show that NUAK1 phosphorylates LATS1 at S464 and this has a role in controlling its stability. In summary, our work highlights a novel role for NUAK1 in the control of cellular senescence and cellular ploidy.

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Introduction

Normal human diploid cells have a finite replicative potential, mainly because of telomere erosion. When they reach this critical point they become senescent (Hayflick, 1965). Senescent cells remain metabolically active, show characteristic changes in cell morphology, physiology, and gene expression, and typically show a senescence-associated β-galactosidase (SA-β-gal) activity (Dimri *et al*, 1995) and the appearance of senescent-associated heterochromatin foci (Narita *et al*, 2003). A phenotypically similar end point can be reached in response to different cellular stresses, notably inappropriate oncogenic signalling or oxidative stress. The fact that normal cells undergo senescence in response to oncogenes has led to the hypothesis that senescence, similar to apoptosis (Lowe *et al*, 2004; Dimri, 2005), is an intrinsic genetic programme with tumour-suppressive properties (Braig *et al*, 2005; Chen *et al*, 2005; Collado *et al*, 2005; Dimri, 2005).

A corollary of the above observations is that oncogenes and tumour suppressor alterations can compromise the senescence programme, thus favouring cell immortalisation and cancer. This is exemplified by our current understanding of the Rb and p53 tumour suppressor networks, which are pivotal in controlling the occurrence of senescence and tumour development (Sherr, 1996; Sionov and Haupt, 1999; Hanahan and Weinberg, 2000; Yamasaki, 2003; Campisi, 2005). Interference with the Rb or p53 pathway through mutation, overexpression, or depletion of various components of these pathways or by overexpression of oncogenic proteins (including the oncoviral proteins E6 and E7) results in bypassed senescence and extension of the replicative lifespan of normal human cells (Jacobs *et al*, 1999; Carnero *et al*, 2000; Lundberg *et al*, 2000; Ohtani *et al*, 2001; Brummelkamp *et al*, 2002; Shvarts *et al*, 2002; d'Adda di Fagagna *et al*, 2003; Gil *et al*, 2004; Herbig *et al*, 2004).

Aneuploidy caused by a weak mitotic spindle checkpoint or by cytokinetic defects results in premature senescence (Baker *et al*, 2004; Takahashi *et al*, 2006). To our knowledge, however, nobody has ever reported having increased the replicative potential of normal cells by enforcing genomic stability safeguards. Strong enforcement of genomic stability safeguards, such as by LATS1 or BubR1, by constitutive expression can even inhibit cell growth (Yang *et al*, 2001; Shin *et al*, 2003; Iida *et al*, 2004). Whether slightly enhanced genomic stability can increase the replicative potential of normal human cells thus remains an open question.

To identify new genes regulating the replicative potential of normal human cells, we have performed a loss-of-function genetic screen, using a retroviral shRNA library. This screen has revealed that the level of NUAK1 has an effect on the replicative potential of normal human cells and this might be due to the level of aneuploidie. This suggests that the maintenance of the normal DNA content can increase the replicative potential of human cells.

Results

NUAK1 depletion prevents senescence

WI-38 normal human diploid fibroblasts (HDFs) approaching senescence were infected with an shRNA retroviral library

*Corresponding author. CNRS UMR 5238, Centre Léon Bérard, 28, rue Laënnec 69373 LYON Cedex 08, France. Tel.: +33 4 78 78 27 10; Fax: +33 4 78 78 27 20; E-mail: BERNARDD@lyon.fnclcc.fr
[7]These authors contributed equally to this work

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(Berns *et al*, 2004). After puromycin selection, the cells were split every week for 3 weeks. Cells infected with the empty (control) vector stopped growing, whereas some cells infected with library pools continued to grow. Genomic DNA was extracted from these cells and an shRNA construct targeting NUAK1 conferring extension of the cellular replicative lifespan was identified, along with shRNAs directed against PLA2R (Augert *et al*, 2009), topoisomerase 1 (Humbert *et al*, 2009), and another shRNA that is still under characterisation. To analyse the role of NUAK1 in replicative senescence, we generated two independent retroviral shRNA vectors, each targeting NUAK1. We then verified the ability of these shRNAs to downregulate endogenous NUAK1 protein levels (Figure 1A). When HDFs were infected with either of the two NUAK1 knockdown shRNAs, they reversed senescence, as monitored using colony assay and by their loss of senescent morphology (Figure 1B). This effect was further confirmed using growth curve analysis in which we observed an extension of replicative lifespan of approximately eight population doublings in NUAK1 knockdown cells (Figure 1C). This increase in replicative potential was accompanied with a decrease in the number of senescent cells, as observed by SA-β-Gal staining or SAHF analysis in WI-38 cells infected with pRS/NUAK1 when compared with cells infected with an empty vector (Figure 1D and E). Ultimately, the NUAK1 knockdown cells entered a senescence state. Indeed, the cells then stopped growing, showed an SA-β-Gal staining and the typical senescent morphology, and were immortalised by hTert expression (Supplementary Figure S1).

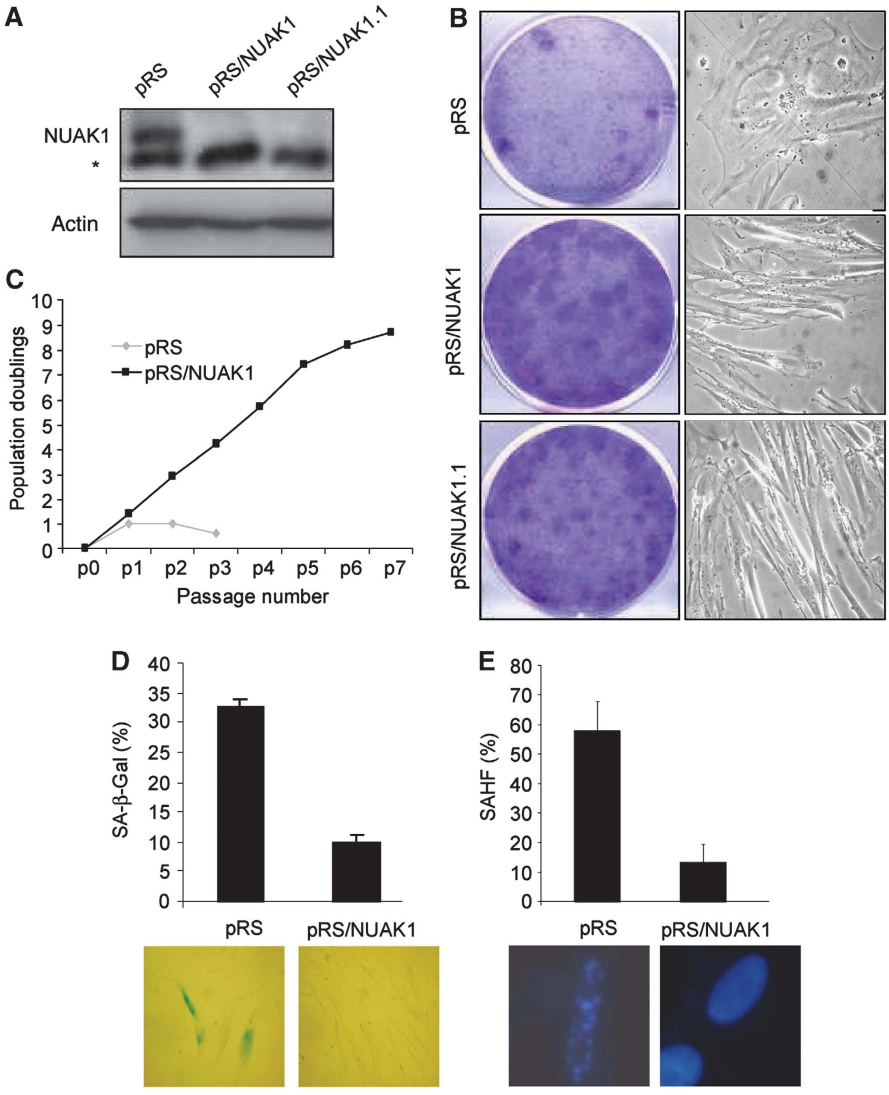

Figure 1 NUAK1 downregulation extends the replicative lifespan. (**A**) WI-38 cells were infected with control pRS, pRS/NUAK1, or pRS/NUAK1.1 (these constructs encode two different shRNAs directed against NUAK1) and selected by puromycin. Cell extracts were prepared and resolved using SDS–PAGE. * denotes a non-specific band. The efficiency of the two shRNAs to downregulate endogenous NUAK1 protein levels was monitored using immunoblot analysis with an anti-NUAK1 antibody. (**B**) WI-38 cells were infected with a retroviral vector (pRS control, pRS/NUAK1, or pRS/NUAK1.1) and selected with puromycin. Selected cells were seeded at low density and stained after 2 weeks with crystal violet. Alternatively, transmitted images were taken 5 days after seeding to monitor any change in cellular morphology. (**C**) WI-38 cells were infected with pRS or pRS/NUAK1 and selected with puromycin. The cells were split every 5 days and the cell numbers were counted. Cumulative population doublings were showed at each passage. (**D**, **E**) Infected and selected WI-38 cells were analysed for SA-β-Gal activity or SAHF positivity. Analysis was performed when control cells entered senescence.

Collectively, these results indicate that the downregulation of NUAK1 expression results in replicative lifespan extension.

Constitutive NUAK1 expression triggers premature senescence

As downregulation of NUAK1 extends the replicative lifespan, we next analysed whether the endogenous NUAK1 level increases during replicative senescence. To verify this, we measured the NUAK1 transcript level using Q–PCR and protein level using immunoblot analysis in proliferating- versus senescing-cultured HDFs. NUAK1 mRNA and protein increased strongly in late-passage HDFs when compared with early-passage ones (Figure 2A).

To confirm that NUAK1 modulates the occurrence of senescence, we analysed the effect of NUAK1 overexpression. Early-passage HDFs were infected with a retroviral vector expressing a flag-tagged human NUAK1 or with a control retroviral vector. Ectopic expression of NUAK1 was checked using western blotting with an anti-flag tag antibody (Figure 2B). The growth potential of NUAK1-transduced

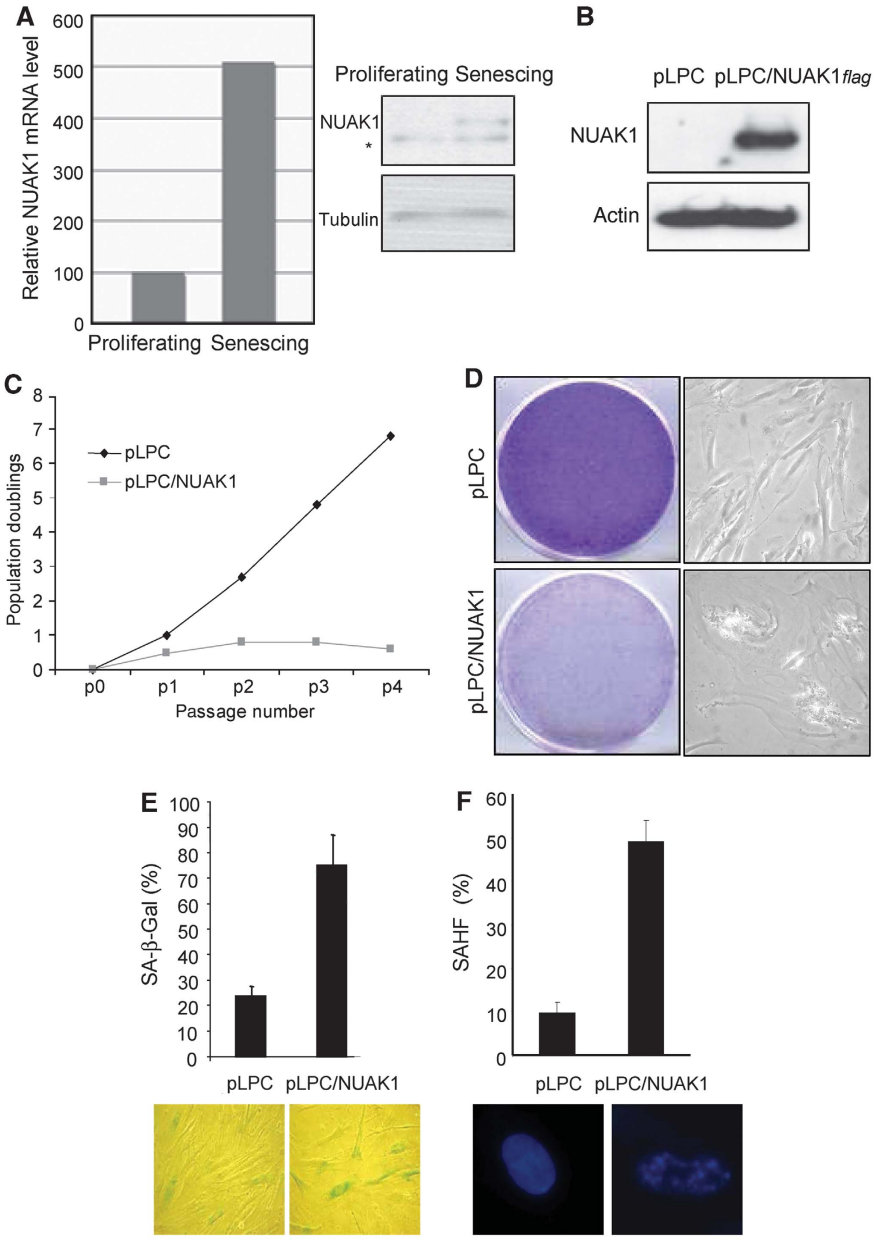

Figure 2 NUAK1 overexpression induces premature senescence. (**A**) RNA and protein were prepared from proliferating and senescing WI-38 cells. After reverse-transcription, real-time quantitative PCR was used to determine NUAK1 mRNA expression. Relative NUAK1 mRNA levels are shown. Alternatively, NUAK1 protein levels were measured by immunoblot analysis using an anti-NUAK1 antibody. * denotes a non-specific band. (**B**) WI-38 cells were infected with the pLPC or pLPC/NUAK1*flag* retroviral vector. After puromycin selection, cells were used to perform different assays. Cell extracts were prepared and resolved using SDS–PAGE. Constitutive expression of flag-tagged NUAK1 levels was checked using immunoblot analysis with an anti-flag-tag antibody. Actin protein levels were used as a loading control. (**C**) Cells were split and counted every 5 days. Cumulative population doublings were calculated and showed after each passage of pLPC-or pLPC/NUAK1*flag*-infected cells. (**D**) Cells were seeded at low density and left to grow for 5 days and phase contrast images were taken to monitor cell morphology. Alternatively, after 2 weeks, cells were fixed with PFA and stained with crystal violet. Representative plates are shown. (**E**, **F**) SA-β-Gal and SAHF assays were performed to analyse senescence of pLPC-infected (control) and pLPC/NUAK1 *flag*-infected HDFs.

cells was examined by means of growth curves and a colony formation assay. NUAK1 expression was found to block cell growth and induce a characteristic senescent morphology (Figure 2C and D). This growth arrest was due to premature senescence induction, as evidenced by the increased proportion of SA-β-Gal-positive and SAHF-positive cells among the NUAK1-overexpressing cells, as compared with control cells (Figure 2E and F). These results reveal that both down-regulation and overexpression of NUAK1 have a major effect on the cellular replicative lifespan by regulating senescence.

NUAK1-induced senescence requires activation by LKB1 kinase

Phosphorylation of NUAK1 at T211 by the LKB1 kinase has been shown to activate its kinase activity (Lizcano *et al*, 2004). The phosphorylation of NUAK1 at S600 by Akt kinase could also regulate NUAK1 (Suzuki *et al*, 2003b). We generated retroviral vectors encoding T211A or S600A mutation and checked their expression using immunoblot (Figure 3A). Interestingly, whereas NUAK1 S600A mutant kept its ability to phosphorylate an AMARA peptide, the NUAK1 T211A mutant lost it (Figure 3B). Accordingly, only the NUAK1 S600A mutant was still able to block cell growth, whereas the NUAK1 T211A mutant was not (Figure 3C). We next wondered whether or not activation of NUAK1 through the T211 involved LKB1 kinase (Lizcano *et al*, 2004). To evaluate this, we stably expressed in WI-38 cells a control vector, or an NUAK1 vector, or an NUAK1 and an LKB1 dominant-negative (LKB1DN) vectors, or an NUAK1 and an shRNA directed against LKB1 (shLKB1) encoding vectors. We first verified the expression of all the proteins (Figure 3D). The growth-inhibitory effect of NUAK1 was largely reverted when LKB1 activity was inhibited by either expression of a dominant-negative form or by its knockdown (Figure 3E). These results indicate that the effect of NUAK1 oversenescence required its phosphorylation by LKB1.

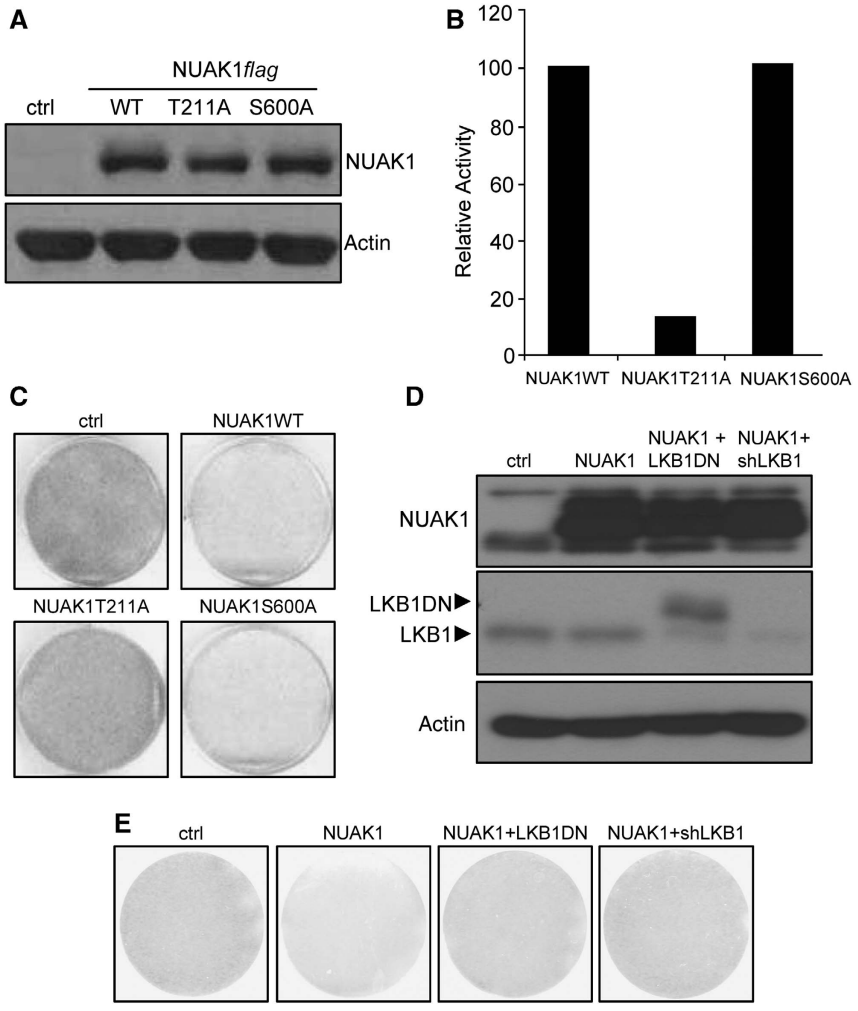

Figure 3 NUAK1 activation by LKB1 is required for NUAK1-induced senescence. (**A**) WI-38 cells were infected with the indicated constructs and puromycin selected. Cellular extracts were prepared and analysed using immunoblotting. Wild type and mutants of NUAK1 expression levels were checked with an anti- flag-tag antibody. Anti-actin antibody was used to monitor protein loading. (**B**) HEK 293 cells were transfected with the indicated vectors. After immunoprecipitation with an anti-flag-tag antibody, NUAK1 kinase activity towards the AMARA peptide was measured. The activity was normalised to 100% to NUAK1-transfected cells. (**C**) After infection and selection, the cells were seeded at low density. After 2 weeks, the cells were fixed and stained by crystal violet. (**D**) WI-38 cells were infected with ctrl or NUAK1 encoding vectors (neo resistance) together with ctrl or LKB1DN or shLKB1 encoding vectors (puro selection) and selected. Cellular extracts were prepared and analysed using immunoblotting. NUAK1 expression was analysed using an anti-flag-tag antibody, and LKB1 expression was analysed using an anti- LKB1 antibody. Anti-actin antibody was used to monitor protein loading. (**E**) After infection and selection as in D, cells were seeded at low density. The cells at 2 weeks after they were fixed and stained by crystal violet are shown. A full-colour version of this figure is available at *The EMBO Journal* Online.

NUAK1-induced senescence requires the kinase activity of NUAK1

To gain insight into the mechanism by which NUAK1 controls the occurrence of senescence, we examined whether the NUAK1 kinase activity mediates this control. We generated a retroviral vector encoding a kinase-dead mutant (K84A) (Lizcano *et al*, 2004; Suzuki *et al*, 2006), and verified its expression using immunoblotting (Figure 4A). We next tested whether this mutant had lost its kinase activity towards the AMARA peptide, a known NUAK1 substrate. As expected, the kinase-dead mutant was unable to phosphorylate a serine residue in the AMARA peptide (Figure 4B). To examine the effect of this mutant on senescence, we compared the ability of NUAK1-expressing and NUAK1K84A-expressing cells to form colonies. As expected, NUAK1 expression was found to inhibit colony formation when compared with control cells (Figure 4C). Interestingly, the kinase-dead mutant K84A was unable to block colony formation and even seemed to favour cell growth (Figure 4C). To further confirm these effects we performed growth curve analysis. The NUAK1K84A-expressing cells showed an extended replicative lifespan when compared with control infected cells, suggesting that it might act as a dominant-negative form (Figure 4D). Nevertheless, the cells entered the senescence state after few additional passages, a senescence that was avoided by hTert constitutive expression (Supplementary Figure S2). We conclude that the kinase activity of NUAK1 is required for NUAK1-induced senescence.

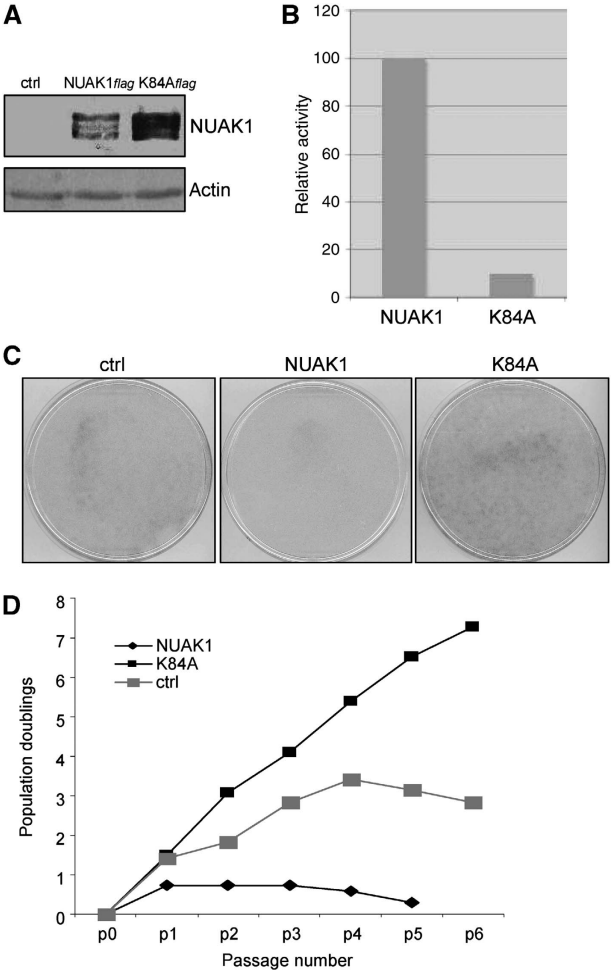

Figure 4 NUAK1 kinase activity is required for senescence induction. (**A**) WI-38 cells were infected with a control vector or with a vector expressing wild-type or K84A NUAK1 mutant. After puromycin selection, cell extracts were prepared and analysed using immunoblotting. Wild-type and mutant NUAK1 expression levels were checked with an anti- flag-tag antibody. Anti-actin antibody was used to monitor protein loading. (**B**) HEK 293 cells were transfected with a vector encoding wild-type or K84A mutant NUAK1. After immunoprecipitation with an anti-flag-tag antibody, NUAK1 kinase activity towards the AMARA peptide was measured. The activity was normalised to 100% to NUAK1-transfected cells. (**C**) WI-38 cells were infected by the indicated constructs and selected with puromycin. The cells were next seeded at low density, allowed to grow, and stained with crystal violet as in Figure 2. (**D**) WI-38 cells were infected with the indicated vectors and selected with puromycin. Growth was monitored up to 6 passages and curves were drawn. A full-colour version of this figure is available at *The EMBO Journal* Online.

NUAK1 induces gross aneuploidies

To identify the pathways and the target through which NUAK1 controls senescence, we first examined the status of the two main senescence-regulating pathways: the p53 and the Rb pathways. Quite surprisingly, p53 and p21 proteins levels were not modified by NUAK1 knockdown or overexpression. In addition, E6 expression was not able to revert NUAK1-induced senescence (Supplementary Figure S3A–C). Rb was found hyper- or hypo-phosphorylated in NUAK1 overexpressed or knockdown cells (Supplementary Figure S3A and B). However, the inhibition of the Rb pathway by E7 was not sufficient to revert NUAK1-induced senescence (Supplementary Figure S3C). The modification observed on Rb is thus insufficient to explain the effect of NUAK1 and could thus be only a mark of the proliferative state.

As genomic instability is reported to cause premature senescence (Baker *et al*, 2004; Takahashi *et al*, 2006), we next examined whether NUAK1 might regulate senescence by affecting the DNA content. We first performed FACS-based cell-cycle analysis of control and NUAK1-overexpressing cells. NUAK1-expressing cells shifted from a mainly 2n DNA content (G1 phase) to a 3–4n DNA content (Figure 5A). In addition, numerous NUAK1-expressing cells had more than 4n DNA content according to the FACS analysis (Figure 5A). Interestingly, cells expressing an shRNA directed against NUAK1 show a normal DNA content profile in contrast with control senescent cells that show a shift of cells in 3–4n DNA content (Supplementary Figure S4). We then wondered whether NUAK1-expressing cells or control senescent cells showing 3–4n DNA content might be blocked in late S-G2/M. This possibility was ruled out on the basis of the observation that cyclin A, a cyclin accumulating in the late S-G2/M phases, was downregulated in NUAK1-expressing cells or in control senescing cells (Figure 5B). From these experiments we conclude that NUAK1-expressing cells or control senescent cells have an aberrant DNA content.

A decrease in LATS1 and subsequent block of cytokinesis has been implicated in senescence (Takahashi *et al*, 2006). The LATS1 level was found to be lower in NUAK1-expressing cells and higher in NUAK1-depleted cells than in respective control cells (Figure 5B). Microscopic analysis confirmed that NUAK1-expressing cells had greater proportion of polynucleated cells when compared with control cells (30 versus 8%; Figure 5C). Interestingly, 5–10% of the mitotic NUAK1-expressing cells contained more than 100 chromosomes,

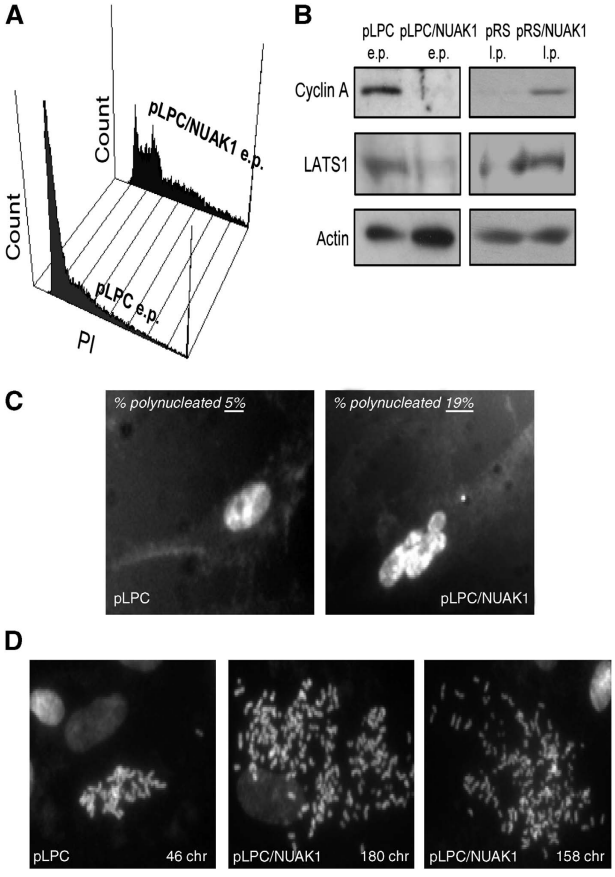

Figure 5 NUAK1 regulates the DNA content. (**A**) At 10 days after infection with a pLPC or a pLPC/NUAK1*flag* vector and puromycin selection, WI-38 cells were fixed with ethanol, stained with propidium iodide, and analysed using FACS to determine the DNA content. (**B**) Cell extracts were prepared from early-passage (e.p.) pLPC-infected and pLPC/NUAK1-infected and late-passage (l.p.) pRS- and pRS/NUAK1-infected WI-38 cells. Levels of the cyclin A and LATS1 proteins were checked using immunoblotting and actin level was used as a loading control. (**C**) WI-38 cells were fixed with PFA and stained with Hoechst. Percentages of polynucleated cells were estimated and Hoechst-stained images are shown. (**D**) Chromosome spreading experiments. At 7 days after infection and selection, cells were treated with colcemid. Cell images with normal and aberrant chromosome numbers are shown.

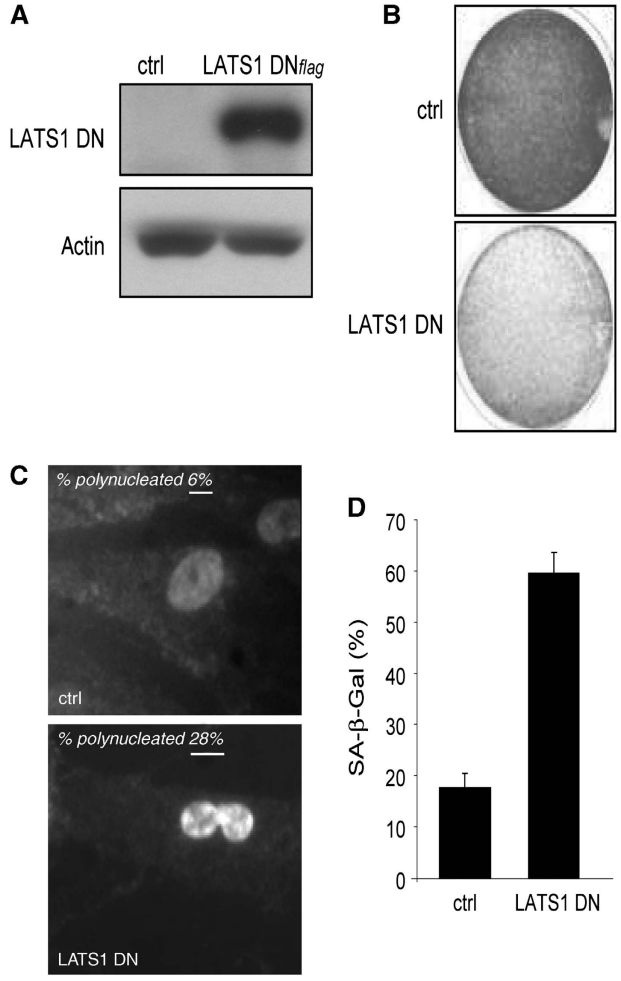

Figure 6 LATS1 downregulation induces gross aneuploidies and premature senescence. (**A**) WI-38 cells were infected with a control vector or with a vector encoding a dominant-negative form of LATS1. After puromycin selection, cell extracts were prepared and expression of LATS1 DN was checked by immunoblotting using anti-flag-tag antibody. Actin level was used as a loading control. (**B**) Infected cells were seeded at low density. After 10 days of culture, the cells were stained with crystal violet. (**C**) The nuclei of infected cells were stained with Hoechst dye. Percentages of polynucleated cells were estimated and Hoechst-stained images are shown. (**D**) SA-β-Gal assays were performed on cells infected with the LATS1DN-encoding construct and on control cells. A full-colour version of this figure is available at *The EMBO Journal* Online.

whereas control cells never showed such an aberrant number of chromosomes (Figure 5D). As this assay required cell entry into mitosis, we think that we underestimated the number of NUAK1-expressing cells with a high chromosome number as the majority of these cells were not growing. LATS1 downregulation might allow cells to undergo aberrant mitosis without cytokinesis (Yang *et al*, 2004). This would explain the aberrant DNA content observed upon constitutive NUAK1 expression. In contrast, downregulation of NUAK1 seems to lead to sustained levels of LATS1 to thus avoid the appearance of aberrant DNA content when compared with control senescing cells.

LATS1 inhibition induces premature senescence

We next examined whether LATS1 inactivation alone might be sufficient to produce the phenotype induced by ectopic NUAK1 expression (i.e., aberrant DNA content and senescence). We infected HDFs with a dominant-negative form of LATS1 (LATS1 DN) (Takahashi *et al*, 2006). LATS1 DN expression was confirmed using immunoblot analysis (Figure 6A). A growth arrest was observed when compared with control infected cells, as judged using colony formation assays (Figure 6B). Similar to constitutive NUAK1 expression, the LATS1 DN expression induced gross aneuploidies (Figure 6C) and the appearance of SA-β-Gal activity (Figure 6D). In addition, LATS1 DN expression in shNUAK1-expressing cells was still able to block cell growth, suggesting that LATS1 might be a downstream target of NUAK1 (Supplementary Figure S5). Thus, inactivation of LATS1 mimics the effect of NUAK1 on aberrant DNA content appearance and senescence.

NUAK1 interacts, phosphorylates, and controls LATS1 levels

As a deficiency of LATS1 causes aneuploidies and premature senescence, we next analysed the relationships between

NUAK1 and LATS1by performing co-immunoprecipitation assays on extracts from cells co-expressing NUAK1 and LATS1. Interestingly, NUAK1 was detected in the LATS1 immunoprecipitate (Figure 7A). We next repeated the immunoprecipitation experiment with endogenous proteins. Once again, NUAK1 protein was found in the LATS1 immunoprecipitate (Figure 7B).

We next transiently co-expressed NUAK1 and LAST1 in HEK 293 cells to analyse the effect of NUAK1 on LATS1 protein levels. Interestingly, LATS1 protein levels decreased when co-expressed with NUAK1, whereas LATS1 mRNA levels remained unchanged (Figure 7C). NUAK2, which belongs to the same sub-family of NUAK1 in the ARK family, was also found to regulate the LATS1 protein levels, whereas AMPKα2, which does not belong to the same sub-family, lacked this ability (Figure 7C). Interestingly, expression of the NUAK1 kinase-dead mutant reverted the effect of NUAK1 WT expression on LATS1 protein levels (Supplementary Figure S6).

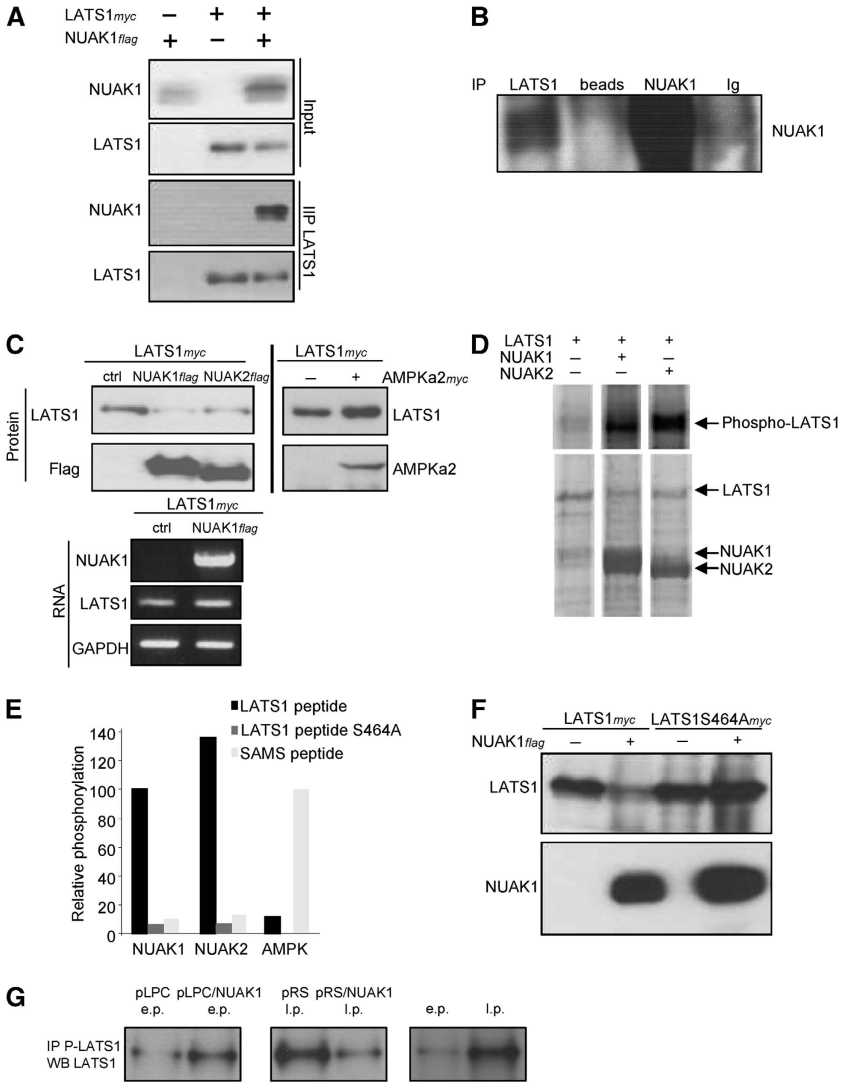

Figure 7 NUAK1 regulates LATS1 levels through phosphorylation at S464. (**A**) HEK 293 cells were transfected with the NUAK1*flag*, LATS1*myc*-encoding vectors, or with both. Immunoprecipitation was performed with an anti-flag antibody at 1 day after transfection and proteins of interest were monitored using immunoblotting with the indicated antibodies. (**B**) WI-38 cells closed to senescence were used to prepare cell extracts. Immunoprecipitation was performed with LATS1 or NUAK1 antibodies. IgG or beads alone were used as controls. The immunoprecipitates were analysed by immunoblotting using an anti-NUAK1 antibody. (**C**) HEK 293 cells were co-transfected with the LATS1*myc*-encoding vector and either with control vector or the NUAK1*flag*- or NUAK2*flag*- or AMPKα2*myc*-encoding vectors. At 1 day after transfection, protein or RNA extracts were prepared. Protein expression was analysed using immunoblotting with the indicated antibodies. RNA expression was analysed using RT–PCR. (**D**) HEK 293T cells were transfected with flag-tagged LATS1, NUAK1, and NUAK2. They were affinity purified on M2-flag resin and eluted with flag-peptide. Eluted LATS1 protein was phosphorylated alone or with NUAK1 or with NUAK2 and separated on SDS–PAGE. LATS1 phosphorylation by NUAK1 and NUAK2 (Phosphoimage, top) and the protein levels (Coomassie stain) are shown. (**E**) HEK 293 cells were transfected with vectors encoding either NUAK1*flag*- or NUAK2*flag*-encoding vectors and immunoprecipitated with a flag resin. Recombinant AMPK was prepared and activated as described in the Materials and methods section. The ability of these three kinases to phosphorylate the LATS1 peptide, LATS1 S464A mutant peptide, or SAMS peptide was measured. (**F**) HEK 293 cells were transfected with the LATS1*myc*- or LATS1S464A*myc*-encoding vector or with or without NUAK1 flag encoding vector. At 2 days after transfection, cell extracts were prepared and the protein levels were monitored using immunoblotting with the indicated antibodies. (**G**) A total of 5 million WI-38 cells were used in each condition to prepare cell extracts (e.p. for early passage and l.p. for late passage). Immunoprecipitations were performed using the anti-phosphoLATS1 antibody. Half of each IP was used for the immunoblot using a LATS1 antibody. A full-colour version of this figure is available at *The EMBO Journal* Online.

These results also confirmed that NUAK1 kinase-dead mutant behaved as an NUAK1 dominant-negative form (Figure 4C and D).

As NUAK1 interacts and regulates LATS1 levels, we next wanted to know whether purified NUAK1 protein would be able to phosphorylate full-length LATS1 protein. Interestingly, NUAK1 and its closest member NUAK2 were both able to phosphorylate LATS1 (Figure 7D). We then synthesised a LATS1 peptide containing a consensus ARK motif around the S464 (predicted to be phosphorylated in the Swiss Prot database) and found that NUAK1 as well as NUAK2, but not AMPK, were able to phosphorylate it (Figure 7E). Mutation of S464 to A in the LATS1 peptide completely abolished phosphorylation by NUAK1 and NUAK2 (Figure 7E), confirming that NUAK1 can specifically phosphorylate S464.

Importantly, we also found that NUAK1 expression had no effect on the protein levels of S464 to A mutant form of LATS1 protein (Figure 7F). To further confirm this phosphorylation and its significance during the senescence regulated by NUAK1, we generated a phospho-specific antibody directed against the phosphor S464 of LATS1. As expected, this antibody was unable to recognise the S464A LATS1 mutant (Supplementary Figure S7). Interestingly, we observed an increase in the endogenous LATS1 S464 phosphorylation during replicative or NUAK1-induced senescence and a decrease in pRS/NUAK1-infected cells during escape of replicative senescence (Figure 7G). Thus, NUAK1 is able to regulate the LATS1 protein levels directly through phosphorylation at S464.

Discussion

In a search for new genetic events that are involved in senescence of normal human cells, we have isolated NUAK1 as a modulator of senescence. A total of 13 proteins belong to the ARK (AMP-activated protein kinase-related kinase) family. They can be classified into five subfamilies: AMP-activated protein kinase (AMPK), salt-induced kinase (SIK), microtubule-affinity-regulating kinase (MARK), brain-specific kinase (BRSK), and SNF1-like kinase 1 (NUAK). These proteins show various biological activities, from controlling cell polarity (MARK proteins) to sensing metabolic changes (AMPK proteins) (Drewes *et al*, 1997; Kahn *et al*, 2005). Some of them are also involved in basic processes, such as controlling cell death and proliferation (Blazquez *et al*, 2001; Meisse *et al*, 2002; Inoki *et al*, 2003; Kimura *et al*, 2003; Li *et al*, 2003; Jones *et al*, 2005). Apart from the evidence suggesting that NUAK1 may participate in inducing invasion and metastasis (Suzuki *et al*, 2003a; Kusakai *et al*, 2004a, b), little is known about its biological function. In this study we show that NUAK1 downregulation increases the replicative potential of HDFs, whereas NUAK1 constitutive expression decreases their replicative potential by inducing premature senescence.

Importantly, AMPKα2 modulates the replicative potential of MEFs through the p53 pathway (Jones *et al*, 2005). Our results suggest that NUAK1 regulates senescence by a mechanism not involving p53, as neither the overexpression nor the knockdown of NUAK1 affected p53 activity in WI-38 cells (Supplementary Figure S3). WI-38 cells are immortalised by expressing the hTert enzyme (Augert *et al*, 2009) or show replicative senescence delay by expressing E6 or E7 oncoproteins (Supplementary Figure S3D). Interestingly, NUAK1 expression blocks growth of E6-expressing cells as well as of hTert-immortalised cells, showing that NUAK1 is not acting through the hTert/telomere length/p53 pathway (Supplementary Figure S3C). Altogether, these results suggest that NUAK1 is acting independently of the hTert pathway, or downstream of telomere shortening signalling through an alternate p53-independent pathway. AMPK and NUAK1 have different target specificities, as AMPK is unable to downregulate the level of LATS1 or to phosphorylate S464 of the LATS1 peptide. In contrast, NUAK2, which belongs to the same sub-family as NUAK1, phosphorylates full-length LATS1 protein or LATS1 peptide and decreases LATS1 levels. This similarity between NUAK1 and NUAK2 was further confirmed as NUAK2 was able to induce premature senescence and downregulation of LATS1 in WI-38 cells (Supplementary Figure S8). Nevertheless, the mechanism of NUAK2 regulation seems to be different from that evoked by NUAK1. This was supported by the observation that unlike NUAK1 mRNA levels that increased during replicative senescence, there was no significant change in NUAK2 mRNA levels during replicative senescence (Supplementary Figure S9). In addition, when we induced senescence through RASV12 expression, we only observed slight variations in NUAK1 and NUAK2 mRNA levels (Supplementary Figure S9). Taking all these observations together, we conclude that mainly NUAK1 is involved in the regulation of the replicative senescence.

A decrease in LATS1 levels is known to induce premature senescence and genomic instability through blocking cytokinesis (Takahashi *et al*, 2006). Our FACS analyses and metaphase spread experiments show that NUAK1 expression induces gross aneuploidies, causing a strong increase in the DNA content per cell. This phenotype correlates with decreased LATS1. Furthermore, premature senescence and aberrant DNA content can be induced by the expression of a dominant-negative form of LATS1. Interestingly, in MCF10a immortal cells, NUAK1 was also found to induce a strong aneuploidy (Supplementary Figure S10A). Nevertheless, in these cells the aneuploidy provoked cell death instead of senescence (Supplementary Figure S10B and C), resulting in a decreased ability to form colonies (Supplementary Figure S10D). Altogether, these results suggest that depending of the cell type the aneuploidy induced by NUAK1 might have different consequences: senescence or cell death.

Hara and collaborators (Takahashi *et al*, 2006) strongly suggest a function for LATS1 in regulating senescence. These researchers propose that there is no return to proliferation in human senescent cells (in contrast with mouse cells) because of an irreversible cytokinesis blockage and aneuploidy (Takahashi *et al*, 2006). Interestingly, the knockdown of NUAK1 after 4 days of its constitutive expression does not allow the cells to re-proliferate, suggesting that NUAK1 also induces an irreversible cell-cycle arrest (Supplementary Figure S11). Hara and collaborators (Takahashi *et al*, 2006) have implicated LATS1 downregulation in irreversible cell-cycle arrest in normal human cells. In their elegant system, they looked at the role of aberrant DNA content accumulation after senescence induction. Our results also suggest a function for LATS1 and aberrant DNA content in the regulation of senescence, but in our system LATS1 and aberrant DNA

content accumulation could also contribute to the initiation of senescence. Indeed, NUAK1 downregulation can preserve cells from gross aneupoidies and extend their replicative lifespan, whereas NUAK1 upregulation induces gross aneuploidies and senescence. According to our data, LATS1 participates in both initiation and enforcement of senescence but the molecular mechanism involved in the downregulation of LATS1 may be different. Indeed, we did not detect any effect of the proteasome inhibitor MG-132 over LATS1 downregulation by NUAK1 (Supplementary Figure S12) and we also found that the mutation of S464 to A effects LATS1 levels in contrast with the findings of Takahashi *et al* (Takahashi *et al*, 2006). It is thus possible that there are two different mechanisms acting through different upstream kinases, resulting in LATS1 downregulation, aneuploidy, and senescence. Nevertheless, the experimental systems that are used are different and hence make it difficult to derive a definitive conclusion by comparing these results.

In conclusion, our paper joins emerging evidence in suggesting that aberrant DNA content might be, similar to aberrant DNA replication, DNA damage, or oxidative stress, one of the triggers contributing to elicit senescence, and that NUAK1 modulation of LATS1 levels is critical for the process.

Materials and methods

Cell culture

WI-38 cells were grown in DMEM (Invitrogen). HEK 293, 293T (ATCC), and the packaging cell line 293 GP (Clontech) were grown in RPMI (Invitrogen). Both media were supplemented with 10% FCS (Hyclone) and gentamycin (Invitrogen).

Vectors

Pools of shRNAs cloned in the pRetroSUPER vector were used for screening (Berns *et al*, 2004). The shRNA sequences inserted into the pRetroSUPER vector to generate the pRS/NUAK1 and pRS/NUAK1.1 constructs are, respectively, 5′-CATCCTCTGATTCTA GGTG-3′ and 5′-GAAGTTATGCTTTATTCAC-3′. NUAK1*flag* cDNA was excised from pcDNA3.1/NUAK1*flag* (Suzuki *et al*, 2003b) and inserted into the pLPC retroviral vector (pLPC/NUAK1*flag*) or in LNCX2 retroviral vector (LNCX2/NUAK1*flag*). NUAK2 cDNA was TOPO cloned essentially with a 5′ flag tag as described (Lizcano *et al*, 2004). pBabe-flag-LKB1 DN was described elsewhere (Shaw *et al*, 2004). The shRNA sequence inserted into the pRS to generate the pRS/LKB1 is 5′-CGGGACTGACGTGTAGAACAA-3′.

The single mutants NUAK1 K84A (kinase dead) were prepared by using pLPC/NUAK1*flag* as template with the Mutagenesis kit (Stratagene) as instructed by the manufacturer. The primers used K84A forward 5′-GGTTGCTATAAGATCCATTCGTAAGGACAAGCT TAAGGATGAACAAG-3′, K84A reverse 5′-CTTGTTCATCCTAAGCTTT GTCCTTACGAATGGATCTTATAGCAACC-3′, T211A forward 5′-TAAG TTCTTACAAGCGTTTTGTGGGAGTC-3′, T211A reverse 5′-GACTCCC ACAAAACGCTTGTAAGAACTTA-3′, S600A forward 5′-CCAGCGC ATCCGCGCCTGCGTCTCTGCAG-3′ and S600A reverse 5′-CTGCAGA GACGCAGGCGCGGATGCGCTGG-3′. The vectors encoding LATS1 *flag*, LATS1 DN *flag*, and LATS1*myc* have been described elsewhere (Hirota *et al*, 2000; Yang *et al*, 2004; Takahashi *et al*, 2006).

Infection and genetic screening

Retrovirus was produced by transfection of 293 GP packaging cells (Clontech) according to the manufacturer's recommendations. For screening, WI-38 cells close to senescence were infected with the pRS control vector or with a library pool. Cells were initially selected with 500 ng/ml puromycin and later cultured in the presence of 200 ng/ml puromycin. WI-38 cells were split every week for 3 weeks until clones appeared. Genomic DNA was then prepared and nested PCRs were performed to identify the positive shRNA. The primer pairs used were: PCR1 reverse 5′-GAGACGTGC TACTTCCATTTGTC-3′ and forward 5′-CCCTTGAACCTCCTCGTTC GACC-3′, PCR2 reverse 5′-TGTGAGGGACAGGGGAG-3′ and forward 5′-ACCTCCTCGTTCGACCC-3′. A total of 20 PCR cycles were performed with the PCR1 primer pair. Afterwards, 10% of the PCR product was subjected to 20 additional PCR cycles with the PCR2 primer pair. The resulting PCR products were cloned with the TOPO TA cloning kit (Invitrogen) and finally sequenced.

Growth curves, colony formation assays, senescence-associated β-galactosidase staining, and senescent-associated heterochromatin formation

After infection and puromycin selection, 90 000 cells were seeded per well in a six-well plate. Every 5–8 days, the cells were split and 90 000 cells were seeded per well into six-well plates. The number of population doublings was calculated at each passage. For colony formation assays, 60 000 cells were seeded and left to grow for 2 weeks. The cells were then fixed with 4% paraformaldehyde and stained with a crystal violet solution. SA-β-Gal and SHAF analyses were performed as described Dimri *et al* (1995) and Narita *et al* (2003), at 2 or 3 days after seeding 90 000 cells per well in six-well plates.

Cell-cycle analysis

For cell-cycle analysis, the cells were fixed in ice-cold 70% ethanol, washed in PBS, and treated with 10 μg/ml RNaseA for 30 min at 37 °C. Propidium iodide (Sigma) was added to the samples (final concentration: 10 μg/ml) before the analysis of at least 5×10^3 cells with an Epics Elite Cytometer (Coulter).

Quantitative RT–PCR

RNA was extracted from cells with the help of the RNeasy kit from Invitrogen. cDNAs were made from RNA polyA with Superscript II according to the manufacturer's recommendations (Invitrogen). Q-PCR was performed with the following primers: NUAK1 forward 5′-GACATGGTTCACATCAGACGA-3′, NUAK1 reverse 5′-CAATAGTGC ACAGCAGAGACG-3′, Control RPS14 forward 5′-GACCAAGACCCC TGGACCT-3′ and Control RPS14 reverse 5′-GAGTGCTGTCAGAGGG GATG-3′.

Immunoblotting

Immunoblot analyses were performed as described in Bernard *et al* (2003). Membranes were incubated with the antibodies directed against the following antigens: flag tag (F3165, Sigma), cyclin A (H-432, Santa Cruz Biotechnology), NUAK1 (Abgent), LATS1 (A300–477A, Bethyl, or G-16, Santa Cruz Biotechnology), LKB1 (sc-32245, Santa Cruz Biotechnology), and actin (A5316, Sigma). Antibody against the phospho S464 of LATS1 was prepared by injecting a phospho S464 peptide (H2N—IPV RSN S_{464} FN NPL G—CONH2). Rabbit phospho-specific antibody was purified by its ability to bind the phospho peptide but not the non phosphorylated peptide (Euromedex). The nitrocellulose membranes were then incubated with the corresponding secondary antibodies (Amersham) and the signal revealed using the ECL kit (PerkinElmer Life Sciences).

Phosphorylation assay

HEK 293T cells were transfected with flag-tagged NUAK1, NUAK2, or LATS1 DNA by means of either calcium phosphate or jetPEI. After 36–48 h, the cells were washed three times with ice-cold PBS and collected by scraping into 0.7 ml lysis buffer containing 25 mM HEPES pH 7.5, 1% Triton X-100, protease inhibitors (Roche; 1 tablet/50 ml), phosphatase inhibitors (50 mM sodium fluoride and 5 mM sodium pyrophosphate), 100 mM NaCl, and 1 mM DTT and kept on ice. Harvested cells were dispersed by four passages through a 21-G needle and were kept on ice for approximately 20 min. The lysate was clarified by centrifuging at 14 000 r.p.m. for 20 min and the supernatant was collected. The clarified lysate was incubated with M2-flag resin (50 μl/500 μl lysate) overnight at 4 °C. The protein-bound resin was washed twice with lysis buffer containing 150 mM NaCl and once with lysis buffer. Flag-resin-bound NUAK1 was eluted with 100 μl elution buffer (25 mM HEPES pH 7.5, 1% Triton X-100, protease and phosphatase inhibitors, 10% glycerol, and 300 ng/μl flag peptide) by shaking at 4 °C for 3–6 h. *In vitro* kinase assays with the NUAK1 and AMPK kinases (purified and activated with CamKKbeta as described by Sanders *et al*, 2007) were carried out with AMARA (AMARAASAAALRRR), SAMs (HMRSAMSGLHLVKRR), LATS1 (PNIPVRSNS_{464}FNNPLGPRRR), and LATS1S464A (PNIPVRSNA_{464}FNNPLGPRRR) peptides, with a 25 μl mixture containing 50 mM HEPES, pH 7.5 and 1% Triton X-100, 1 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM [^{32}P]-ATP, and

1 mM peptide. The reaction mixtures were incubated at 30 °C for 20 min. Transfer of [^{32}P]-phosphate to the peptide substrate was measured by placing 20 μl of the reaction mixture onto P81 phosphocellulose paper and the paper was washed in 1 mM phosphoric acid three times and subjected to scintillation counting.

NUAK1 and NUAK2 phosphorylation of full-length LATS1: Flag peptide eluted LATS1 protein was incubated at 37 °C in 25 mM HEPES pH 7.5, 1% Triton X-100, protease and phosphatase inhibitors, 10% glycerol and 0.5 μl 32P ATP (6000 Ci/mmol, Perkin-Elmer) either alone or with flag peptide eluted NUAK1 or NUAK2 for 20 min in a 30-μl reaction. Reactions were stopped with SDS–PAGE sample buffer and separated on NUPAGE (Invitrogen) gels in Tris-Glycine buffer. Gels were stained with Simply Blue stain (Invitrogen) and destained with water, dried, and phosphor imaged.

Chromosome spreading

The cells were incubated for 1 h in a Karyomax Colcemid (Invitrogen Corporation), trypsinised, and incubated in a 60 mM KCl hypotonic buffer. The cells were fixed with freshly made methanol/acetic acid solution (3:1 v/v), spread onto frozen slides, and then air-dried. DNA was stained with 1 μg/ml Hoechst 33258 for 3 min. The chromosomes were observed and counted under an epifluorescence microscope (Zeiss, axioplan 2) with a Hoechst filter.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure S1

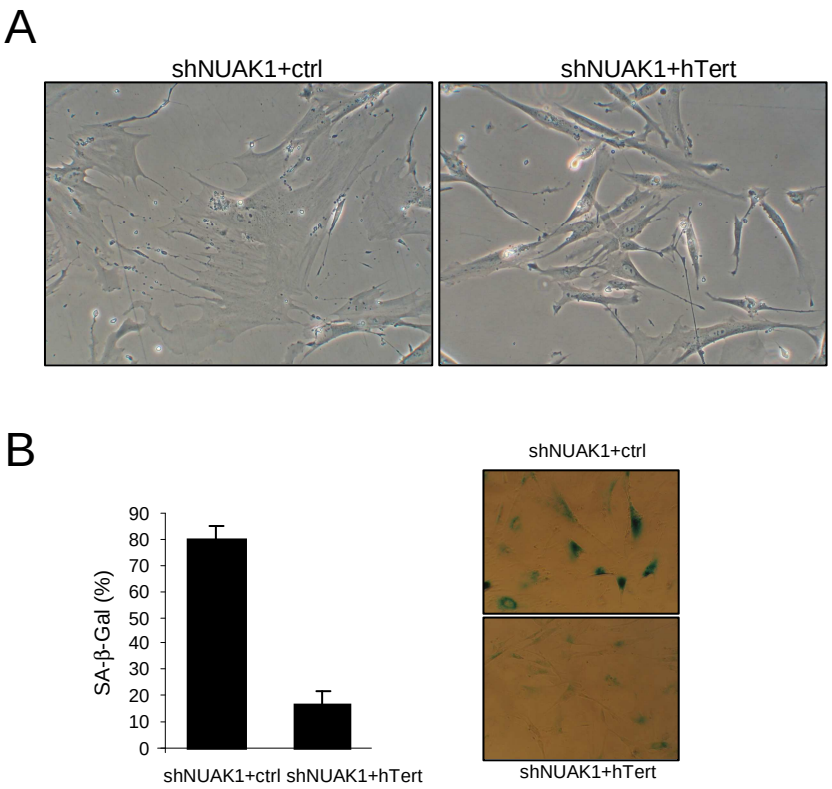

Figure S2

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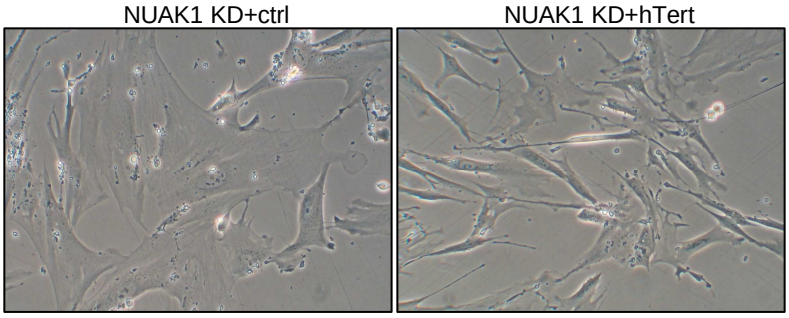

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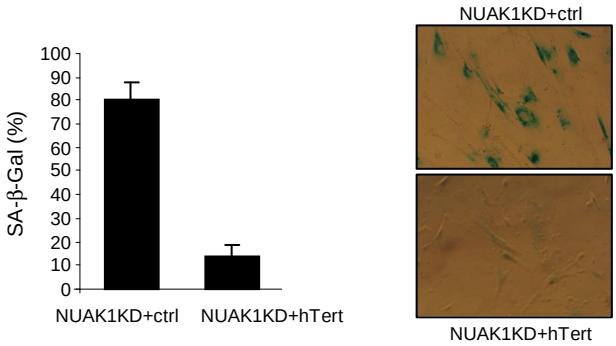

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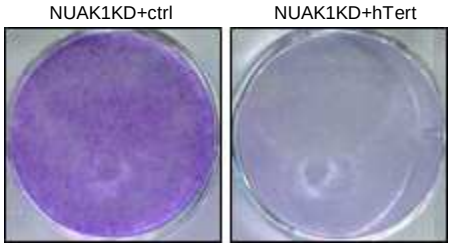

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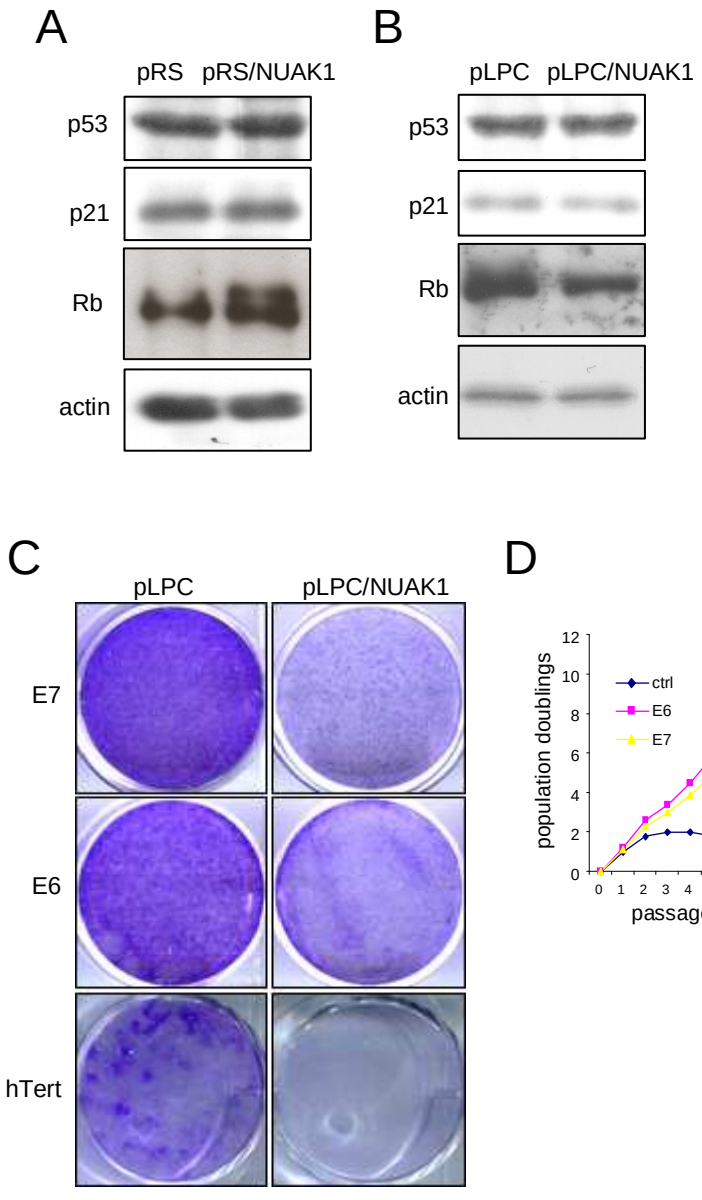

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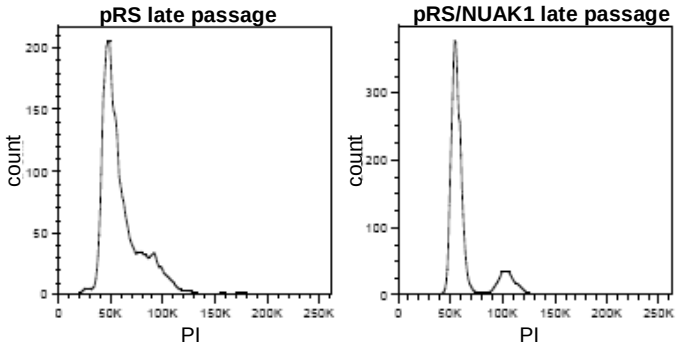

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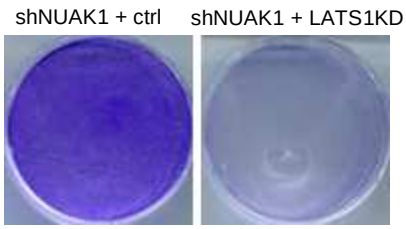

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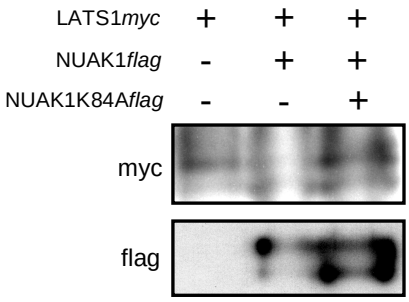

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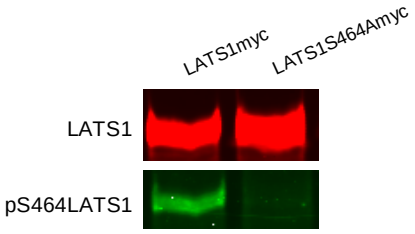

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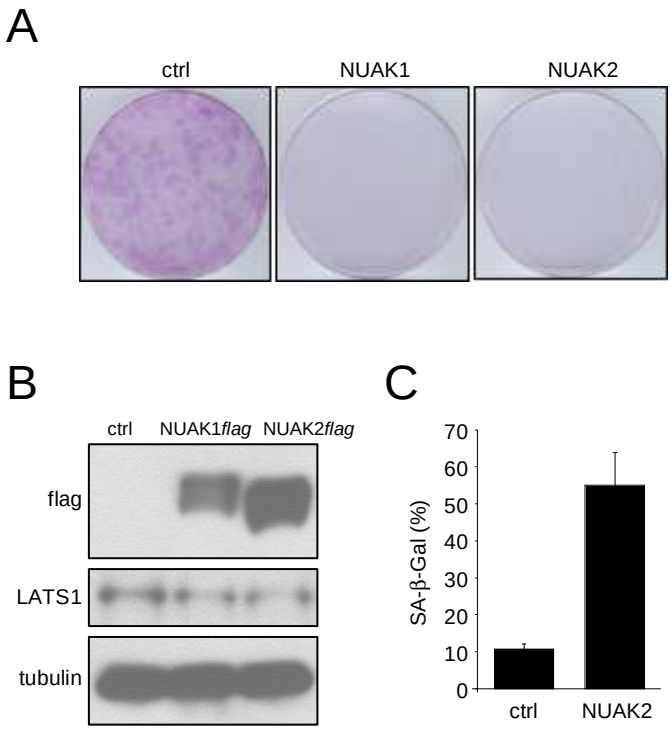

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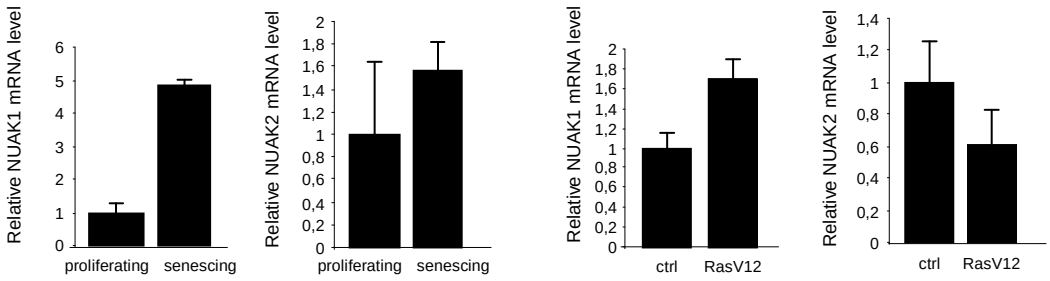

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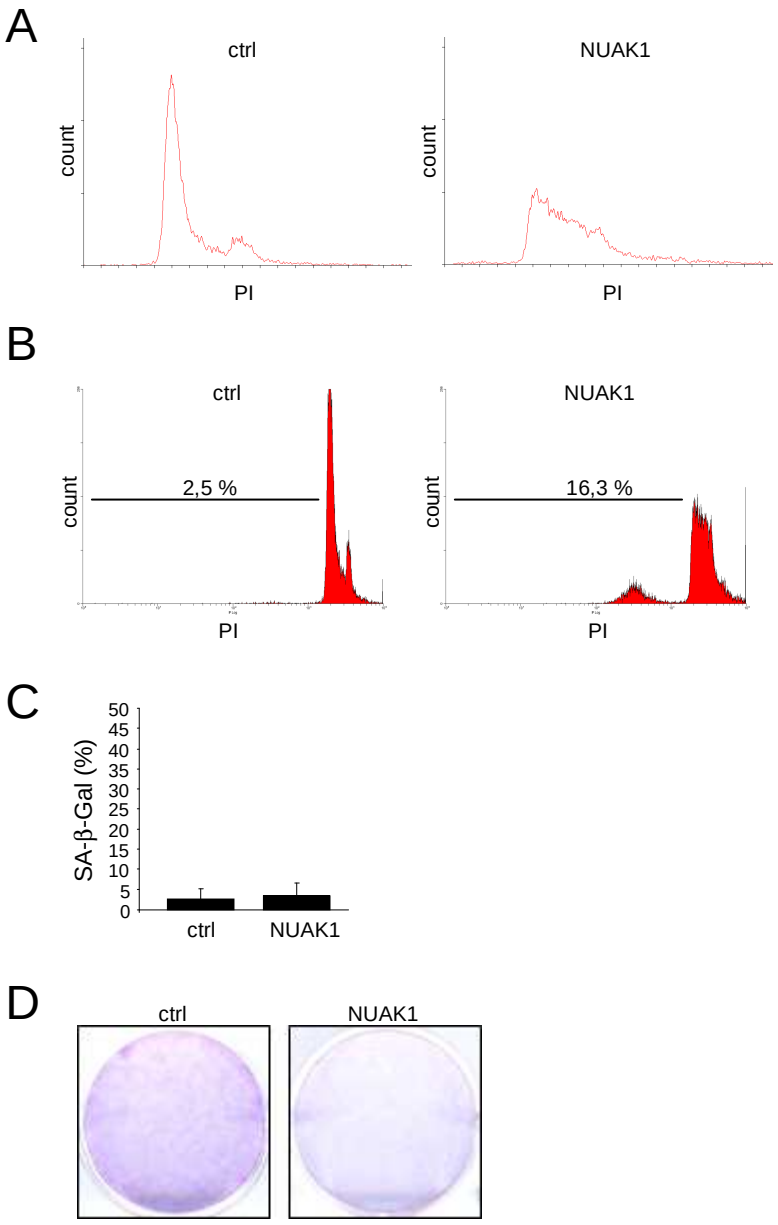

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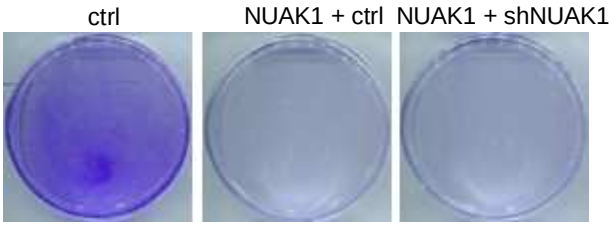

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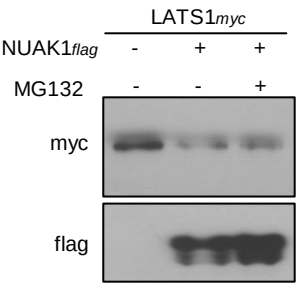

Cell

Chemokine Signaling via the CXCR2 Receptor Reinforces Senescence

Juan C. Acosta,[1,5] Ana O'Loghlen,[1,5] Ana Banito,[1] Maria V. Guijarro,[2] Arnaud Augert,[3] Selina Raguz,[1] Marzia Fumagalli,[4] Marco Da Costa,[1] Celia Brown,[1] Nikolay Popov,[1] Yoshihiro Takatsu,[1] Jonathan Melamed,[2] Fabrizio d'Adda di Fagagna,[4] David Bernard,[3] Eva Hernando,[2] and Jesús Gil[1,*]

[1]Cell Proliferation Group, MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Campus, W12 0NN London, UK
[2]Department of Pathology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA
[3]UMR 8161, Institut de Biologie de Lille, CNRS/Universites de Lille 1 et 2/Institut Pasteur de Lille, IFR 142, Lille, France
[4]IFOM Foundation–FIRC Institute of Molecular Oncology Foundation, 20139 Milan, Italy
[5]These authors contributed equally to this work
*Correspondence: jesus.gil@csc.mrc.ac.uk
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SUMMARY

Cells enter senescence, a state of stable proliferative arrest, in response to a variety of cellular stresses, including telomere erosion, DNA damage, and oncogenic signaling, which acts as a barrier against malignant transformation in vivo. To identify genes controlling senescence, we conducted an unbiased screen for small hairpin RNAs that extend the life span of primary human fibroblasts. Here, we report that knocking down the chemokine receptor CXCR2 (IL8RB) alleviates both replicative and oncogene-induced senescence (OIS) and diminishes the DNA-damage response. Conversely, ectopic expression of CXCR2 results in premature senescence via a p53-dependent mechanism. Cells undergoing OIS secrete multiple CXCR2-binding chemokines in a program that is regulated by the NF-κB and C/EBPβ transcription factors and coordinately induce CXCR2 expression. CXCR2 upregulation is also observed in preneoplastic lesions in vivo. These results suggest that senescent cells activate a self-amplifying secretory network in which CXCR2-binding chemokines reinforce growth arrest.

INTRODUCTION

Replicative senescence was first described in primary human fibroblasts that had reached the end of their proliferative life span in tissue culture (Hayflick and Moorhead, 1961). Senescent cells undergo an apparently irreversible growth arrest but remain metabolically active and display characteristic changes in cell morphology, physiology, and gene expression (Campisi and d'Adda di Fagagna, 2007). In the fibroblast model, one of the principal determinants of senescence is the erosion of the telomeres that occurs at every cell division. This eventually registers as DNA damage and triggers cell cycle arrest via activation of the p53 pathway (d'Adda di Fagagna et al., 2003). The definition of senescence has since been broadened, and it is now accepted that other forms of DNA damage and cellular stress, whether caused by the presence of an activated oncogene, unscheduled DNA replication, oxidative stress, or suboptimal culture conditions can all provoke a senescence phenotype irrespective of telomere status (Collado et al., 2007). This latter phenomenon is referred to as oncogene-induced senescence (OIS), premature senescence or stasis, to distinguish it from classical replicative senescence. However, the implementation of senescence represents an integrated response to a diverse range of signals.

Despite the experimental focus on tissue culture models, the wider significance of senescence in vivo has become a matter of record. The most compelling examples are manifestations of OIS in benign or premalignant lesions carrying single activated oncogenes (reviewed in Narita and Lowe, 2005). Senescence is therefore a first-line defense against potentially dangerous mutations (Lowe et al., 2004), locking the afflicted cells into a permanent state of arrest (Mooi and Peeper, 2006). Progression to malignancy would correlate with escape from or impairment of senescence. However, this pivotal mechanism of tumor suppression comes at a cost to the organism, because it sets limits to the proliferative and regenerative potential of normal tissues. Cellular senescence is therefore intimately associated with aging (Collado et al., 2007).

Senescent cells undergo characteristic changes in gene expression. Some of these changes, such as activation of p53 or the upregulation of the cyclin-dependent kinase (CDK) inhibitors, $p16^{INK4a}$ and $p21^{CIP1}$, relate directly to the establishment and maintenance of growth arrest; however, it is striking to note that senescent cells produce increased amounts of secreted proteins, including extracellular proteases and matrix components, growth factors, cytokines, and chemokines (Mason et al., 2006; Shelton et al., 1999). Consequently, senescent cells can alter the tissue microenviroment and affect neighboring cells through paracrine signaling. For example, senescent fibroblasts can stimulate angiogenesis (Coppe et al., 2006), alter differentiation (Parrinello et al., 2005), and promote growth and tumorigenesis of epithelial cells (Krtolica et al., 2001). Factors secreted by senescent cells also contribute to tumor clearance by signaling to the immune system (Xue et al., 2007). In addition, it was recently shown that

plasminogen activator inhibitor-1 (PAI-1), a secreted protein that has been regarded as a marker for senescence, is a transcriptional target of p53 that directly contributes to the establishment of senescence (Kortlever et al., 2006). Similarly, a study published while this work was under revision (Wajapeyee et al., 2008) shows that the insulin-like growth factor binding protein 7 (IGFBP7) mediates senescence induced by oncogenic BRAF in normal melanocytes. It is therefore plausible that additional factors secreted by senescent cells could influence cell growth.

Among the candidates are the multiple chemokines released by senescent cells. Chemokines are a family of small chemotactic cytokines that mediate communication between different cell types and have multiple roles in health and disease (Mantovani et al., 2006). For example, the proinflammatory chemokine IL-8/CXCL8 modulates endothelial cell migration and promotes angiogenesis, tumorigenesis, and metastasis (Yuan et al., 2005). Ras-dependent secretion of IL-8 enhances tumor progression by promoting vascularization through paracrine signaling (Sparmann and Bar-Sagi, 2004). Chemokines bind to receptors of the GPCR superfamily. The CXCR2 receptor binds to angiogenic CXC chemokine family members, containing a glutamic acid-leucine-arginine motif (ELR+). Thus, in addition to IL-8, CXCR2 binds CXCL1, 2, and 3 (GROα, β, and γ), CXCL5 (ENA-78), CXCL6 (GCP2), and CXCL7 (NAP2), whereas CXCR1 binds only to GCP2, NAP2, and IL-8.

Here, we report the identification of a small hairpin RNA (shRNA) targeting CXCR2 in a functional screen for bypass of replicative senescence in human fibroblasts. In addition, cells undergoing OIS secrete multiple CXCR2-binding chemokines in a manner dependent on NF-κB and C/EBPβ. These results suggest that senescent cells activate a self-amplifying secretory program in which CXCR2 ligands reinforce growth arrest. Importantly, evidence for elevated expression of CXCR2 in preneoplastic lesions, together with a tumor-associated mutation and loss of expression in advanced cancers, would be consistent with the view that malignancy reflects escape from senescence.

RESULTS

Downregulation of CXCR2 Expression Extends Cellular Life Span

Identifying genes that regulate senescence can reveal novel tumorigenic mechanisms, and several notable examples have been uncovered in different contexts (Gil et al., 2004; Narita et al., 2006; Rowland et al., 2005). In the current study, we used the NKI shRNA library targeting 7914 known genes (Berns et al., 2004) in a functional screen for extension of the life span of primary human fibroblasts (Figure 1A). The library is in a 96-well format, with each well containing three independent shRNAs against the same gene in the pRetroSuper (pRS) vector. Pooled DNA from each 96-well plate was used to infect IMR-90 cells. Importantly, we used near-senescent IMR-90 cells (Figure S1A), which have very limited growth potential, with the expectation that agents that interfere with senescence would allow the outgrowth of proliferative colonies.

Among the constructs that extended replicative life span in this setting (such as shRNAs targeting p53, Rb, and three other constructs that we are in the process of validating) was an shRNA against CXCR2 (Figure 1B). Retesting confirmed that depletion of CXCR2 with either of two independent shRNAs delayed senescence in IMR-90 cells but did not immortalize them (Figures 1B and 1C). Both shRNAs caused almost complete extinction of CXCR2 expression as judged by quantitative RT-PCR (qRT-PCR) (Figure 1D) and immunofluorescence (Figure 5C). These findings are not unique to IMR-90 cells as knock-down of CXCR2 also extended the life span of other primary cells (WI-38 and human mammary epithelial cells; Figures S1B–S1D).

CXCR2 Depletion Diminishes OIS and the DNA-Damage Response

Because replicative senescence reflects an amalgam of different signaling events, we asked whether knockdown of CXCR2 could also influence OIS. For this purpose, we took advantage of IMR-90/MEK:ER fibroblasts that express a switchable version of MEK1, a downstream effector of Ras (Collado et al., 2005). Upon addition of 4-hydroxytamoxifen (4OHT), these cells undergo growth arrest (Figures S2A and S2B). Depletion of CXCR2 with shRNA allowed a limited number of cells to evade MEK-induced arrest (Figure 1E). Consistent results were achieved with two independent shRNAs. The percentage of cells incorporating BrdU also increased upon knockdown of CXCR2 as measured 2 days after MEK activation (Figure 1F). Similar results were observed following transfection of controls and CXCR2 siRNAs into BJ fibroblasts infected with a retrovirus encoding H-Ras^{G12V} (Figures S2C and S2D).

Because CXCR2 influences oncogene-induced and replicative senescence, and DNA-damage response signaling plays a core role in both (Campisi and d'Adda di Fagagna, 2007), we investigated whether CXCR2 modulates the DNA damage checkpoint. To this end, we exposed IMR-90 cells infected with a control or an shRNA vector targeting CXCR2 to X-irradiation. Whereas control cells encountered a DNA damage checkpoint, as shown by diminished BrdU incorporation, cells with either CXCR2 or p53 knocked down did not arrest significantly (Figure 1G). Moreover, upon CXCR2 knockdown, ATM activation was impaired as indicated by reduced number and decreased intensity of DNA-damage response (DDR) foci containing the phosphorylated active form of ATM in irradiated cells (Figure 1H). Similarly, pST/Q antibodies recognizing phosphorylated ATM and ATR targets gave reduced signals in cells depleted of CXCR2 expression (Figure S2E). These results are consistent with CXCR2 affecting senescence by influencing the activation of the DNA damage checkpoint.

Premature Senescence Induced by Expression of CXCR1 or CXCR2 Is Dependent on p53

To complement the findings with CXCR2 shRNAs, we next asked whether ectopic expression of CXCR2 or its paralog CXCR1 would impair cell proliferation. When viruses expressing CXCR1 or CXCR2 (Figure 2A) were used to infect IMR-90 cells, both caused retarded growth culminating in premature senescence (Figure 2B). CXCR2 overexpression also slowed the growth of WI-38 and human mammary epithelial cells (Figure S3). Although in human fibroblasts the CXCR2-induced arrest was not as dramatic as that caused by H-Ras^{G12V}, the cells showed reduced incorporation of BrdU (Figure 2C) and displayed

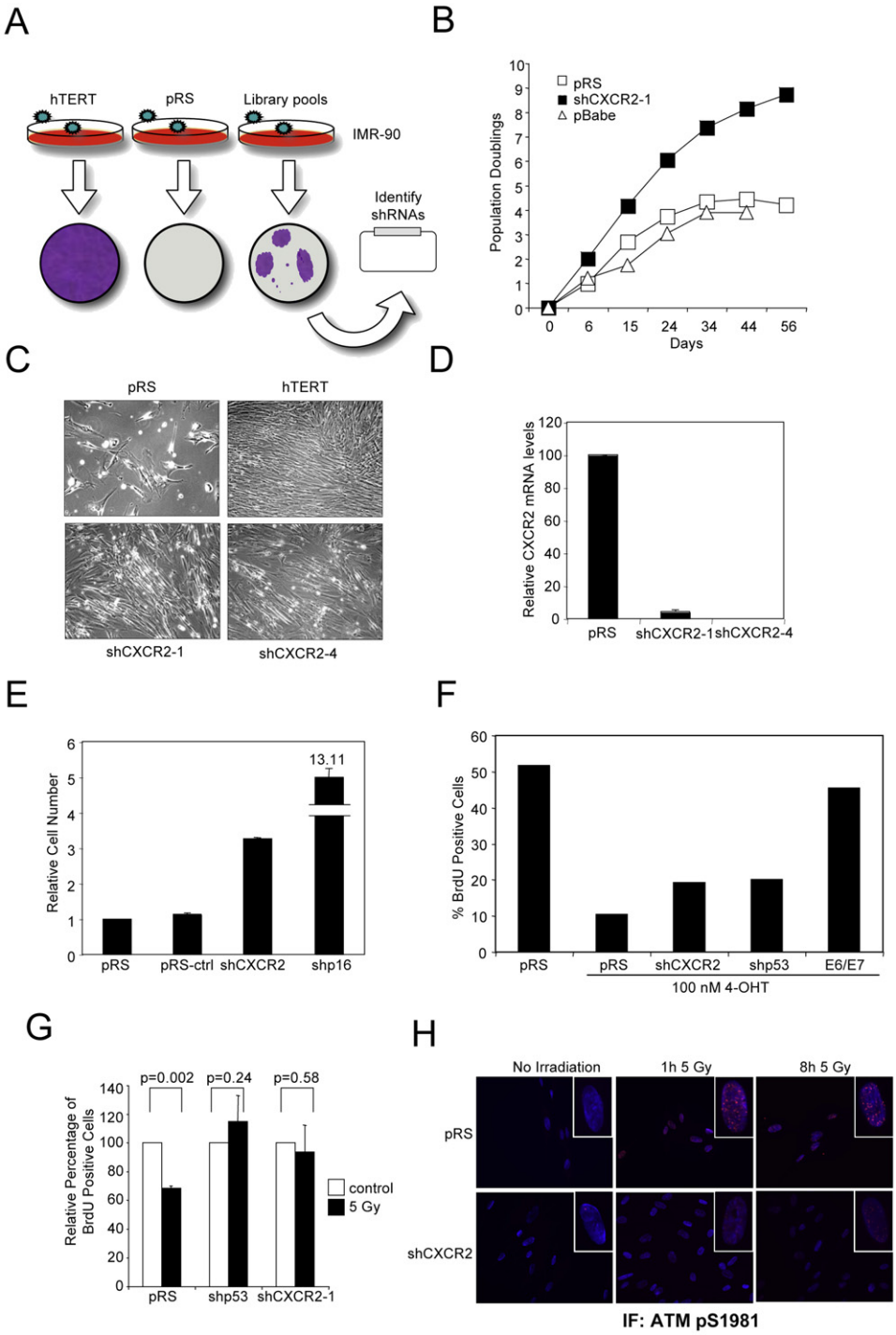

Figure 1. Depletion of CXCR2 Extends Cellular Life Span and Blunts Oncogene-Induced Senescence

(A) Outline of the genetic screening for identifying shRNAs extending life span of IMR-90 cells.

(B) IMR-90 cells were infected with pRS, pBabe, and shCXCR2-1 after selection growth curves were performed.

(C) Microphotographs showing the effect of two independent constructs targeting CXCR2 (1 and 4) over growth and morphology of IMR-90 cells.

(D) Confirmation of the levels of CXCR2 knockdown by qRT-PCR. Error bars represent standard deviation.

(E) IMR-90/MEK:ER cells were infected with the indicated vectors, selected and 10^5 cells seeded per 10 cm dish. Next day, 100 nM 4-OHT was added. After 15 days, plates were fixed and stained with crystal violet. Plot represents the relative cell number normalized to pRS-infected cells. For clarity, the scale has been split. Error bars represent the standard deviation.

characteristic features of senescence. For example, the proportion of cells staining positively for senescence-associated β-galactosidase (β-Gal) activity and displaying senescence-associated heterochromatin foci was elevated in cultures transduced with CXCR1 or CXCR2 compared with controls (Figure 2D). Similarly, although the overall percentages were small, a higher proportion of the CXCR2-transduced cells displayed DDR foci. In addition, the number of DDR foci and their intensity was more elevated in cells expressing CXCR2 (Figure 2E).

As the DNA damage pathway activates p53, we explored the role of p53 in CXCR2-mediated arrest by genetic means. To this end we analyzed the effects of CXCR2 in WI-38 human fibroblasts in which p53 or pRb functions were disrupted using the HPV E6 and E7 proteins, respectively. The data confirm the importance of p53 but imply that the Rb pathway is also involved in establishing the CXCR2-induced arrest in human cells (Figure 2F). Consistent with the role of p53, depletion of CXCR2 in different primary cells resulted in reduced levels of p53, $p21^{CIP1}$, and other p53 targets as assessed via western blot analysis (Figure S4). To substantiate these findings, we took advantage of mouse embryo fibroblasts (MEFs) genetically deficient for p53, $p16^{Ink4a}$/$p19^{Arf}$, or $p21^{Cip1}$. Infection of wild-type MEFs (passage 1) with retroviruses encoding human CXCR1 or CXCR2 caused a rapid growth arrest, whereas control cells continued proliferating (Figure 2G). If anything, the effects of CXCR1 or CXCR2 were more profound than in IMR-90 cells. Similar results were obtained when the assays were performed in *Ink4a/Arf*$^{-/-}$ or *p21*$^{-/-}$ MEFs and the cells arrested with the morphological characteristics of senescence (Figure S5). In contrast, *p53*$^{-/-}$ MEFs were refractory to CXCR1 or CXCR2 expression and continued proliferating (Figure 2G). Interestingly, in *p53*$^{-/-}$ MEFs, ectopic expression of CXCR2 resulted in cells that lost contact inhibition and produced anchorage-independent colonies, although smaller than with $H\text{-}Ras^{G12V}$ (Figure S5F). Taken together, these results suggest that CXCR2-induced senescence is p53-dependent.

Coordinated Upregulation of CXCR2 and Their Ligands during Senescence

Having shown that altering CXCR2 expression can either delay or promote senescence, we next asked whether the endogenous levels of CXCR2 change during senescence. In human fibroblasts, CXCR2 expression was low (undetectable by immunofluorescence) at early and middle passage but increased during replicative senescence, as detected by qRT-PCR and immunofluorescence (Figures 3A and 3B and Figure S6). Similarly, the levels of CXCR2 messenger RNA were approximately 6-fold higher in cells undergoing $H\text{-}Ras^{G12V}$-induced senescence compared with controls (Figure 3C). Upregulation of CXCR2 following $H\text{-}Ras^{G12V}$ expression was also demonstrable by immunofluorescence (Figure 3D).

If the receptor is upregulated, it was important to know whether any of the CXCR2-binding chemokines were expressed during OIS. To this end, we used antibody arrays (Ray Biotech) to monitor the levels of 90 secreted factors (Figure S7) in the medium from control IMR-90/LXSN and IMR-90/MEK:ER cells treated with and without 100 nM 4OHT for 72 hr. Approximately 12-14 secreted factors were significantly expressed during OIS, including the proinflammatory cytokines IL-6 and IL-1α (Figure 3E). This is consistent with previous studies (Mason et al., 2006). Of the 37 chemokines represented on the arrays, only 8 showed elevated expression in cells undergoing OIS (Figure 3E). This could be an overestimate, because the array did not have an antibody that discriminates GROβ and GROγ from GROα. Interestingly, this set included all CXCR2 ligands except GCP2. Because antibody arrays are semiquantitative, and have a limited dynamic range (saturated for the IL-8 and GROα, β, γ antibodies), we performed qRT-PCR and enzyme-linked immunosorbent assay (ELISA) to measure IL-8 and GROα. The results from the ELISAs showed that 48 hr after treatment of IMR-90/MEK:ER, cells with 4OHT, secretion of IL-8, and GROα increased dramatically (Figures 3F and 3G). In accordance with published reports (Ancrile et al., 2007; Sparmann and Bar-Sagi, 2004), IL-8 and GROα also increased in response to MEK activation, even in the absence of senescence (Figure S8). Analyses using qRT-PCR confirmed a significant increase of all CXCR2 ligand transcripts, including GCP2, in cells undergoing OIS, with a more prominent induction of IL-8 and ENA-78 (Figure 3H). These data indicated a coordinated upregulation of CXCR2 and its ligands at senescence, occurring at the RNA level.

NF-κB and C/EBPβ Regulate the Expression of CXCR2 Ligands during OIS

To elucidate the signaling pathways involved in the induction of IL-8 and other CXCR2 ligands during OIS, we first asked whether different chemical inhibitors could block their production. As a positive control, treatment with the MEK inhibitor PD98059 prevented the induction of IL-8 following addition of 4OHT, in IMR-90/MEK:ER cells (Figure 4A). Inhibition of p38 MAPK with 10 μM SB202190 or inhibition of IκB kinases with 10 μM BAY 11-7082 also blunted the upregulation of IL-8 (Figure 4A). Using antibody arrays, we observed that the induction of IL-8, most CXCR2 ligands, and other chemokines depended on NF-κB (Figure 4B). Bioinformatic predictions showed that sites for NF-κB and C/EBP, among others, were present in the promoters of most CXCR2 ligands (data not shown).

Because of precedents linking Ras and MEK to the activation of NF-κB and C/EBPβ (Finco et al., 1997; Nakajima et al., 1993), and the role of C/EBPβ in OIS (Sebastian et al., 2005), we focused on these factors and asked whether they were being activated during OIS. Analysis of NF-κB function showed that there is active NF-κB in the nucleus upon MEK induction (Figure 4C). In addition,

(F) IMR-90/MEK:ER cells were infected with the indicated viruses. After selection, cells were left untreated or treated with 100 nM 4OHT. After 5 days, a 16 hr pulse of BrDU was given, the cells were fixed, and BrdU was quantified.

(G) IMR-90 cells infected with control vector or shCXCR2-1 were irradiated with 5 Gy and pulsed with BrdU 1 hr after irradiation for 8 hr. Later, BrdU-positive cells were quantified and normalized to the respective control cells. Error bars represent the standard deviation.

(H) Same cells as in (G) were analyzed by immunofluorescence using ATM pS1981 antibody.

The experiments shown in (B-F) were performed independently at least three times. Experiments shown in (G) and (H) were performed independently twice with similar results.

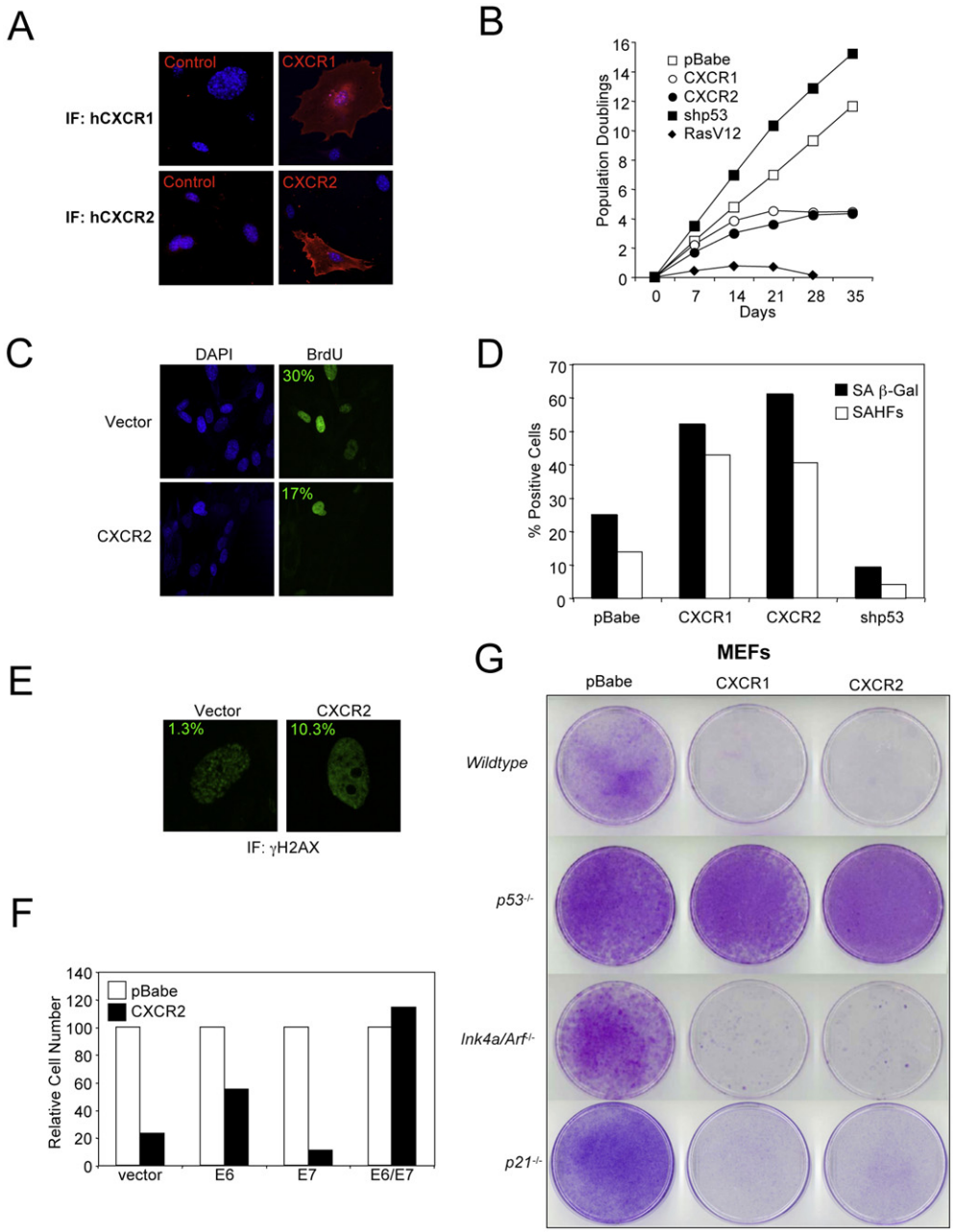

Figure 2. Ectopic Expression of CXCR2 or CXCR1 Causes Premature Senescence Dependent of p53

(A) MEFs were infected with vectors expressing human CXCR1 or CXCR2 or controls. Expression of human CXCR1 (upper panel) or CXCR2 (lower panel) was assessed via immunofluorescence.

(B) IMR-90 cells were infected at passage 17 with the indicated vectors, selected, and growth curves were performed.

(C) Percentage of BrdU-positive cells in IMR-90 cells infected with the vector or overexpressing CXCR2.

(D) Percentage of the indicated cells positive for SAβ-Gal activity and senescence-associated heterochromatin foci (SAHFs).

(E) Percentage of γH2AX-positive cells in IMR-90 cells infected with the vector or overexpressing CXCR2.

(F) Human diploid fibroblasts infected with the indicated vectors were seeded in 10 cm dishes. The plates were fixed 10–15 d after seeding and stained with crystal violet. Crystal violet was extracted and quantified.

(G) MEFs of the indicated genotypes were infected with pBabe, CXCR1, CXCR2, and H-Ras^{G12V} retroviruses, selected, and 10^5 cells were seeded per 10 cm dishes. The plates were fixed 10–15 d after seeding and were stained with crystal violet.

The experiments showed were performed independently at least twice with similar results.

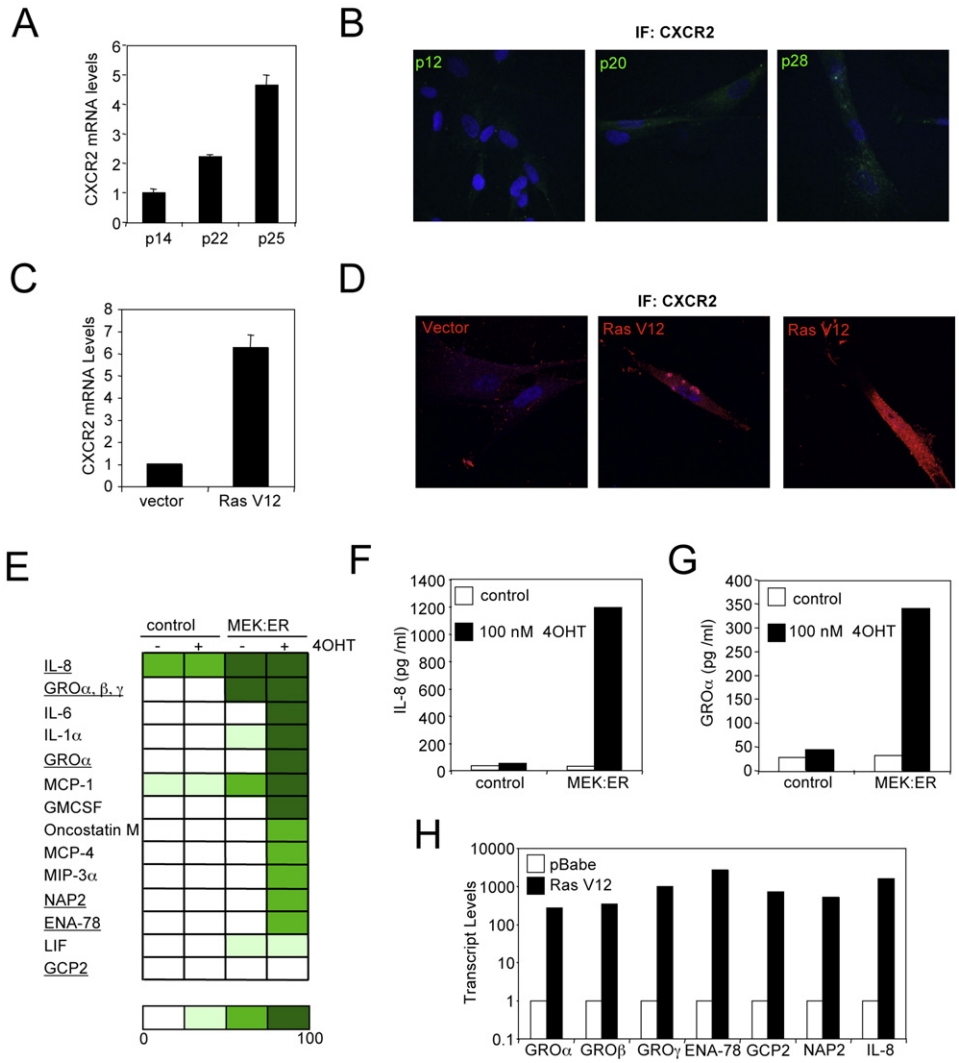

Figure 3. Coordinated Upregulation of CXCR2 and Their Ligands during Senescence

(A) Analysis of CXCR2 transcript levels during serial passage of IMR-90 cells. Error bars represent the standard deviation.

(B) IMR-90 cells at the indicated passages were fixed and subjected to CXCR2 immunofluorescence.

(C) Analysis via qRT-PCR of CXCR2 transcript levels in IMR-90 cells infected with pBabe or H-Ras^{G12V}. Error bars represent the standard deviation.

(D) IMR-90 cells infected with pBabe or H-Ras^{G12V} were subjected to CXCR2 immunofluorescence.

(E) Antibody arrays were used to measure the secretion of 90 factors by IMR-90/LXSN or IMR-90/MEK:ER cells grown in DMEM 0.5% fetal bovine serum and treated with (+) or without (–) 100 nM 4OHT for 72 hr. The arrays were scanned and quantified; the levels were normalized to internal positive controls present in each membrane and split into four groups (0%-25%; 25%-50%; 50%-75%; 75%-100%, or more) referred to the positive control expression in order to represent the semiquantitative results as a heat map. Presented are the factors upregulated during OIS. CXCR2-binding chemokines are underlined.

(F, G) The amount of IL-8 (F) or GROα (G) secreted by IMR-90/MEK:ER or control cells treated with or without 100 nM 4OHT during 48 hr was quantified via ELISA. The experiments were performed independently at least four times with similar results.

(H) The levels of CXCR2 ligands expressed in IMR-90 cells infected as indicated were quantified via qRT-PCR. Similar results were observed in three independent experiments.

the expression of two key NF-κB transducers, RelA and IKKβ increases during OIS (Figure 4D and Figure S9D). To substantiate the role of NF-κB, we designed two shRNAs targeting RelA (Figure 4E). Depletion of RelA decreased the production of IL-8, and most CXCR2 ligands during OIS (Figures 4F and 4G). Finally, we also detected by chromatin immunoprecipitation binding of RelA to the promoters of IL-8 and GROγ, suggesting that the observed regulation of these chemokines by NF-κB was direct (Figure 4H).

Besides NF-κB, the activation of C/EBPβ also increased in response to MEK induction and the C/EBPβ messenger RNA itself was also upregulated during OIS (Figure S9). Depletion of C/EBPβ using shRNA constructs reduced the induction of IL-8, GROα, and NAP2 during OIS (Figures S10A and S10B). The effect over IL-8 secretion was confirmed using independent siRNAs targeting C/EBPβ (Figure S10F). Interestingly, binding sites for C/EBPβ and NF-κB are present in neighboring positions

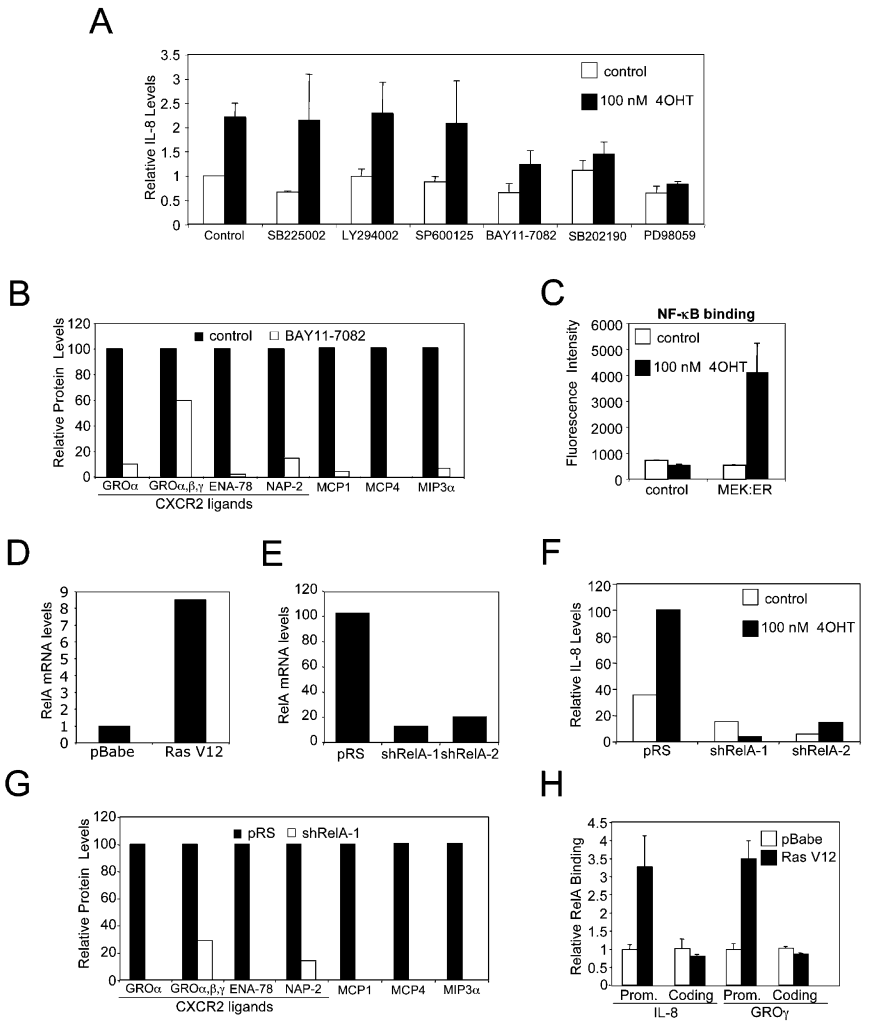

Figure 4. NF-κB Controls the Expression of CXCR2 Ligands during Senescence

(A) IMR-90/MEK:ER cells were treated with SP600125 (JNK inhibitor, 10 μM), PD98059 (MEK inhibitor, 20 μM), SB202190 (p38 inhibitor, 10 μM), BAY 11-7082 (IKK inhibitor, 10 μM), LY294002 (PI3K inhibitor, 2 μM), or SB225002 (CXCR2 inhibitor, 200 nM). One hour later, 100 nM of 4OHT or an equivalent volume of vehicle was added. Twenty-four hours later, supernatants were collected and IL-8 was measured via ELISA. The experiment was performed independently three times, and the mean is represented. Error bars represent the standard deviation.

(B) IMR-90/MEK:ER cells were treated with 10 μM BAY 11-7082 or vehicle (control). One hour later, 100 nM of 4OHT was added. Seventy-two hours later, supernatants were collected and used to probe antibody arrays. The values for the different chemokines present in the control membrane were taken as 100%.

(C) Nuclear extracts from IMR-90/LXSN or IMR-90/MEK:ER left untreated or treated with 100 nM 4OHT for 72 hr were used to measure NF-κB activity as explained in Experimental Procedures. The experiment represented triplicate samples, and two independent experiments were performed with similar results. Error bars represent the standard deviation.

(D, E) IMR-90 cells were infected with the indicated vectors, and expression of RelA was analyzed via qRT-PCR.

(F) IMR-90/MEK:ER cells were infected with the indicated vectors. Cells were selected, seeded, and switched to 0.5% fetal bovine serum, and 24 hr later 100 nM 4OHT was added. Supernatants were collected 24 hr after 4OHT treatment, and IL-8 was measured via ELISA.

(G) Supernatants collected at 72 hr from the same experiment as in (F) were used to probe antibody arrays.

(H) Chromatin immunoprecipitation analyzing the binding of RelA/p65 to the promoter of CXCR2 ligands. Specific binding is represented as the ratio of p65 versus Histone H3 binding and normalized to the binding in control cells. Similar results were obtained in two independent experiments. Error bars represent the standard deviation.

in the IL-8 and GROα promoters and these transcription factors regulate synergistically IL-8 and GROα during viral infection and stress (Hoffmann et al., 2002).

CXCR2 Is Activated in Senescence

As CXCR2 ligands are upregulated during senescence, we analyzed their functional involvement in the process. We generated retroviral vectors to ectopically express IL-8 and GROα. Significantly, expression of IL-8 or GROα reduced proliferation of late passage IMR-90 cells (Figure 5A and Figures S11A and S11B). Conversely, depletion of IL-8 expression using an shRNA (Figure S11C) extended the life span of IMR-90 cells (Figure 5B). To investigate whether IL-8 depletion affected CXCR2 activation, we analyzed the subcellular localization of CXCR2. The location of CXCR2 in endosomes can be considered as a surrogate marker for CXCR2 activation (Sai et al., 2006 and Figure S12) and correlates with other effects caused by CXCR2 activation such as production of reactive oxygen species (ROS) (Figure S13). CXCR2 localization in endosomes diminished when comparing control cells with cells expressing sh-IL-8 (from 71% to 25%) (Figure 5C), suggesting that sh-IL-8 extends cellular life span by restraining CXCR2 activity.

Knockdown of either IL-8 or GROα using shRNAs alleviated OIS in IMR-90 expressing MEK:ER as measured by enhanced BrdU incorporation (Figure 5D) or rescue of cell growth (data not shown). Although the effects were relatively modest (as with CXCR2 knockdown) they were consistently observed with two independent shRNAs against IL-8 or GROα (Figure 5D and data not shown). Interestingly, antibodies neutralizing IL-8 and GROα also alleviated OIS, as did treatment with a chemical CXCR2 inhibitor (SB 225002) (Figure 5E). This suggests that secreted CXCR2 ligands can reinforce senescence. In cells undergoing H-Ras^{G12V}-triggered OIS, CXCR2 was mainly in endosomes (70%), indicative of activation (Figure 5F), whereas CXCR2 localized mainly in the membrane following treatment with either SB 225002 or with neutralizing antibodies targeting IL-8 and GROα. Treatment with an MEK inhibitor that downregulates the expression of IL-8 and other ligands (Figure 5F) resulted

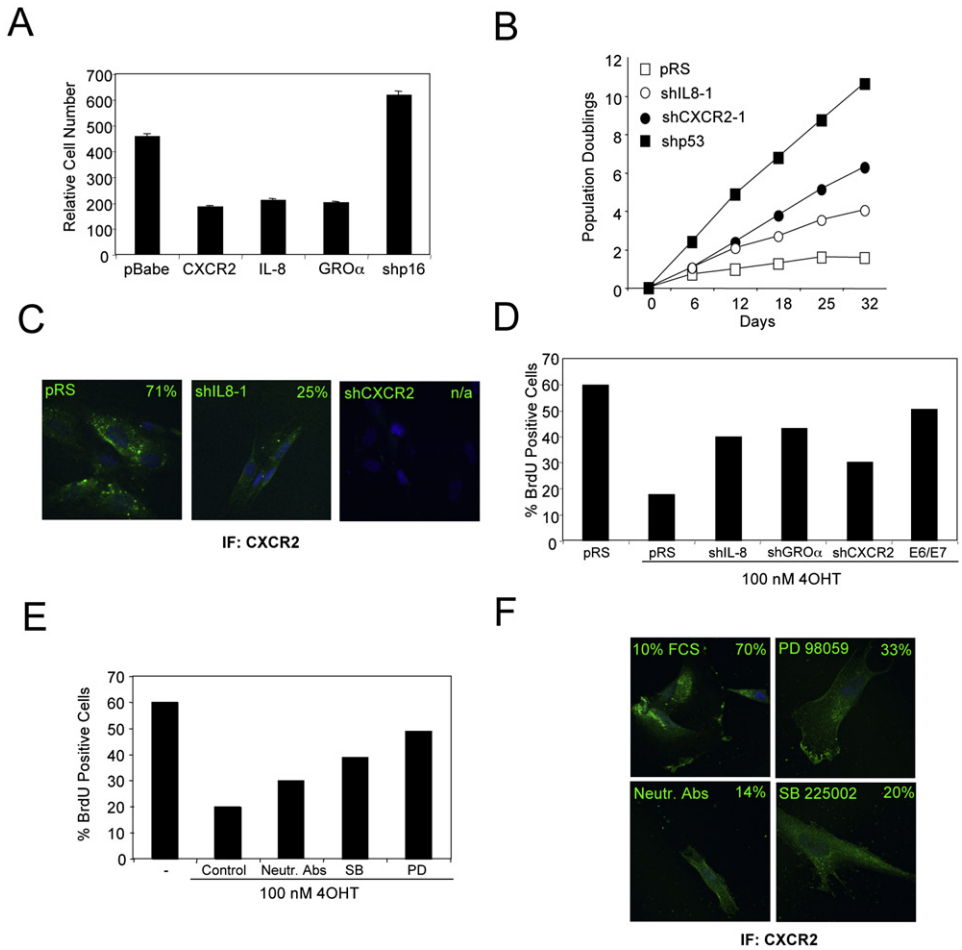

Figure 5. Role for CXCR2 Ligands in Mediating Senescence

(A) IMR-90 cells were infected with the indicated retroviruses. After selection, 10^5 cells were seeded in a 10 cm dish, and after 15 days plates were fixed and stained with crystal violet that was extracted and quantified. Error bars represent the standard deviation.

(B) IMR-90 cells were infected with the indicated retroviruses after selection growth curves were performed.

(C) IMR-90 cells infected with the indicated vectors were subjected to CXCR2 immunofluorescence at late passage. The percentage of cells showing preferential localization of CXCR2 in endosomes as explained in Figure S12 is shown.

(D) IMR-90/MEK:ER cells were infected with the indicated retrovirus. After selection, cells were plated and treated with vehicle or 100 nM 4OHT. Forty-eight hours after treatment, cells were pulsed with BrdU for 16 hr, and BrdU incorporation was quantified. Similar results were obtained in three independent experiments.

(E) IMR-90/MEK:ER cells were treated as indicated. Final concentrations added were: 10 μg/ml for each IL-8- and GROα-neutralizing antibody (Neutr. Abs), 200 nM SB225002 (SB), and 20 μM PD98059 (PD). Twenty-four hours after treatment, cells were pulsed with BrdU for 16 hr and BrdU incorporation was quantified.

(F) IMR-90 cells infected with RasV12 and treated as indicated were subjected to CXCR2 immunofluorescence. Concentrations added were as in (E) and they were kept for 4 hr. The percentage of cells showing preferential localization of CXCR2 in endosomes as explained in Figure S12 is shown.

in strong localization of CXCR2 in the cytoplasmic membrane. These results show a clear correlation between CXCR2 activation and induction of senescence. In addition, the ability of neutralizing antibodies to alleviate OIS suggests that secreted CXCR2 ligands may have a role in senescence reinforcement.

CXCR2 Expression Is Elevated in Preneoplastic Lesions

The upregulation of CXCR2 and their binding chemokines during OIS prompted us to ask whether the components of the CXCR2 network, CXCR1, CXCR2 and their ligands, might also be upregulated in preneoplastic lesions associated with senescent cells. For example, topical treatment of mouse skin with DMBA/TPA leads to the appearance of benign papillomas. These premalignant lesions display a high proportion of senescent cells and the expression of markers of senescence, such as *Arf* or *Dcr2* is elevated compared to control skin (Collado et al., 2005 and Figure 6A). The expression of CXCR2 and its ligands was also significantly increased in papillomas (Figure 6A). Interestingly, IL-6 and its receptor are also upregulated in papillomas (Figure S14), suggesting that a coordinated expression of receptor-ligand pairs could be a recurrent theme during senescence.

To extend these analyses to a clinical setting, we monitored CXCR2 expression in a panel of 30 prostate intraepithelial neoplasia (PIN) samples by immunohistochemical staining. Preneoplastic PIN lesions are enriched in senescent cells (Chen et al., 2005, and Figure S15A) and their progression to malignant

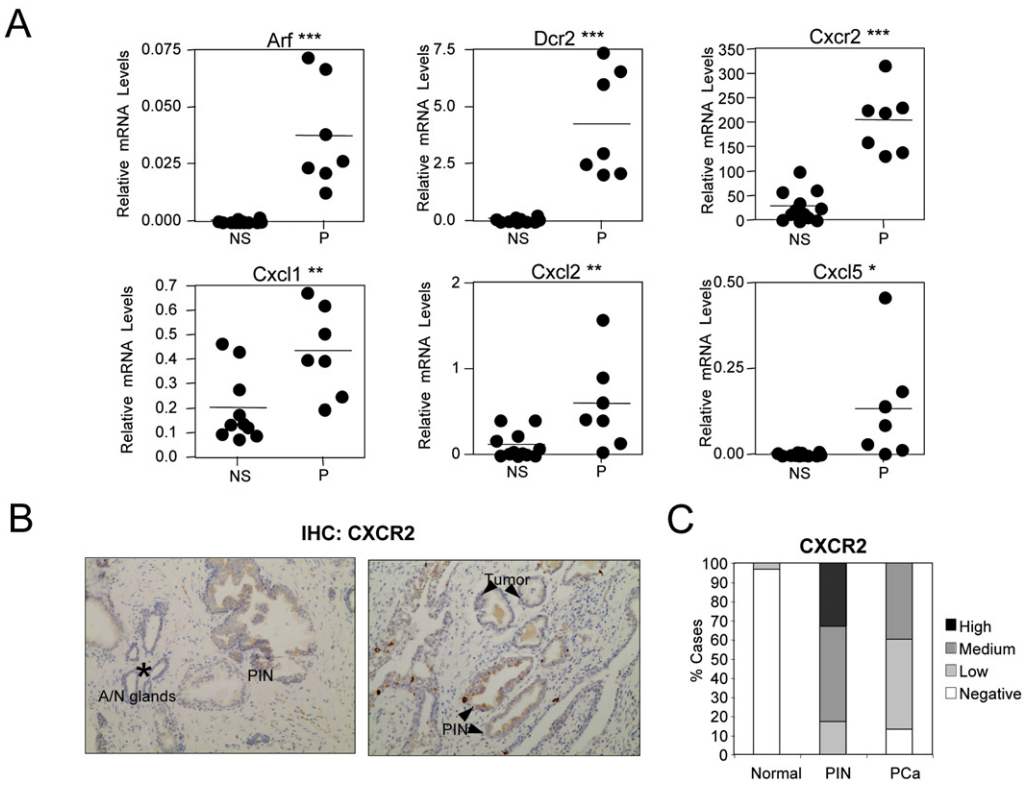

Figure 6. CXCR2 Expression Is Elevated in Premalignant Lesions

(A) qRT-PCR analysis of Arf, Dcr2, Cxcr2, Cxcl1, Cxcl2, and Cxcl5 transcript levels in samples from normal skin (NS) or DMBA-TPA-induced mouse papillomas (P). The p values correspond to a nonparametric, unpaired t test. *p < 0.05. **p < 0.001. ***p < 0.0001.

(B) Immunohistochemistry showing CXCR2 staining in sections from prostate. PIN, prostate intraepithelial neoplasia; A/N glands, atrophic/normal glands.

(C) CXCR2 staining. In 29/30 cases, the stain in PIN glands was above that in normal glands, generally negative. In four cases were PCa was present in the same sections, it was negative for CXCR2 staining or weakly positive. In 8/14 cases analyzed, we observed that PIN glands stain more intensely than the PCa in the same section, in 2/14 the CXCR2 staining was similar in PCa and PIN, with the rest of cases showing heterogeneous CXCR2 staining.

prostate adenocarcinoma (PCa) is thought to require cooperating mutations that allow bypass of senescence. Epithelial cells from normal prostate glands did not stain positive for CXCR2 expression (only scattered cells, likely of neuroendocrine origin, did). In contrast, the PIN lesions were positive for CXCR2. In about a third of these cases, the staining was relatively low but consistently above normal gland levels (Figures 6B and 6C). When glands displaying PCa were present, they stained for CXCR2 less intensely than PIN (8/14) (Figures 6B and 6C). We also analyzed the expression of CXCR1 (Figures S15B and S15C) with qualitatively similar results. In general, the levels of CXCR1 staining were higher in PIN than that of CXCR2, but its distribution was more heterogeneous among glands and even inside an individual gland.

A CXCR2 Mutant Present in Lung Adenocarcinoma Cells Alleviates OIS

Reasoning that further proof for a pathological role for CXCR2 in senescence in vivo would come if inactivating mutations in human tumors existed, we searched the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/). An analysis of 40 cancer cell lines identified a point mutation in CXCR2 (G354W) in the lung adenocarcinoma cell line NCI-H1395. To investigate whether the substitution affects CXCR2 function, we expressed the $CXCR2^{G354W}$ allele in IMR-90 cells. Immunofluorescence and FACS showed that steady-state levels of the $CXCR2^{G354W}$ allele were higher than those of CXCR2 wild-type (Figure 7A and Figures S16A and S16B). Under standard tissue culture conditions, the $CXCR2^{G354W}$ allele localized mainly in the membrane, while CXCR2 wild-type was present mostly in endosomes (Figure 7B). Interestingly, when IMR-90 cells expressing CXCR2 wild-type were treated with a CXCR2 antagonist that prevents its internalization and recycling, the levels and localization of CXCR2 wild-type resembled those of the $CXCR2^{G354W}$ allele (Figure 7A). Moreover, stimulation of IMR-90 cells with IL-8 or GROα failed to internalize $CXCR2^{G354W}$ (Figure 7B) and did not increase ROS levels, contrary to cells expressing CXCR2 wild-type (Figure S13). These results are consistent with defective internalization of the $CXCR2^{G354W}$ allele that results in diminished signaling.

Next, we compared the effects of $CXCR2^{G354W}$ and $CXCR2^{WT}$ on cell proliferation. Whereas IMR-90 cells expressing wild-type CXCR2 suffered premature senescence, cells expressing the $CXCR2^{G354W}$ allele did not (Figure 7C). Similar results were obtained in MEFs (Figures S16C and S16D). Interestingly, the NCI-H1395 cell line bears a mutation in B-RAF (G469A) that

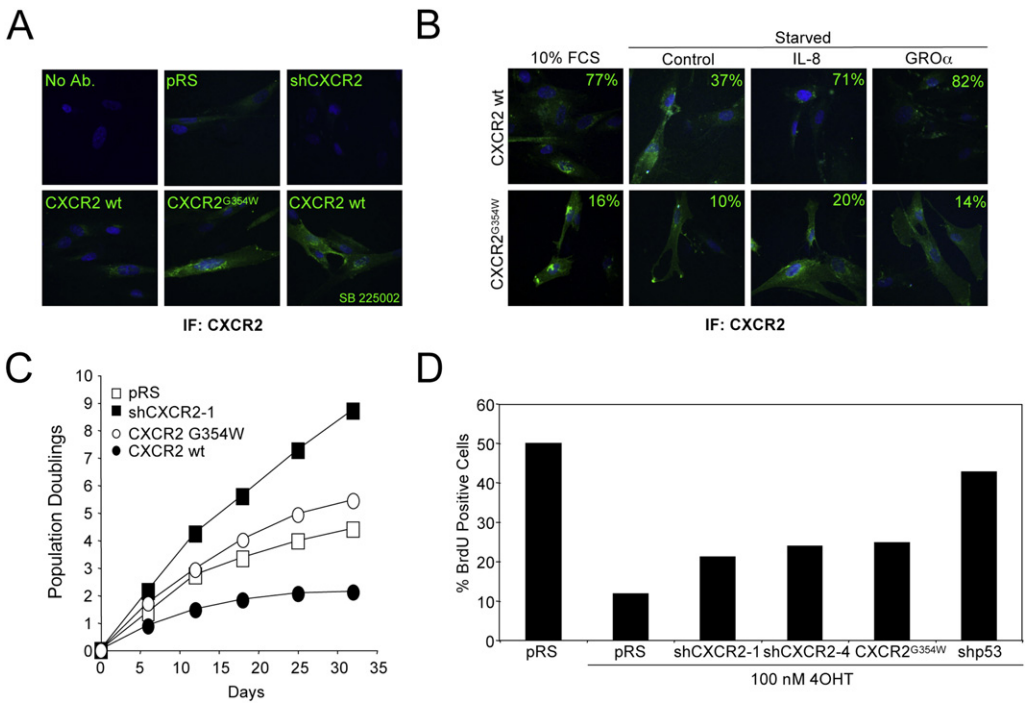

Figure 7. A CXCR2 Mutation Present in Lung Adenocarcinoma Alleviates Senescence

(A) The levels of CXCR2 in IMR-90 cells infected with the indicated vectors were analyzed via immunofluorescence. When indicated, cells were treated for 16 hr with 200 nM SB225002.

(B) CXCR2 immunofluorescence in IMR-90 cells infected with CXCR2 wild-type and CXCR2^{G354W} treated as indicated. Cells were serum starved overnight and treated with 100 ng/ml IL-8 or 250 ng/ml GROα for 1 hr. The percentage of cells showing preferential localization of CXCR2 in endosomes as explained in Figure S12 is shown.

(C) IMR-90 cells were infected at passage 17 with the indicated retroviral vectors, selected, and growth curves were performed.

(D) IMR-90/MEK:ER cells were infected with the indicated retrovirus. After selection, cells were plated and treated with control vehicle or 100 nM 4OHT. Forty-eight hours after treatment, cells were pulsed with BrdU for 16 hr, and BrdU incorporation was quantified. Three independent experiments yielding equivalent results were performed.

renders it constitutively active (Wan et al., 2004) and could induce OIS. To investigate the effect of the G354W mutation on OIS, we infected IMR-90/MEK:ER cells with the CXCR2^{G354W} allele. Expression of this allele alleviated OIS to a similar extent as shRNAs targeting CXCR2 (Figure 7D). It is tempting to speculate that the presence of the CXCR2^{G354W} allele might have allowed the lung adenocarcinoma NCI-H1395 cells to bypass BRAF^{G469A}-induced OIS.

DISCUSSION

The significance of senescence in physiological and pathological settings, such as aging and cancer, has gained firm ground. In recent years, multiple studies have shown that senescent cells accumulate in premalignant lesions and during aging (Collado et al., 2007). It is therefore important to learn more about the mechanisms underpinning the establishment and maintenance of senescence and the consequent behavior of the senescent cell. Despite steady progress in probing the roles of the p53 and Rb pathways, little is known about other mechanisms that might contribute to the senescent phenotype.

One of the characteristic features of senescent cells is that they produce increased quantities of various secreted proteins. Factors secreted by senescent cells exert multiple effects on neighboring epithelial cells (Coppe et al., 2006; Krtolica et al., 2001; Parrinello et al., 2005) and direct the immune system to clear a senescent lesion (Xue et al., 2007). Secreted factors such as PAI-1 (Kortlever et al., 2006) and IGFBP7 (Wajapeyee et al., 2008) also contribute to senescent growth arrest. Here we show that fibroblasts undergoing OIS upregulate the chemokine receptor CXCR2 and its ligands, and that by experimentally manipulating these levels, it is possible to promote or delay senescence. This suggests the existence of a positive feedback loop involving chemokine signaling via CXCR2 that acts to reinforce senescence. Similar results are reported in an accompanying paper by Kuilman et al. (2008; this issue of *Cell*), which shows that IL-6 is expressed by senescent cells and is required for the induction and maintenance of cell cycle arrest of cells exposed to oncogenic stress. The effects exerted by CXCR2 in vitro, though modest, are conserved across a wide range of primary cell types. The physiological relevance of CXCR2 signaling during OIS is further backed by our observations in preneoplastic lesions and tumors (Figures 6 and 7).

Two of the prominent ligands for CXCR2 are IL-8 and GROα. IL-8 has multiple paracrine and autocrine effects. As a paracrine agent, it modulates endothelial cell migration and is

a chemoattractant for neutrophils. As an autocrine agent, IL-8 promotes the growth of different cancer cell types. Elevated expression of IL-8 is observed in experimental settings (Sparmann and Bar-Sagi, 2004) and human malignancies, and has been linked to increased angiogenesis and vascularization, metastatic spread, and poor prognosis (Yuan et al., 2005). Similar inferences have been drawn for GROα, first described as an autocrine factor stimulating the growth of melanoma cells (Bordoni et al., 1990). Although these growth-promoting functions appear at odds with the growth arrest that we observe in primary cells, such paradoxical behavior has become common.

The classic example of a protein having pro-oncogenic or anti-oncogenic activity depending on cellular context is H-Ras^{G12V} (Serrano et al., 1997), but similar conclusions have been drawn for HMGA (Narita et al., 2006) and KLF4 (Rowland et al., 2005). By analogy, overstimulation of CXCR2 activity, by increasing the levels of the receptor or its ligands, or as a consequence of upstream oncogenic signals, elicits a senescence phenotype in primary cells. In cells in which the senescence machinery is compromised, such as *p53*$^{-/-}$ MEFs (Figure 2G and Figure S5) or immortal NIH 3T3 cells (Burger et al., 1999), autocrine CXCR2 signaling becomes pro-oncogenic, enabling anchorage independent growth. As shown with other oncogenes (eg H-Ras^{G12V}, Serrano et al., 1997) the requirements for canceling CXCR2-induced senescence in human cells are more strict. Turning this around, cells in which CXCR2 signaling has been compromised are also less able to engage senescence in response to oncogenic signals from Ras or MEK.

The DNA-damage response is central to replicative and oncogene-induced senescence. Our data show that expressing CXCR2 increases DNA damage, and conversely its depletion results in diminished activation of the DDR. How CXCR2 activity influences the DDR and p53 would need further investigation, but we hypothesize that an increase in ROS levels (Figure S13) might be involved. ROS levels can influence OIS (Lee et al., 1999) and telomere-induced DNA damage during replicative senescence (Passos et al., 2007). Therefore, CXCR2 seems to control senescence via a different mechanism that other secreted factors such as PAI-1, which regulates the PI3-kinase pathway (Kortlever et al., 2006), or IGFBP7, which impacts on MAPK signaling (Wajapeyee et al., 2008).

Another interesting issue is whether CXCR2 ligands could induce senescence in a paracrine fashion. Our experiments indeed showed that neutralizing antibodies against IL-8 and/or GROα alleviated OIS. Given the observed increase in the expression of both CXCR2 and its ligands during senescence, we hypothesize that secreted CXCR2-binding chemokines mainly reinforce senescence in cells that have already upregulated CXCR2 as opposed to spreading senescence to proliferative neighboring cells. We cannot discount that upregulation of CXCR2 and its ligands could act in part through an intracellular or autocrine activation of the receptor. Whether secreted CXCR2 ligands contribute to senescence in normal cells by acting in a paracrine way, as it has been described for IGFBP7, will require further investigation. Overall, the ability of CXCR2-binding chemokines to reinforce senescence and to promote clearance of incipient tumors via recruitment of immune cells (as shown by Xue et al., 2007) argues for a potent tumor suppressor effect for CXCR2 signaling.

The extent to which IL-8, GROα, and other CXCR2-binding chemokines are upregulated during replicative and particularly oncogene-induced senescence is striking. The IL-8 gene contains an enhancer that can be bound cooperatively by NF-κB and C/EBPβ, and our analyses suggest a role for both in regulating IL-8 and other CXCR2 ligands during OIS. NF-κB is activated in response to Ras or MEK (Finco et al., 1997), as is C/EBPβ (Nakajima et al., 1993). Although it is presently unclear how CXCR2 expression is controlled during the implementation of senescence, it is also an NF-κB target (Maxwell et al., 2007). Overall, the regulation of both CXCR2 and its ligands seems key for reinforcing senescence. Taken together, the combined data implicate NF-κB and C/EBPβ in controlling the secretory program associated with oncogene-induced senescence.

CXCR2 is upregulated not only in senescent cells in culture but also in preneoplasic lesions in vivo. We illustrate this point with DMBA/TPA-induced papillomas in the mouse, and in PIN, regarded as precursor of human PCa (Figure 6). Although it would clearly be interesting to extend these analyses to additional tumor types, mining of published datasets presents a mixed picture. Elevated levels of CXCR2 transcripts have been reported in lung carcinoid tissue (Bhattacharjee et al., 2001) while lower levels of CXCR2 expression in head and neck squamous cell carcinomas coexist with upregulation of most CXCR2 ligands (Ginos et al., 2004; Toruner et al., 2004). The second would be consistent with the idea that increased levels of CXCR2 are associated with senescence in premalignant lesions and that more advanced cancers develop as a result of failure of senescence. Loss of CXCR2 expression could be viewed either as evidence for or as a causal factor in the bypass of senescence. Support for the latter comes from the identification of a CXCR2 inactivating mutation in the lung adenocarcinoma cell line NCI-H1395. Although we have to caution that only a single cell line with mutations in CXCR2 has so far been reported in COSMIC, we still believe that it provides an important proof of principle. Expression of the CXCR2^{G354W} allele not only does not induce premature senescence unlike CXCR2 wild-type, but also alleviates OIS.

In summary, we report here that the chemokine receptor CXCR2 and many of its ligands are upregulated during senescence. They form part of a chemokine network reinforcing growth arrest in a p53-dependent manner. The relevance of this network is unveiled as different preneoplastic lesions show enhanced expression of CXCR2, and its downregulation or mutation may be necessary for progression of some cancer types.

EXPERIMENTAL PROCEDURES

Cell Culture, Retroviral Infection, and Growth Curves

293T, BJ, WI-38, IMR-90, and NCI-H1395 cells (ATCC) were maintained as indicated. Retrovirus production and infection, growth curves, SA-β-Gal analysis, and isolation and maintenance of MEFs were performed as described previously (Gil et al., 2004). MEFs of different genotypes were obtained from S. Lowe (Cold Spring Harbor Laboratories, USA).

Genetic Screening

IMR-90 cells (passage 20; see Figure S1A), were infected with the appropriate controls or pools of constructs of the NKI RNAi library (Berns et al., 2004). Constructs allowing bypassing senescence were identified by PCR as described in the Supplemental Experimental Procedures.

Retroviral Vectors

Complementary DNAs for human IL-8, GROα, CXCR1, and CXCR2 were cloned via PCR in pBabepuro or pMarX IV puro. The CXCR2^{G354W} mutant was generated via PCR using a primer that incorporates the mutation and cloned into pBabepuro. Retrovirus encoding shRNAs were constructed as described previously (Gil et al., 2004).

Immunofluorescence

Immunofluorescence was performed using confocal laser scanning microscopy (TCS SPI system, Leica). Antibodies used were: CXCR1 (555937; BD), CXCR2 (555932; BD), γH2AX (05-636; Upstate), pST/Q (2851; Cell Signaling), and ATM pS1981 (200-301-400; Rockland). Senescence-associated heterochromatin foci were visualized with DAPI staining.

Quantitative RT-PCR and Taqman Analysis

Total RNA was extracted using the RNeasy minikit (QIAGEN). Complementary DNAs were generated using Superscript II (GIBCO). PCR reactions were performed on an Opticon 2 (Bio-Rad) using SYBR Green PCR Master Mix (Applied Biosystems) or via TaqMan 5′-nuclease methodology using ABI7700 (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems). Expression was normalized to β-actin for mouse or RPS14 for human samples. Sequences for the primers used are included in the Supplemental Experimental Procedures.

Analysis of Gene Expression in Papillomas

Skin papillomas were generated in FVB mice using a classical initiation/promotion protocol (Collado et al., 2005). Papillomas and normal skin were used to prepare total RNA using Trizol (Invitrogen) followed by additional purification with RNeasy minikit (QIAGEN).

Immunohistochemical Analyses

Immunohistochemical analyses of CXCR1 and CXCR2 were performed in 30 PIN and paired prostate adenocarcinoma samples from properly consented patients accrued by the New York University tissue bank (PI: Jonathan Melamed, MD) as described in detail in the Supplemental Experimental Procedures.

Human Chemokine and Cytokines Antibody Arrays

Human chemokine or human cytokine V arrays (Ray Biotech, Inc) were used following the manufacturer's instructions. After developing, films were scanned and the images processed and quantified using ImageJ software (National Institutes of Health). Intensity was normalized to internal positive controls for comparison.

ELISA for IL-8 and GROα

The concentration of chemokines released to the supernatant was measured via specific IL-8 or GROα ELISA (Quantikine ELISA Kit; R&D Systems).

Chromatin Immunoprecipitation

IMR-90 cells were harvested and processed for chromatin immunoprecipitation. Sheared chromatin was sonicated together with 3 μg of RelA/p65 (sc-109; Santa Cruz) or H3 antibodies (ab1791; Abcam). Immunoprecipitated DNA was isolated using Chelex-100 (Bio-Rad) and analyzed by qRT-PCR. Primers used are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental Data include sixteen figures and are available with this article online at http://www.cell.com/cgi/content/full/133/6/1006/DC1/.

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Supplemental Data

Chemokine Signaling via the CXCR2 Receptor

Reinforces Senescence

Juan C. Acosta, Ana O'Loghlen, Ana Banito, Maria V. Guijarro, Arnaud Augert, Selina Raguz, Marzia Fumagalli, Marco Da Costa, Celia Brown, Nikolay Popov, Yoshihiro Takatsu, Jonathan Melamed, Fabrizio d'Adda di Fagagna, David Bernard, Eva Hernando, and Jesús Gil

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Genetic Screening for extension of lifespan in IMR-90.

IMR-90 cells (passage 20, see Sup. Fig. 1A), were infected with the appropriate controls or pools of constructs of the first release of the NKI RNAi library obtained from Cancer Research Technologies/Cancer Research UK (Berns et al., 2004). After selection, cells were split every week until proliferation of cells stopped. Genomic DNA was extracted from library-infected proliferating cells using DNAzol (Invitrogen). shRNA inserts were amplified by PCR using the primers pRS forw CCCTTGAACCTCCTCGTTCGACC and pRS rev GCCTCCCCTACCCGGTAGAATTCG. After PCR amplification, the shRNAs were sequenced and subcloned into pRS for re-testing. So far, we have identified constructs targeting p53, Rb, CXCR2 and 3 others that we are currently validating.

Retroviral shRNA constructs.

Retrovirus encoding shRNAs targeting, CXCR2, IL-8, GROα, RELA and C/EBPβ were constructed by cloning oligonucleotides in pRS as previously described (Gil et al., 2004). The sense shRNA target sequences are as follows:

sh-CXCR2-1	GGACCGTCTACTCATCCAA
sh-CXCR2-4	GACAGCAGGCCTTCCTTTG
sh-IL8-1	TTGAACTAACAATCCTAGT
sh-IL8-2	TACAAGATTCCTGGTTAAA
sh-GROα-2	CATTTCTCATGTTGAAACT
sh-GROα-3	ATGTTCTCCAGTCATTATG
sh-RELA-1	CCGGATTGAGGAGAAACGTAA
sh-RELA-2	AAGGACATATGAGACCTTCAA
sh-C/EBPβ-1	CACCCTGCGGAACTTGTTCAA

Immunoblot.

Immunoblot analyses were performed using the following antibodies: p53 (DO-1, Santa Cruz Biotechnology), p21 (C-19, Santa Cruz Biotechnology), p16 (C-20, Santa Cruz Biotechnology), MDM2 (2A10) and actin (A5316, Sigma).

Immunohistochemical analyses on PIN samples.

Stainings were performed on formalin-fixed, paraffin-embedded tissue sections with the above described antibodies against CXCR1 and CXCR2, using the standard avidin-biotin immunoperoxidase staining procedure. Thus, paraffin-embedded tissues were sectioned at 5-µm thickness, deparaffinized and quenched with 3% hydrogen peroxide for 15 minutes. Antigen unmasking was performed by heat-retrieval with citrate buffer for 15 minutes. Sections were incubated with the respective primary antibody overnight at 4°C in a humid chamber, then incubated for 30 minutes with biotinylated anti-mouse (Vector Labs.) and developed with Vectastain ABC complex (Vector labs.) for 30 minutes. Diaminobenzidine was used as the chromogen and hematoxylin for counterstaining. The intensity of the staining was scored from 0 (negative) to 3 (strong). The distribution of the staining in the glands (proportion score) ranged from 0 (0%) to 5 (100 %). Both scores were added to generate a total score (0 to 8). A total score of 0

was considered negative; 1 or 2 was considered low; 3-5, medium and 6-8 high.

Primers used for quantitative RT-PCR and Taqman analysis.

Primer sets were as follows: hCXCR2 (GCTCTGACTACCACCCAACC and AGGACACCTCCAGAAGAGCA), hIL-8 (AAGGAAAACTGGGTGCAGAG and ATTGCATCTGGCAACCCTAC), hGROα (GAAAGCTTGCCTCAATCCTG and CACCAGTGAGCTTCCTCCTC), hRPS14 (TCACCGCCCTACACATCAAACT and CTGCGAGTGCTGTCAGAGG), hC/EBPß (Quantitect primer assay: Hs_CEBPB_2_SG, Qiagen), hRelA (TTGAGGTGTATTTCACGGGACC and GCACATCAGCTTGCGAAAAGG), hGCP2 (AGAGCTGCGTTGCACTTGTT and GCAGTTTACCAATCGTTTTGGGG), hIKBKB (ACAGGGGGATTTGGAAATGT and CACATTGGGGTGGGTCAG), mCxcr2 (GCTCACAAACAGCGTCGTAG and GAATAAGGGCATGCCAGAG), mDcr2 (AGCTAACCCAGCCCATAATCGTC and AGTTCCCTTCTGACAGGTACTGGC), mArf (GCCGCACCGGAATCCT and TTGAGCAGAAGAGCTGCTACGT), mCxcl1 (CTGGGATTCACCTCAAGAACATC and CAGGGTCAAGGCAAGCCTC), mCxcl2 (CCAACCACCAGGCTACAGG and GCGTCACACTCAAGCTCTG) mCxcl5 (TGCGTTGTGTTTGCTTAACCG and AGCTATGACTTCCACCGTAGG), mIL6 (CAAGAAAGACAAAGCCAGAGTC and GAAATTGGGGTAGGAAGGAC), mIL6R (CACAACGAAGCGTTTCACAG and GCTGCCAGCTGACTTTGAG) and mß-actin (GGCACCACACCTTCTACAATG and GTGGTGGTGAAGCTGTAGCC).

TaqMan 6-carboxy-fluorescein (FAM) Gene Expression Assays (Applied Biosystems) used were: hGROα (CXCL1: Hs00236937_a1), hGROβ (CXCL2: Hs00601975_a1), hGROγ (CXCL3: Hs00171061_a1), hENA-78 (Hs00171085_a1), hIL-8 (Hs0017103_a1) and hNAP2 (PPBP: Hs00234077_m1).

Primers used for ChIP experiments.

Primers used for ChIP were as followed: IL-8, for promoter region (-118 to +159,

GGTTTGCCCTGAGGGGATG and ACAGAGCTGCAGAAATCAGGAAGGCT) and for coding region (+2200 to +2426, TAGCAAAATTGAGGCCAAGG and AAACCAAGGCACAGTGGAAC) and GROγ, for promoter region (-267 to +72, CCCAGCTCTTTCCTCCAACC and TCGGCGAACCCTTTTTATGC) and for coding region (+2473 to +2690, ATTTTAACATGGGCGTCTGC and CCCAACCTGTCTTTTGTGCT).

siRNA transfections.

Cells were transfected with 100nM siRNA using HiPerfect. Transfection efficiency was controlled using siGLO, a fluorescently label siRNA (Dharmacon).

Transcription factors binding activity.

NF-κB and C/EBP activities were measured using a Luminex-based assay (Maligen Biosciences). The assay is based on transcription factors present in nuclear extracts binding to biotinylated dsDNA probes specific for NF-κB binding (sense 5'-AGGGGACTTTCCCA-3') or C/EBP binding (sense 5'-ACATTGCACAAT-3'). After incubation with a digestion reagent, oligonucleotides were captured onto fluorescently-labeled microspheres. Signals were detected with a Luminex instrument. C/EBPβ activation was measured using the TransAM^{TM} kit (Active Motive Europe) which use specific probes combined with antibodies detecting C/EBPβ. 0.5-1 μg of nuclear extracts was used for the assay.

Measurement of reactive oxygen species (ROS) production.

Cells were stained with DCFDA (Molecular Probes, Eugene, OR) as recommended by the manufacturer. Cells were loaded with 5 μg/mL H_2DCFDA for 5 minutes, washed twice in PBS, and immediately analyzed by fluorescence-activated cell sorting or microscopy.

Figure S1. Depletion of CXCR2 extends the lifespan of different cell types.

(A) Growth curves (beginning at passage 12) summarizing the IMR-90 cell passages used in the experiments of this investigation. Growth curves of IMR-90 cells. In black it is shown the starting point for the screening and extension of lifespan experiments: cells were infected around passage 20, and upon selection growth curves and other experiments started at passage 22, close to senescence. In red are shown the schema for premature-senescence experiments (i.e. Fig. 2 or Fig. 5A), where infections were performed around passage 17 when IMR-90 cells still have some proliferative potential. Marked as grey dots are the passage numbers (p14, p22, p25) used for qRT-PCR experiments analyzing the expression of CXCR2 during serial passage (Fig. 3A).

(B) WI-38 cells were infected with the indicated retroviruses and colony formation assays were performed. Crystal violet stained plates shown that while control cells close to senescence stop proliferating, knockdown of CXCR2 allows further growth.

(C) Post-stasis human mammary epithelial cells (HMEC) cells were infected with the indicated retroviruses and colony formation assays were performed. Crystal violet stained plates shown that when control cells stop proliferating, knockdown of CXCR2 confers a growth advantage, although more modest that knockdown of p53.

(D) The same cells as in (C) were used to generate growth curves. The experiment shows that shCXCR2 extends the lifespan of HMECs.

Figure S2. CXCR2 knock-down alleviates oncogene-induced senescence and blunts the DNA damage response.

(A) 10^5 of IMR-90/MEK:ER or IMR-90/LXSN cells were seeded in a 10 cm dish and treated without (control) or with 100nM 4-hydroxi-tamoxifen (4OHT). After 15 days, plates were stained with crystal violet.

(B) BrdU incorporation assay. IMR-90/MEK:ER cells were treated with or without 100 nM 4OHT for 48 h and cell cycle analysis was performed (propidium iodide versus

BrdU), confirming that MEK activation results in cell cycle arrest in G1 and cells show a dramatic decrease in BrdU incorporation.

(C) BJ cells were infected with a control vector (pBabe) or a vector expressing H-Ras^{G12V} and selected. 15 days after infection, cells were transfected with 100 nM of a negative control siRNA (siGLO) or siRNAs targeting CXCR2 or p53. 3 days after transfection, cells were pulsed with BrdU for 16h, cells fixed and BrdU quantified.

(D) Analysis of CXCR2 knockdown by qRT-PCR.

(E) IMR-90 cells infected with retrovirus encoding shCXCR2-1 or control vector (pRS) were irradiated with 5Gy. 1 or 8 hours after irradiation the DNA damage response was evaluated by immunofluorescence using antibodies against phosphorylated ATM/ATR targets (pS/TQ). Representative pictures of non irradiated and irradiated cells are presented to compare the intensities.

Figure S3. Expression of CXCR2 causes growth arrest in different cell types.

(A) IMR-90 cells were infected with a control vector (pBabe) or a vectors expressing CXCR1 or CXCR2. After infections cell were selected, equal numbers seeded and fixed and stained with crystal violet after 10 days.

(B) WI-38 cells were infected with a control vector (pBabe) or a vector expressing CXCR2. After infections cell were selected, equal numbers seeded and fixed and stained with crystal violet after 10 days.

(C) HMEC cells were infected with a control vector (pBabe) or a vector expressing CXCR2. After infections equal number of cells were seeded, fixed and stained with crystal violet after 10 days.

Figure S4. Depletion of CXCR2 results in lower levels of p53.

Western blot analyzing the expression of the indicated proteins in WI-38 **(A)**, IMR-90 **(B)** and HMEC **(C)**. An unspecific band is marked with *.

Figure S5. The growth arrest induced by expression of CXCR2 is dependent of p53.

(A-D) MEFs of the following genotypes (A) wild-type; (B) $p53^{-/-}$; (C) $Ink4a/Arf^{-/-}$; (D) $p21^{-/-}$ were infected with pBABE, CXCR1, CXCR2 and Ras^{G12V} retroviruses, selected and 10^4 cells seeded in a 24 well plate. Plates were fixed and stained with crystal violet every 2 days and growth curves generated.

(E) Representative pictures of the indicated MEFs (corresponding to the experiments described here and in Fig. 2G) are shown.

(F) Growth in soft agar. *$p53^{-/-}$* or *$Ink4a/Arf^{-/-}$* MEFs were infected with the indicated viruses. Cells were selected, and after selection 10^4 cells were grown in soft agar in a 6 well plate. Representative pictures are shown.

Figure S6. CXCR2 levels increase during serial passage in WI-38 cells.

Analysis of CXCR2 transcript levels during serial passage of WI-38 cells by qRT-PCR using RSP14 levels for normalization.

Figure S7. List of soluble factors analyzed using antibody arrays. A complete list of the targets of the 89 antibodies present in the antibody arrays (targeting 90 different secreted factors) used in this study (human chemokine antibody array and human cytokine V antibody array, Ray Biotech.).

Figure S8. Activation of MEK in the absence of senescence results in the induction of IL-8 and GROα.

IMR-90/LXSN cells or IMR-90/MEK:ER/E6/E7 cells were seeded, maintained overnight in 0.5% FCS and treated for 48h with 100 nM 4OHT. At that stage supernatants were taken and IL-8 **(A)** or GROα **(B)** quantified by ELISA.

Figure S9. Activation of C/EBP and upregulation of IKKβ during OIS.

(A) Nuclear extracts from IMR-90/LXSN or IMR-90/MEK:ER left untreated or treated with 100 nM 4OHT for 72h were used to detect C/EBP activity using specific DNA-binding probes coupled to fluorescent detection as explained in experimental procedures.

(B) IMR-90/MEK:ER cells were treated with 100 nM 4OHT or an equivalent volume of vehicle (control) and 24 h later C/EBPβ activation was measured in nuclear extracts using a Trans AM EMSA ELISA assay. As a control, nuclear extracts of 293T cells transfected with a control vector or a plasmid expressing C/EBPβ were included.

(C, D) IMR-90 cells were infected with a control vector (pBabe) or H-Ras^{G12V} and the expression of C/EBPβ **(C)**, and the NF-κB activating kinase IKKβ **(D)** were analyzed by qRT-PCR.

Figure S10. Interfering with C/EBPβ expression reduces secretion of CXCR2 ligands in OIS.

(A) IMR-90/MEK:ER cells were infected with the indicated vectors and the expression of C/EBPβ was analyzed by qRT-PCR.

(B) Effect of knocking down C/EBPβ over the secretion of different chemokines during OIS. IMR-90/MEK:ER cells were infected with the indicated vectors and treated with 100 nM 4OHT. 72 hours after treatment, supernatants were collected and antibody arrays were used to measure the protein levels of the indicated secreated factors.

(C) IMR-90/MEK:ER cells were transfected with 100 nM of three independent siRNAs targeting C/EBPβ (Qiagen). Cells extracts were prepared 4 days after transfection and the expression of C/EBPβ was quantified by qRT-PCR using RSP14 levels for normalization.

(D) IMR-90/MEK:ER cells were transfected with 100 nM of a siRNAs targeting RelA which efficacy had been previously proven (Qiagen). Cells extracts were prepared 4 days after transfection and the expression of RelA was quantified by qRT-PCR using RSP14 levels for normalization.

(E) IMR-90/MEK:ER cells were transfected with 100 nM of a siRNAs targeting IL-8 which efficacy had been previously proven (Qiagen). Cells extracts were prepared 4

days after transfection and the expression of IL-8 was quantified by qRT-PCR using RSP14 levels for normalization.

(F) IMR-90/MEK:ER cells were transfected with 100 nM of the indicated siRNAs. 48 hours later cells were switched to 0.5% FCS and 24 h later 100 nM 4OHT was added. Supernatant was collected 24 h after 4OHT treatment and IL-8 levels measured by ELISA.

Figure S11. Effect of IL-8 and GROα over growth of IMR-90 cells.

(A) IMR-90 cells (passage 17) were infected with the indicated retroviruses. After selection, 10^5 cells were seeded per 10 cm dish. Plates were fixed after 10-15 days and stained with crystal violet.

(B) Representative microphotographs showing IMR-90 cells infected with the indicated retrovirus. Cell were selected, seeded at the same density and photographs taken 7-10 days after seeding.

(C) IMR-90 cells were infected with shIL-8-1 retroviruses, selected, and the expression of IL-8 was quantified by qRT-PCR using RSP14 levels for normalization.

(D) IMR-90 cells were infected with shGROα-2 retroviruses, selected, and the expression of GROα was quantified by qRT-PCR using RSP14 levels for normalization.

Figure S12 Analysis of CXCR2 subcellular localization as a surrogate marker for CXCR2 activation.

(A) CXCR2 localization criteria. An example of cells with CXCR2 located on the membrane or cells with spotted location are shown. IMR-90 infected with CXCR2 retroviruses and treated with 200 nM of CXCR2 inhibitor SB 225002 (Membrane 1-2) or 100 ng/mL of IL-8 plus 250 ng/mL of GROα (Spotted) were stained by immunofluorescence with CXCR2 antibodies. This criteria was used for the rest of the immunofluorescence quantifications. To simplify, the rest of cells with a no precise pattern of CXCR2 localization were not scored.

(B) Colocalization of spotted CXCR2 with Transferrin in endosomes. IMR-90 cells infected with CXCR2-Cherry retroviruses were starved over night and incubated for 1 hour with 100 ng/mL IL-8 and 15 ng/mL Transferrin-FITC. After incubation, cells were analized by confocal microscopy.

(C) IMR-90 cells infected with CXCR2-wt and $CXCR2^{G354W}$ were cultured under standard conditions (10% FCS), or starved over night and treated with 100 ng/mL IL-8, 250 ng/ml GROα, a mix of 100 ng/mL IL-8 plus 10 μg/mL of anti-IL8 neutralizing antibody, a mix of 250 ng/mL GROα plus 10 μg/mL of anti-GROα neutralizing antibody, 10% FCS during 1 hour, or 200 nM SB 225002 during 24 hours as indicated. After treatment, cells were stained by immunofluorescence for CXCR2 and the percentage of cells presenting preferential CXCR2 localization in the membranes or endosomes as described in (A) was quantified.

(D) Representative micrographs of IMR-90 cells expressing CXCR2 wt and treated as in (C) are shown.

Figure S13. CXCR2 activation correlates with ROS production in IMR-90 cells.

(A-C) Cells infected with pCherry empty vector (A), CXCR2 wt-Cherry (B) or $CXCR2^{G354W}$-Cherry (C) were either culture under standard conditions (10% FCS); starved over night (starved); treated during 4 hours with a mix of 100 ng/mL IL-8 plus 250 ng/mL GROα (+IL-8 & GROα); or treated for 24 hours with 200 nM SB 225002 (SB 225002) as indicated. After treatment, cells were incubated during 5 minutes with 5 μg/mL of H_2DCFDA and fixed. Confocal representative pictures are shown.

(D) IMR-90 cells infected with pRS vector, shCXCR2, CXCR2-wt or $CXCR2^{G354W}$ were incubated during 5 minutes with 5 μg/mL H_2DCFDA and the fluorescence intensity was measured by FACS. Bars represent geometrical mean intensity of the fluorescence.

Figure S14. Expression of IL6 and IL6r in DMBA-TPA induced mouse papillomas.

Analysis by qRT-PCR of the transcript levels of IL6 and its receptor in samples from normal skin or DMBA-TPA-induced mouse papillomas. * ($p<0.05$)

Figure S15. CXCR1 expression in PCa progression.

(A) Senescence (SA-β-Gal staining) and histopathological (haematoxylin/eosin stain, H&E) analysis of cryosections from human prostate samples showing strong SA-β-Gal staining associated with prostate intraepithelial neoplasia (PIN). Left panel, a photograph showing a prostate section including multiple PIN lesions staining positive for SA-β-Gal is shown. Eosin counterstaining demarcates cellular contours. A closeup from that section is shown in the middle panel. An H&E staining of an equivalent section is shown in the right panel. The staining was performed in samples from 10 different patients with similar results. Thus, senescent cells were found associated with PIN but not with normal tissue or prostate cancer (PCa).

(B) Immunohistochemistry showing CXCR1 staining in sections from prostate. Two representative photographs are shown. In the left, we can observe a high-grade PIN lesion (HGPIN) staining strongly for CXCR1 while the levels in epithelial cells of normal glands are negative or very low in some cases. Note that stromal smooth-muscle cells show diffuse CXCR1 staining. In the right panel we can observe a section showing medium staining of PIN while adjacent PCa is negative for CXCR1.

(C) Summary of the results obtained for immunohistochemical (IHC) staining using CXCR1 antibodies. 30 cases containing PIN were analyzed. In general staining was more intense but also more patchy than for CXCR2. 8 cases stained at low levels, 13 to medium levels and 9 to high levels. 26/30 cases showed a higher intensity of staining for CXCR1 in PIN that in normal glands. In addition 14 cases showing PCa were stained. 6/14 PCa cases were negative, 2 stained at low levels, and 5 stained at medium levels for CXCR1. In at least 11/14 cases, we observed that PIN glands stain more intensely than the PCa lesions present in the same section.

Figure S16. Effects of the CXCR2 G354W allelle over replicative senescence.

(A) IMR-90 cells were infected and selected with a control retrovirus (pBabe) or retroviruses expressing CXCR2 wt or the CXCR2 G354W allelle. After selections cells were fixed, permeabilized and subjected to FACS analysis using antibodies recognizing CXCR2.

(B) IMR-90 cells infected with CXCR2 wt-Cherry or $CXCR2^{G354W}$-Cherry were either culture under standard conditions (Control) or treated for 24 hours with 200 nM SB 225002 (SB 225002) as indicated. Confocal representative pictures are shown.

(C) MEFs were infected with pBabe, CXCR2wt, $CXCR2^{G354W}$, H-Ras^{G12V} and shp16/Arf. After infection, cells were selected and 15,000 cells were seeded in a 24 well plate. Plates were collected and fixed for crystal violet staining every 3 days. Crystal violet was extracted and measured for monitor cell growth.

(D) After selection, 10^5 MEFs infected with the indicated vectors were seeded in a 10 cm dish, and plates fixed and stained 15 days later.

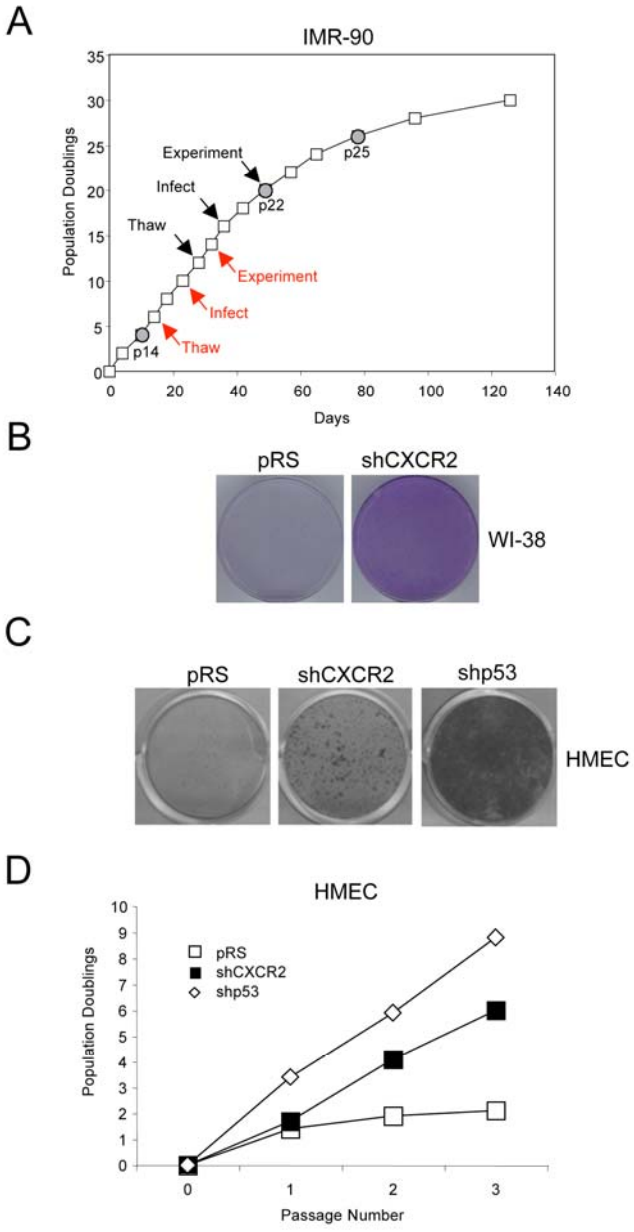
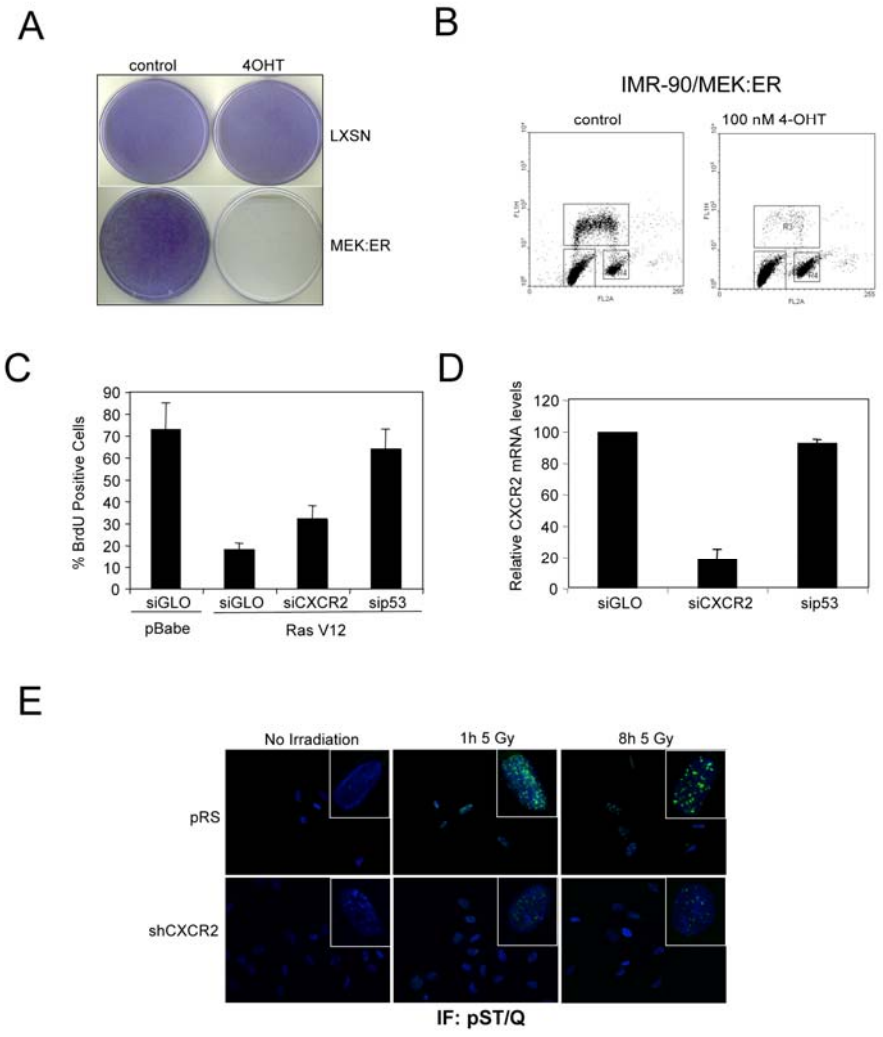


Figure S1 Acosta *et al.*



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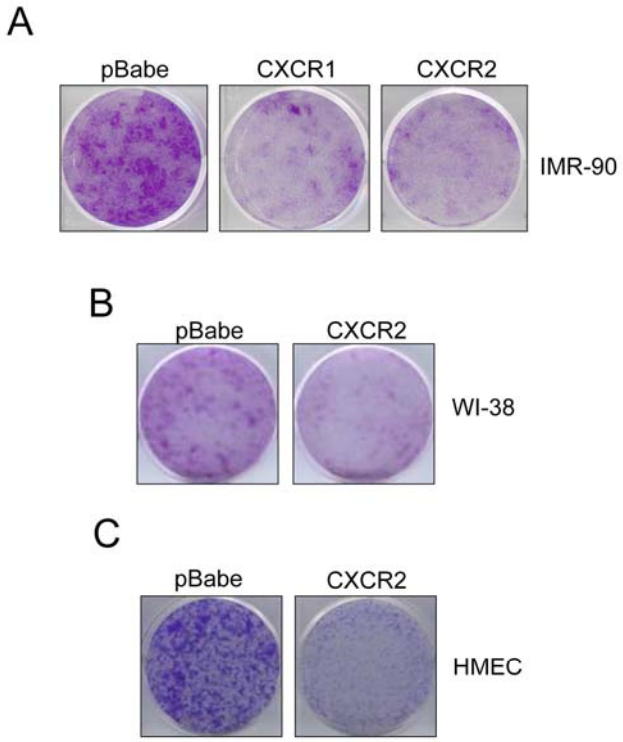

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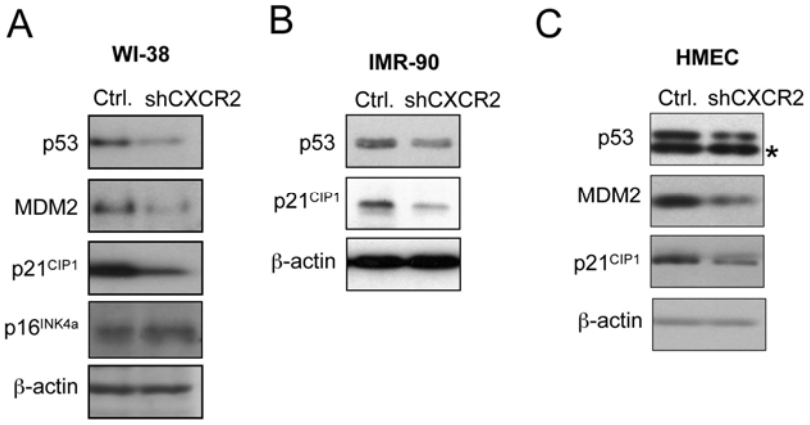

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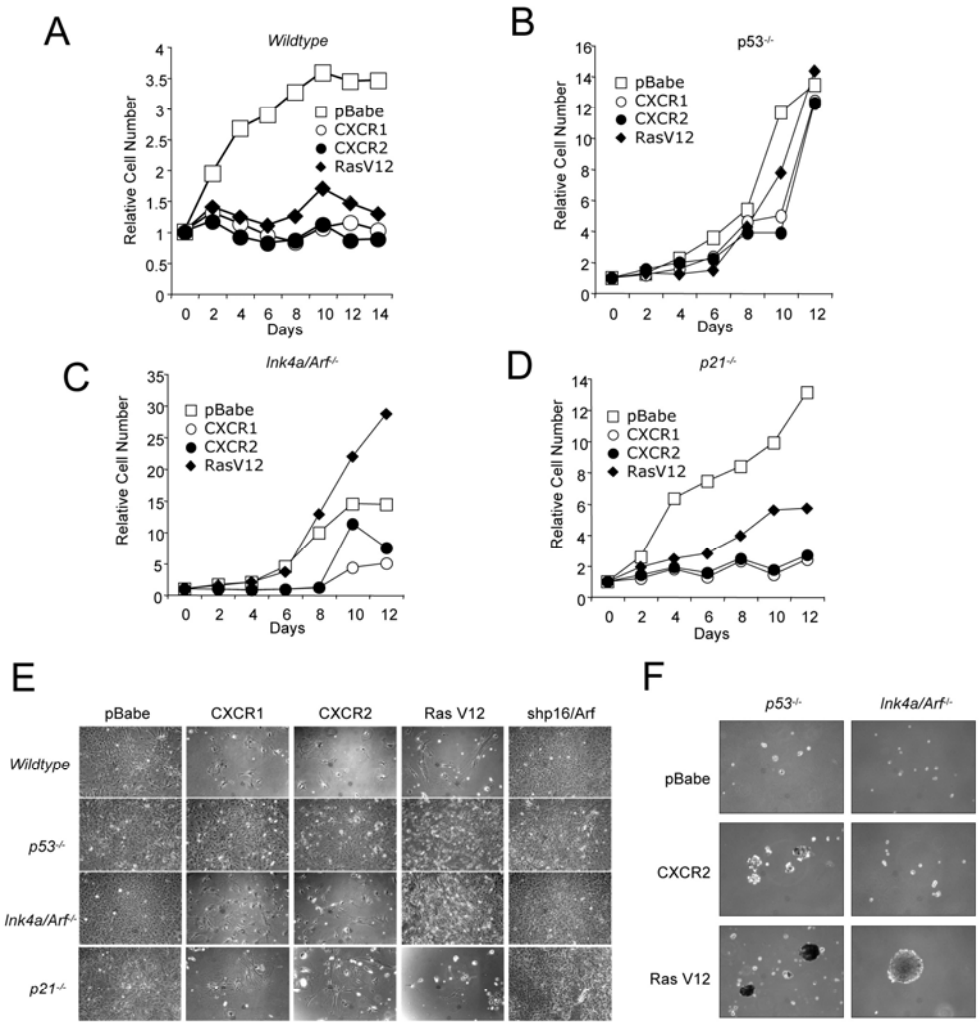

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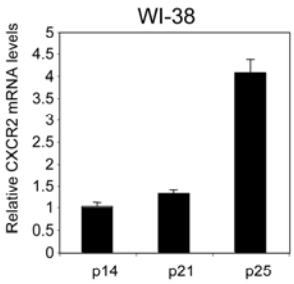

Figure S6 Acosta *et al.*

Angiogenin	IGFBP-2	MIP-1β
BDNF	IL-10	MIP-1δ
BLC	IL-12	MIP-3α
CCL28	IL-13	MIP-3β
CkβB-1	IL-15	MPIF-1
CTACK	IL-16	NAP2
CXCL16	IL-1α	NT-3
EGF	IL-1β	NT-4
ENA-78	IL-2	Oncostatin M
Eotaxin	IL-3	Osteoprotegerin
Eotaxin-2	IL-4	PARC
Eotaxin-3	IL-5	PDGF
FGF-4	IL-6	PIGF
FGF-6	IL-7	RANTES
FGF-7	IL-8	SCF
FGF-9	IP-10	SDF-1
Flt-3 ligand	I-TAC	SDF-1α
Fractalkine	Leptin(OB)	SDF-1β
GCP-2	LIF	TARC
GCSF	LIGHT	TECK
GDNF	Lymphotactin	TGF
GMCSF	MCP-1	TGF-β2
GROα/β/ γ	MCP-2	TGF-β3
GROα	MCP-3	Thrombopoietin
HCC-4	MCP-4	TIMP1
HGF	MCSF	TIMP2
I-309	MDC	TNF-α
IFN-γ	MIF	TNF-β
IGF-1	MIG	VEGF
IGFBP-1	MIP-1α	

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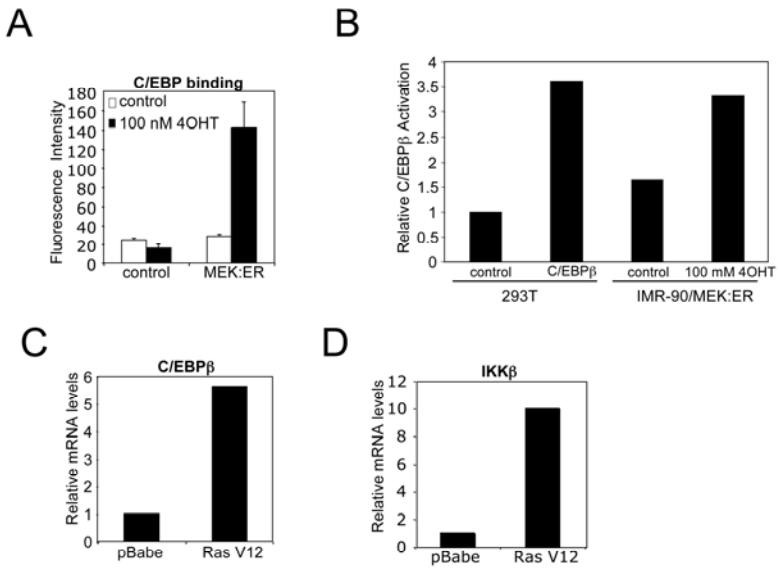


Figure S9 Acosta *et al.*

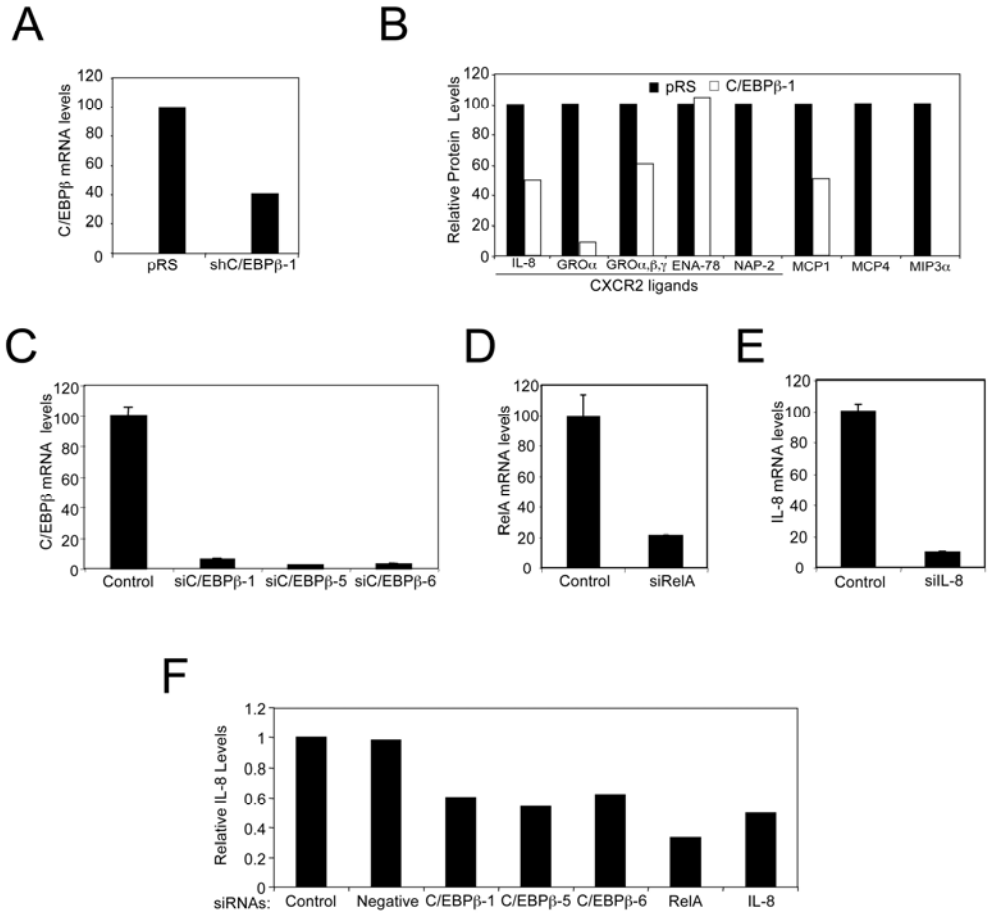
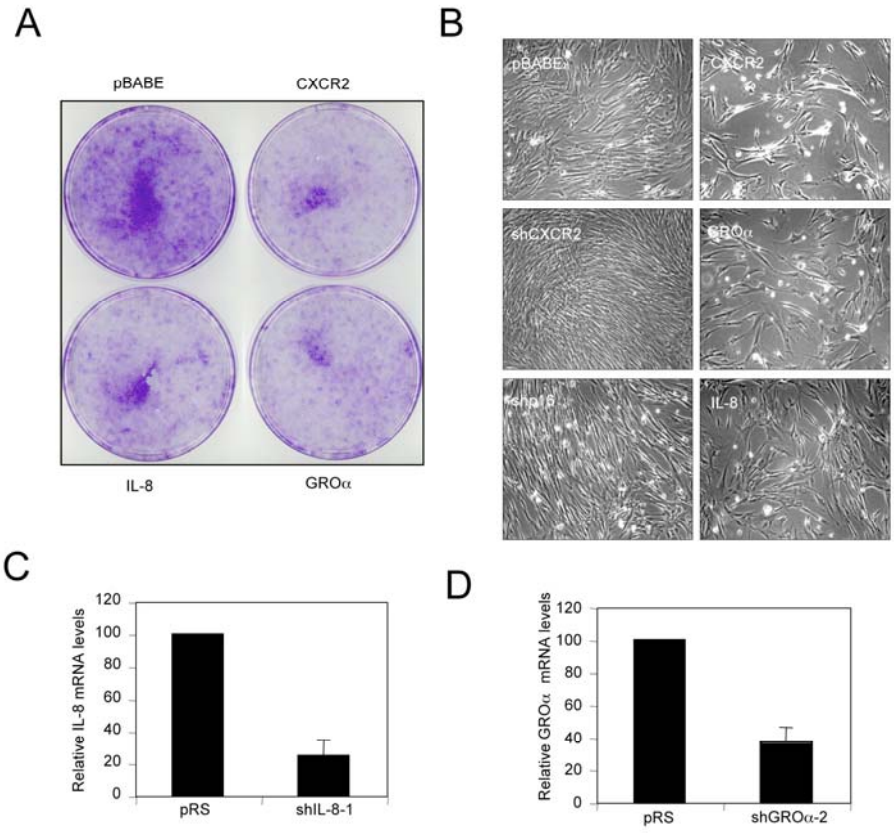


Figure S10 Acosta *et al.*



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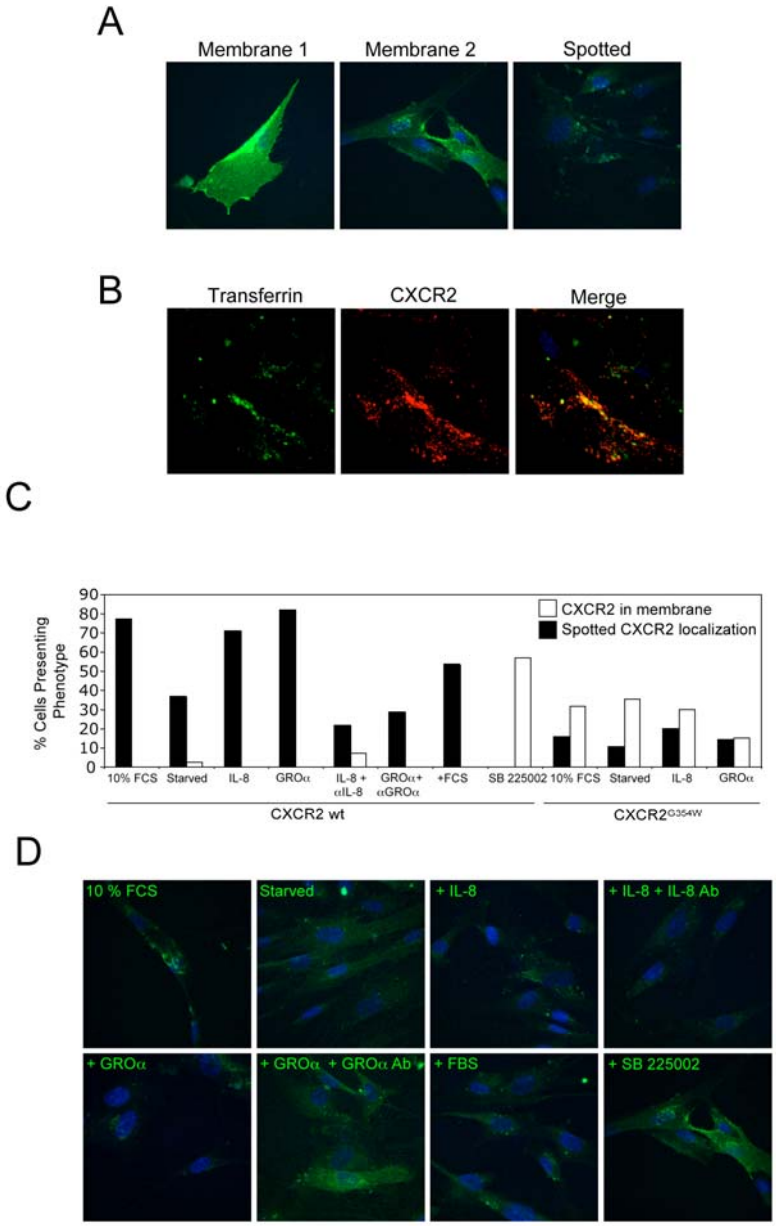

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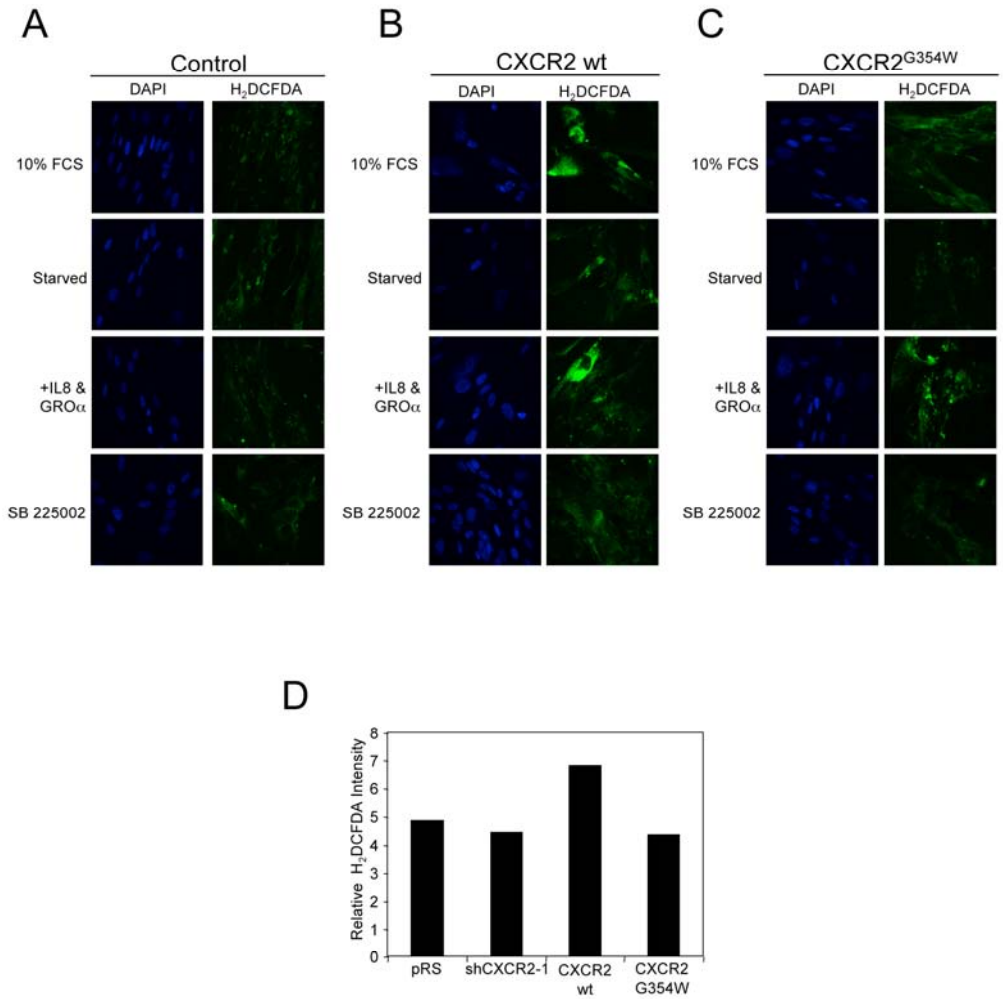

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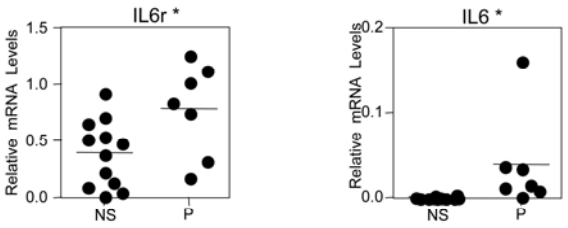

Figure S14 Acosta *et al.*

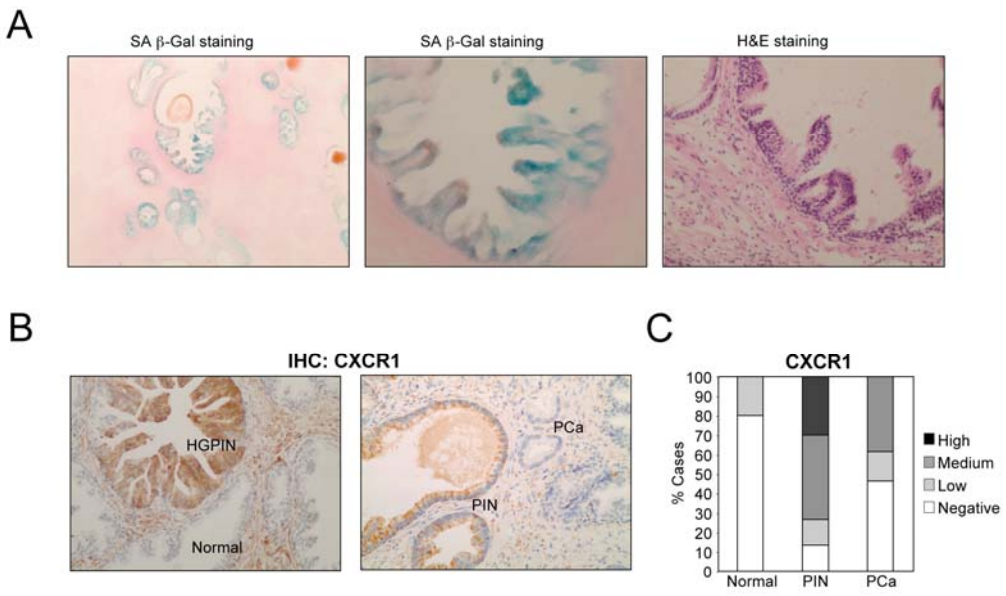

Figure S15 Acosta *et al.*

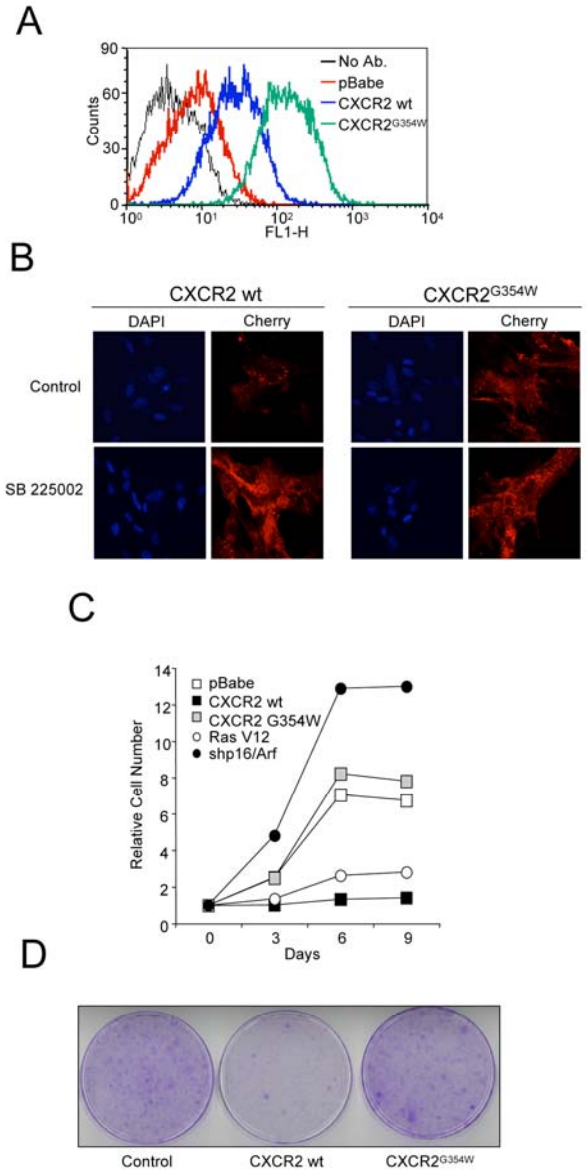

Figure S16 Acosta *et al.*

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Inhibition of lysyl oxidase stabilizes senescence and delays tumorigenesis in human cells and mice

Clotilde Wiel,[1,2,3,4] Arnaud Augert,[1,2,3,4,5] David Vincent,[1,2,3,4] Delphine Gitenay,[1,2,3,4] David Vindrieux,[1,2,3,4] Vanessa Arfi,[1,2,3,4] Hélène Daher,[1,2,3,4] Laurent Bartholin,[1,2,3,4] Etienne Lelievre[5,6], David Bernard,[1,2,3,4,6]

[1]Inserm U1052, Centre de Recherche en Cancérologie de Lyon, F-69000 Lyon, France

[2]CNRS UMR5286, F-69000 Lyon, France

[3]Centre Léon Bérard, F-69000 Lyon, France

[4]Université de Lyon, F-69000 Lyon, France

[5]UMR8161, Institut de Biologie de Lille, CNRS/Universités de Lille 1 et 2, F-59000 Lille, France

Etienne Lelièvre and David Bernard contributed equally to this work

Address Correspondence to: David Bernard, Centre de Recherche en Cancérologie de Lyon, UMR INSERM U1052/CNRS 5286, Centre Léon Bérard, 28, rue Laënnec, 69373 LYON Cedex 08, France. Phone: +33 4 26 55 67 92; Fax: +33 4 78 78 27 20; email : bernardd@lyon.fnclcc.fr.

No conflict of interest

Abstract

Cellular senescence, a stable proliferation arrest accompanied by morphological and biochemical marks, is induced in response to various stresses, including oncogene stress. Oncogene-induced senescence (OIS) results in blocked proliferation and thus constitutes a failsafe program counteracting tumorigenesis. The events enabling a tumor in a benign senescent state to escape from OIS and become malignant are largely unknown. Here we show that lysyl oxidase activity contributes importantly to the decision to maintain senescence or not. In an original human epithelial cell model of reversible senescence, inhibition of lysyl oxidase activity was found to render OIS irreversible, whereas constitutive expression of the Lox or LoxL2 protein favored OIS reversal. The relevance of these *in vitro* observations was supported by *in vivo* findings: in a transgenic mouse model of aggressive pancreatic ductal adenocarcinoma, inhibition of lysyl oxidase activity was found to stabilize senescence, delay tumorigenesis, and increase survival. Mechanistically, lysyl oxidase activity favors the reversal of senescence by regulating the focal adhesion kinase, an integrin-regulated kinase. Altogether, our results demonstrate that in addition to regulating migration, invasion, and metastasis, lysyl oxidase activity participates in primary tumor growth by directly favoring the reversal of senescence.

Introduction

Oncogene-induced senescence (OIS) drives cells into permanent cell cycle arrest, causing them to acquire specific markers (morphology, senescence-associated β-galactosidase activity (SA-β-Gal), for example) in response to aberrant oncogenic signals (1). In responsive cells, the stress generated by oncogene activation counterbalances the proliferation-stimulating potential of this activation by triggering senescence. Various benign tumors (melanoma nevi, prostatic intraepithelial neoplasias, lung adenomas…) caused by oncogene activation accumulate senescent cells. Tumors thus remain in a benign state as long as senescence is sustained. Conversion of senescing benign lesions to malignancy thus involves escape from senescence (2-4).

Benign tumors can be stable for months or years or might never progress towards malignancy (5). After accumulating senescent cells for months or years, they might evade senescence because of somatic mutations occurring intrinsically such as in the pRB and p53 pathways (1, 6-9) or/and because of microenvironmental changes occurring extrinsically, affecting the reversibility/irreversibility of senescence. Although senescence-linked cell cycle arrest is often considered irreversible, it actually appears reversible under some circumstances, in which cells with senescence hallmarks have been shown to re-grow (10, 11). Little is known about the processes controlling the reversibility of senescence, except that the $p16^{INK4a}$/Rb pathway may be involved (10, 11).

Lysyl oxidase (LOX) activity is reported to increase migration, invasion, and metastasis, mainly as a result of stiffening of the extracellular matrix induced by inducing collagen cross linking (12-14). LOX activity is believed to have no effect on cell growth *in vitro* or *in vivo* (12, 13), but in the context of MMTV-neu-induced breast tumors, it appears to stimulate primary tumor growth (15). The oncogenic potential of neu, a tyrosine kinase receptor, may be limited by the activation of the OIS failsafe program (16). We thus

hypothesized that in the MMTV-neu context, LOX activity might regulate cell growth, and thus primary tumor growth, by impacting the failsafe OIS program engaged by the oncogenic activation.

We indeed show that LOX activity is a key regulator of oncogenic-stress-induced senescence, thereby affecting tumorigenesis and survival in mice developing malignant tumors.

Results and Discussion

To study the (ir)reversibility of senescence in human epithelial cells, we used post-stasis human mammary epithelial cells (HMECs) unable to express $p16^{INK4a}$ (17). We were thus hoping that this extinction of $p16^{INK4a}$ favors the senescence reversal (10, 11). The cells were next immortalized by hTert expression (sustaining telomere integrity and thus avoiding replicative senescence - (17)) and stably infected to express an inducible oncogene MEK:ER (HMEC-TM cells), or RAF:ER in some experiments (HMEC-TR cells). In HMEC-TM cells where MEK was activated by 4-hydroxytamoxifen (4-OHT) 3 days treatment (Figure 1A), as indicated by phosphorylation of its substrate ERK - Supplemental Figure 1A, we first checked that OIS was induced at d0. OIS induction by 4-OHT was demonstrated by loss of the two proliferation markers examined (cyclin A and PhosphoH3Ser10) (Supplemental Figure 1A), growth arrest (Supplemental Figure 1B), and the onset of the SA-β-Gal marker (supplemental Figure 1C). As hypothesize, HMEC-TM cells monitored after their entry into senescence (Figure 1B, upper panel) proved able to grow back (Figure 1B, upper panel, d6 and d9) and to lose the senescence hallmark, SA-β-Gal activity (Figure 1C, upper panel). This model is thus a suitable model for uncovering new mechanisms regulating the reversal of senescence.

To investigate a role of LOX activity in regulating senescence, we first inhibited this activity using 3-Aminopropionitrile (BAPN); a specific inhibitor of the LOX activity of all the LOX proteins (Lox, LoxL1, LoxL2, LoxL3 and LoxL4) (18-20). BAPN treatment alone had no effect on HMEC-TM cell growth (Supplemental Figure 2A) as expected. BAPN treatment during the oncogenic stress slightly increased OIS (Supplemental Figure 2B and C). Strikingly, it completely blocked spontaneous senescence reversal, and thus rendered senescence irreversible, as shown by the inability of the treated cells to form colonies, in contrast to control cells (Figure 1B), and by their ability to maintain SA-β-Gal activity when

untreated cells lost it (Figure 1C). Thus, LOX inhibition blocked the reversal of the senescence induced by an oncogenic stress.

Inversely, cells expressing Lox (or LoxL2) or not (Supplemental Figure 3A), were treated with 4-OHT to trigger senescence, released from the oncogenic stress for 3 days, and then examined for reversal of senescence. At d3, although the initial growth arrest (d0) was similar in control and Lox-expressing cells (Supplemental Figure 3B), Lox-expressing cells displayed strong reversal of senescence as compared to control cells, i.e. they produced more colonies (Figure 1D) and fewer of them were SA-β-Gal positive (Figure 1E). Lox catalytic activity was shown to be responsible for the Lox-triggered reversal of senescence, as BAPN addition completely blocked it (Figure 1F). LoxL2, another member of the LOX protein family, was found to share with Lox the ability to cause senescence reversal (Supplemental Figuure 3A and B, Figure 1F). LOX effect appeared to depend on the oncogenic stress, as its constitutive expression alone did not affect cell growth (Supplemental Figure 3C). Together, these data demonstrate that LOX activity can render senescence reversible.

To address the relevance of this interesting *in vitro* observation, we sought an appropriate *in vivo* mouse model. We wanted a model developing malignant tumors upon oncogenic stimulation and lacking $p16^{INK4a}$, so that senescence, if any, is readily reversible (quick benign-to-malignant conversion). We chose a murine model of pancreatic ductal adenocarcinoma (PDAC) where the activation of Kras (Kras-G12D mutant) in the pancreas induces the formation of precancerous ductal lesions and senescence (21) but has to be combined with a $p16^{INK4a/Arf}$ deficiency to potently induce PDAC (22, 23). Pdx1-Cre; LSL-$Kras^{G12D/+}$; $INK4a/Arf^{lox/lox}$ mice (KIA mice) develop macroscopic pancreatic cancer at the frequency of 100% by the age of 6-7 weeks (23). To test the suitability of this model for our purposes, we first examined whether cellular senescence could be detected in KIA mice. We sacrificed WT and KIA mice 45 days after birth. The pancreases of KIA or control animals

were dissected and the normal zone was separated from the tumoral zone (supplemental Figure 4A). In the KIA mice, the normal part (confirmed by HPS staining) showed senescence, as indicated by the presence of SA-β-Gal activity and the absence of proliferation marker Ki67 labeling (Supplemental Figure 4B). In contrast, the tumoral part (confirmed by HPS staining) no longer displayed any SA-β-Gal activity and was Ki67-positive (supplemental Figure 4B). This observation suggests that the onset of aggressive PDAC requires senescence evasion. The pancreases of control animals, BAPN-treated or not, displayed no SA-β-Gal activity and no Ki67 staining (supplemental Figure 4B). This observation strengthens the observation we made *in vitro*, according to which BAPN is not sufficient to induce senescence without an oncogenic stress. KIA mice then represent an attractive model to test senescence reversibility, and more specifically, to test the ability of a LOX activity inhibitor to stabilize OIS, delay tumorigenesis, and increase survival. BAPN injection in KIA mice resulted in pancreases LOX catalytic activity inhibition as measured by collagen deposition (Figure 2A) and importantly increased by about 20% the survival of these mice (Figure 2B). Two BAPN-treated and vehicle-treated KIA mice were sacrificed 45 days after birth, before they reach experimental endpoint, allowing us to analyze the pancreas of those mice. By this time, untreated KIA mice had begun to die, whereas BAPN-treated KIA mice still seemed to be well protected (Figure 2B). As expected, the untreated KIA mice displayed PDAC, with numerous macroscopic multifocal tumors, whereas the BAPN-treated mice displayed no tumor or only 1 isolated macroscopic tumor (Figure 2C). The pancreases of untreated mice were found to be SA-β-Gal negative, whereas those of the BAPN-treated mice were SA-β-Gal positive (Figure 2D). Altogether, these results tally with our *in vitro* results and strongly support an involvement of LOX in regulating senescence stability, *in vivo* tumorigenesis, and survival.

The results of Weaver's group, showing that LOX activity increases primary tumor growth in a model of neu-induced mammary tumors (15) may be consistent with ours. The effect they describe could be due to regulation of senescence reversibility. As they found LOX activity to influence primary tumor growth by regulating integrin-FAK signaling (15), we investigated whether the integrin-FAK pathway might play a role in regulating senescence reversibility. The level of P-FAK (FAK^{Y397}) was found to decrease during OIS (supplemental Figure 5A), to rise upon OIS reversal, and to remain low when LOX was inhibited (Figure 3A). The involvement of the integrin-FAK pathway in senescence reversal was further supported by the observation that sub-cytostatic doses (data not shown) of two FAK inhibitors (FAK Inhibitor 14 and PF 573228) (supplemental Figure 5A) inhibited growth reversal (Figure 3B) and sustained SA-β-Gal senescence marker (Figure 3C and Supplemental Figure 5B). To see whether FAK activation might allow cells to escape from 4-OHT/BAPN-induced irreversible senescence, we used HMEC-TR cells expressing a constitutively active form of FAK (Supplemental Figure 5C). After 4-OHT/BAPN treatment, the ability of these cells to form colonies (Figure 3D) and their loss of the SA-β-Gal senescence marker (Figure 3E and supplemental Figure 5D) showed they could escape from treatment-induced irreversible senescence. These data constitute compelling evidences that FAK signaling is regulated by LOX activity and influences senescence reversal. Accordingly, an integrin-competing peptide RGDS, which acts upstream of FAK, or an LY294002, a PI3K inhibitor which acts downstream of FAK (24), were also blocking senescence reversal (Supplemental Figure 6 and 7). We thus propose a model where the inhibition of the LOX-integrin-FAK-PI3K pathway inhibits reversal of OIS (Figure 3F).

The molecular mechanisms determining the evolution of benign senescing tumors towards malignancy are almost unknown, except for the contribution of the loss of tumor suppressor genes such as p53 and $p16^{INK4a}$, among others (10, 11). We show that in human

epithelial cells displaying spontaneously reversible OIS, LOX activity acts via the FAK-PI3K pathway to favor senescence reversal. Accordingly, we show in a model of aggressive pancreatic ductal adenocarcinoma that inhibition of LOX activity stabilizes senescence, delays tumorigenesis, and increases survival. Our data contribute to deciphering a new role of LOX activity, recently observed by others (15), in regulating primary tumor growth. They further suggest that this role depends on oncogenic stress. The fact that the inhibition of LOX activity affects primary tumor growth in an oncogenic-stress-dependent manner, as our *in vitro* and *in vivo* results suggest, reconciles apparent discrepancies in the literature. LOX activity has indeed been shown to regulate primary tumor growth when coupled with oncogenic stress (15), but not in the absence thereof (12, 13). It will be interesting to see whether LOX activity also regulates cell responses to other cellular stresses driving cells to senesce or not.

Methods

Cell Culture

Human mammary epithelial cells (HMECs) (Lonza) were cultured in MEBM (Promocell) and penicillin/streptamycin (Invitrogen). Virus-producing GP293 cells (Clontech) were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone) and penicillin/streptamycin. Infected cells were selected as appropriate with neomycin (100 µg/ml), puromycin (500 ng/ml), or both.

Plasmids and reagents

4-Hydroxytamoxifen (4-OHT) (Sigma) was used daily for 3 days at 250 nM final concentration. 3-Aminopropionitrile (fumarate salt) (A3134, Sigma) was used daily at 350 µM final concentration for *in vitro* analysis. FAK inhibitor 14 (3414, Tocris Bioscience), PF573228 (3239, Tocris Bioscience), LY294002 (sc-201426, Santa Cruz Biotechnology), and RGDS peptide (H-1155, Biochem) were used daily at the following respective final concentrations: 250 nM, 500 nM, 1 µM, and 50 µM.

The following plasmids were used: pWZL-Neo-Myr-Flag-FAK (Addgene plasmid 20610)(25), pBabe-hygro-hTert was purchased at Addgene (Addgene plasmid 1773)(26), pBAbe-puro-Raf:ER (27) pNLP-Neo-Mek:ER (3). For Lox and LoxL2 cloning, the C-terminal FLAG tagged mouse Lox and LoxL2 cassettes were amplified by PCR using PFU (Stratagene) and mouse Lox and LoxL2 coding sequences in pIRES-hrGFP-1a vector as template (Lelièvre et al. 2008) and introduced into pLPCX vector (Clontech) at NotI and ClaI sites.

Transfection and infection

GP293 cells were transfected with PEI reagent according to the manufacturer's recommendations (Euromedex). Two days after transfection, target HMECs were infected with viral supernatant mixed with fresh medium (1/2) and polybrene (final concentration: 8 µg/mL).

Antibodies

The antibodies used were: anti-phosphoERK (9101, Cell Signaling), anti-phosphoAKT^{Ser473} (9271, Cell Signaling), anti-AKT (9272, Cell Signaling), anti-phosphoFAK^{Tyr397} (3283, Cell Signaling), anti-FAK (3285, Cell Signaling), anti-flag (200472, Stratagene), anti-phosphohistone3^{Ser10} (ab14955, Abcam), anti-cyclinA (H432, sc-751, Santa Cruz technology), anti-Ki67 (clone Tec-3, M7249, DAKO), anti-tubulin (T6199, Sigma), and anti-actin (A5316, Sigma).

Colony formation assays

Fifteen thousand cells were seeded into 6-well plates and treated or not with the indicated compound(s). At the end of the experiments, cells were fixed with 4% PFA for 15 minutes, washed with water, and stained with a crystal violet solution (Sigma).

Mouse engineering and treatment

By crossing Pdx1-Cre;Ink4a/Arf$^{lox/lox}$ (no phenotype) with LSL-Kras$^{G12D/+}$; Ink4a/Arf$^{lox/lox}$ (no phenotype) individuals, we "routinely" generate Pdx1-Cre;LSL-Kras$^{G12D/+}$;Ink4a/Arf$^{lox/lox}$ animals (representing 25% of the total progeny according to the expected Mendelian inheritance) developing macroscopic pancreatic cancer at the frequency of 100% by the age of 6-7 weeks. Mice were treated by intraperitoneal injection of BAPN (100 mg/kg, dissolved in saline) or vehicle.

SA-ß-Gal analysis

Senescence-associated β-galactosidase activity was assayed in HMECs and on pancreatic sections fixed and stained with the Senescence Beta-galactosidase Kit (Cell Signaling) as recommended by the manufacturer.

Histology and Sirius red staining

Pancreatic sections (4-5 μm) were stained with HPS (Hematoxylin-Phloxin-Safron). For Immunohistochemical analysis, paraffin-embedded murine pancreatic tumor tissues were used. Slides were serially sectioned at 4-μm thickness. After deparaffinization and rehydration, the slides were incubated in 5% hydrogen peroxide in sterile water to block endogenous peroxidases. For heat-induced antigen retrieval, tissue sections were boiled in 10 mmol/L citrate buffer pH6 in a microwave oven for 20 minutes. The slides were then incubated at room temperature for one hour with the primary antibody diluted in "low-background" antibody diluent (DAKO Real). After rinsing in PBS, the slides were incubated with a biotinylated secondary antibody bound to a streptavidin peroxidase conjugate (Dako E0468) for one hour at room temperature. Bound antibody was revealed and sections were finally counterstained with hematoxylin.

Paraffin-treated sections of pancreas were stained for one hour in 0.1% picrosirius red solution (Direct Red 80, 365548, Sigma) in picric acid solution (P6744, Sigma), washed twice with acidified water, and then mounted in Eukitt quick-hardening mounting medium (03989, Sigma).

Acknowledgments

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Figure 1

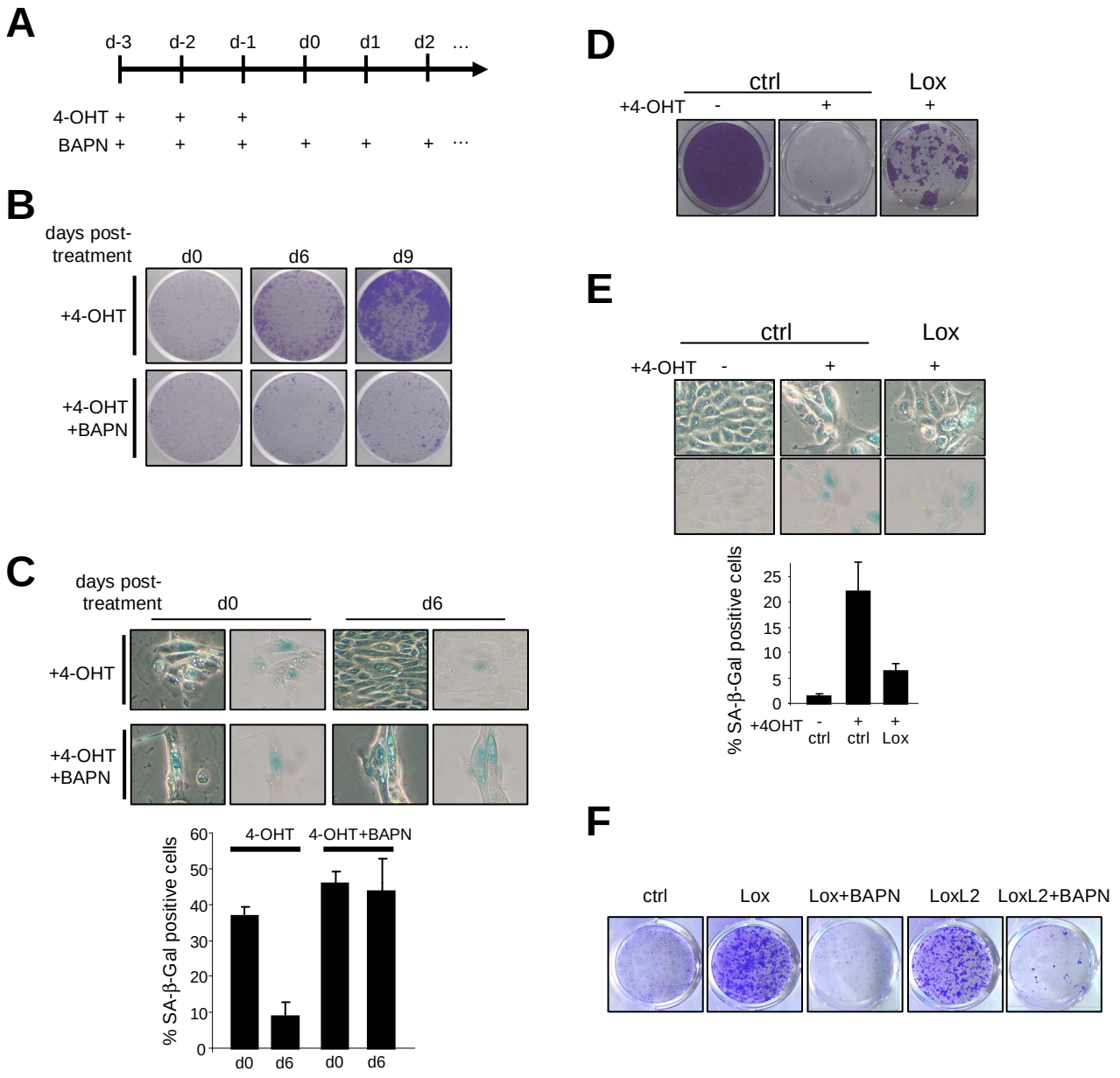
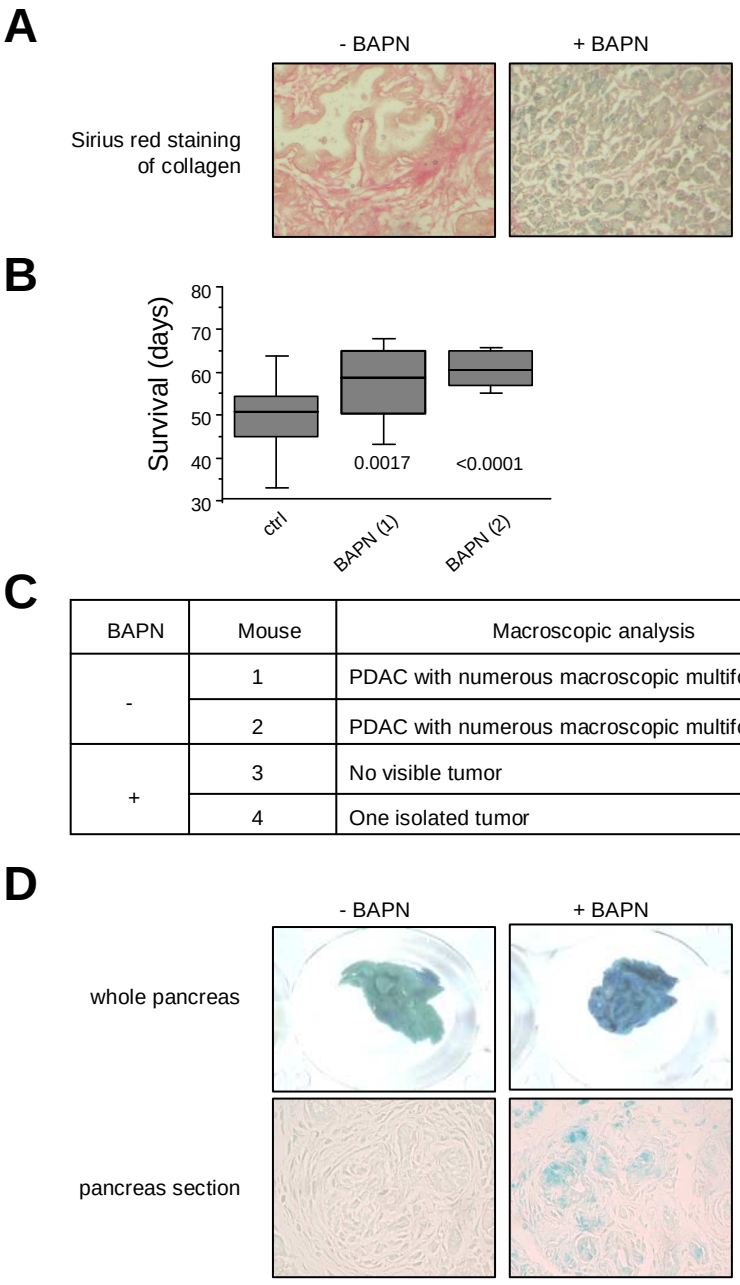


Figure 1: LOX controls the reversal of oncogene-induced senescence. (**A**) Protocol of treatment to induce senescence and study its reversal in HMEC-TM cells. (**B**) HMEC-TM were treated for 3 days with 4-OHT, with or without BAPN. Cells were PFA fixed and crystal violet stained to measure cell growth at various time points. (**C**) Cells were treated as in B, fixed, and assayed for SA-β-Gal activity. (**D**) HMEC-TM cells were infected with a Lox-encoding or control retroviral vector. After puromycin selection, they were treated for 3 days with 4-OHT. After 3 extra days without treatment, cells were fixed, and crystal violet stained. (**E**) Cells were treated as in D, fixed, and assayed for SA-β-Gal activity. (**F**) HMEC-TM cells were infected with a Lox- or LoxL2-encoding or a control retroviral vector. After puromycin selection, cells were treated for 3 days with 4-OHT, with or without BAPN. After 3 days without 4-OHT treatment, the cells were fixed and crystal violet stained.

Figure 2



BAPN	Mouse	Macroscopic analysis
-	1	PDAC with numerous macroscopic multifoci tumors
	2	PDAC with numerous macroscopic multifoci tumors
+	3	No visible tumor
	4	One isolated tumor

Figure 2: LOX inhibition delays pancreatic carcinogenesis and stabilizes senescence in vivo. (**A**) Pdx1-Cre;LSL-KrasG12D/+; Ink4a/Arflox/lox (KIA) mice were injected or not with BAPN, 3 times a week from day 25 after birth. Mice were sacrificed, pancreases fixed and a sirius red staining was performed to measure collagen deposits. (**B**) KIA mice were injected (n=11) or not (n=75) with the LOX inhibitor BAPN. Protocol 1: Mice were injected from day 25 after birth, 3 times weekly (n=5). Protocol 2: Mice were injected from day 21 after birth, every day (n=6). (**C**) Two mice of protocol 1 were sacrificed and Analyzed for the presence of macroscopic pancreatic tumors. (**D**) The pancreases of the two mice sacrificed in B were assayed for SA-β-Gal activity.

Figure 3

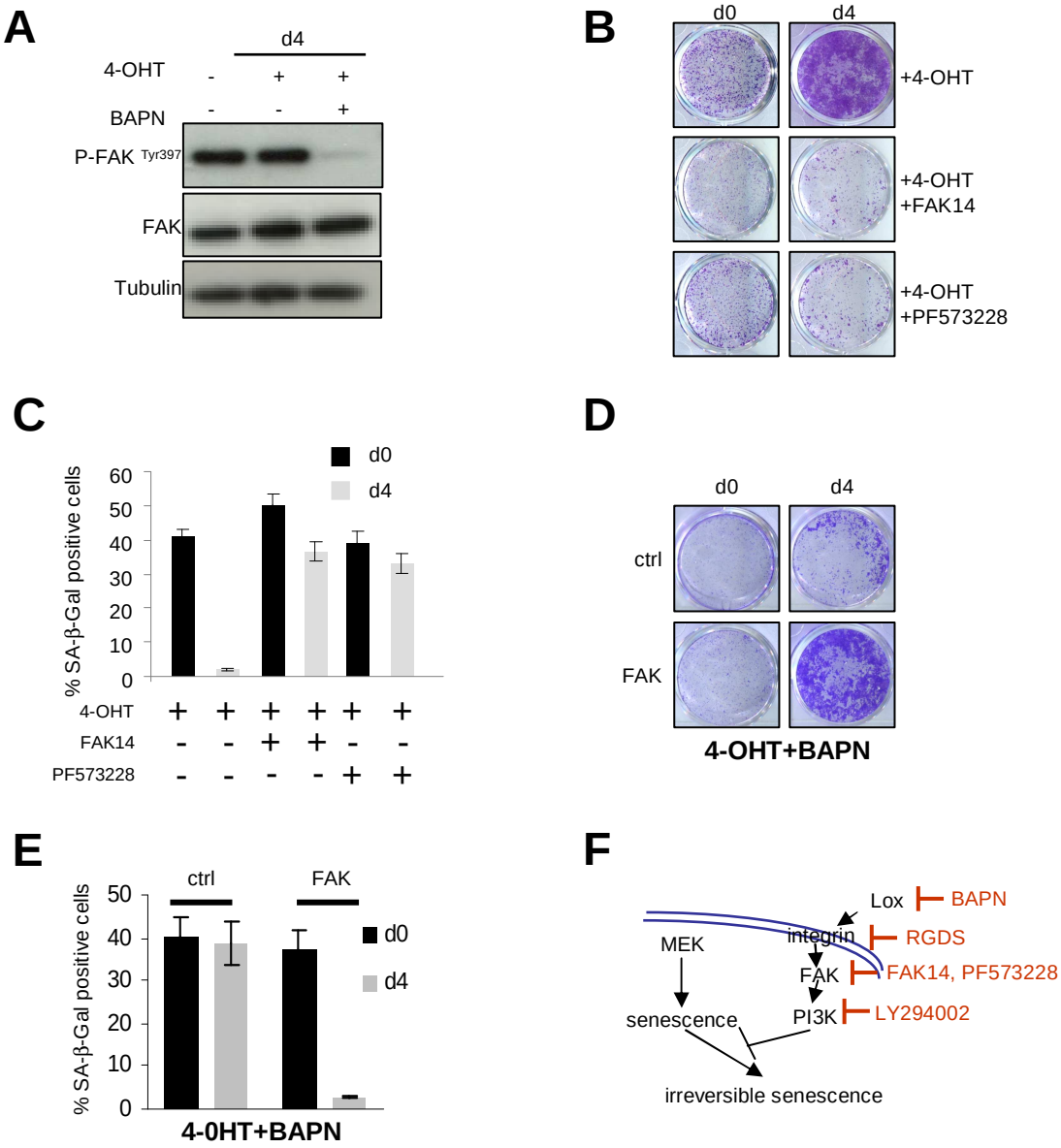
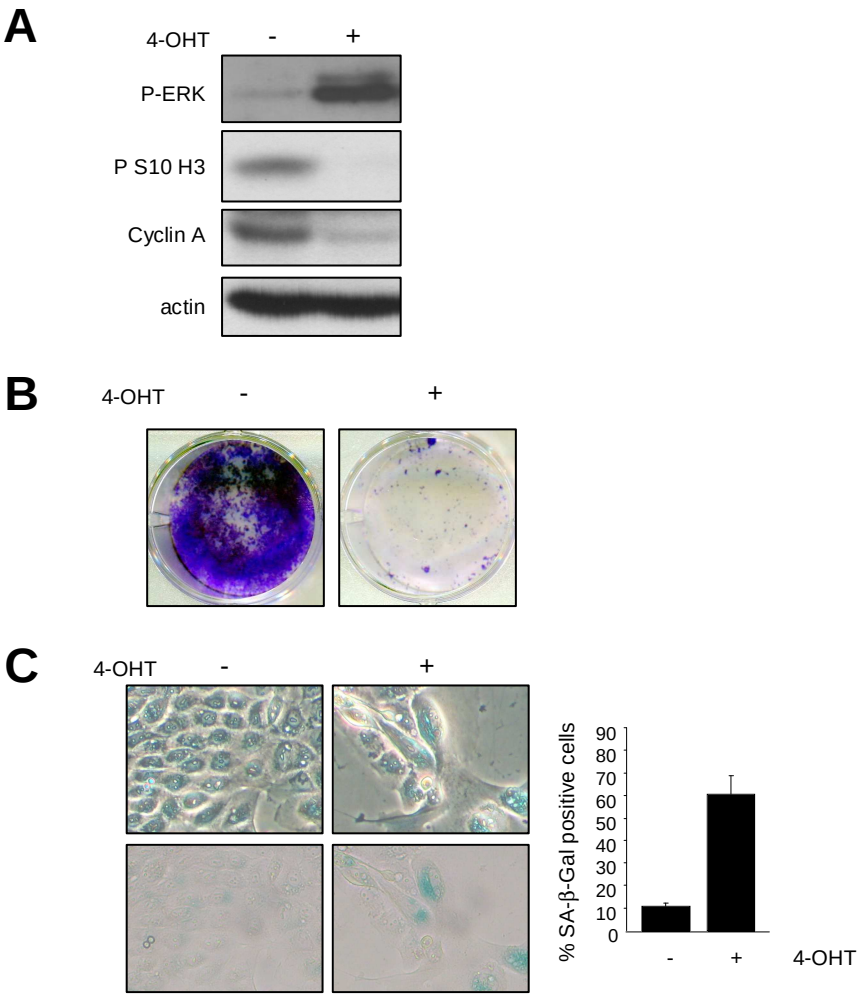


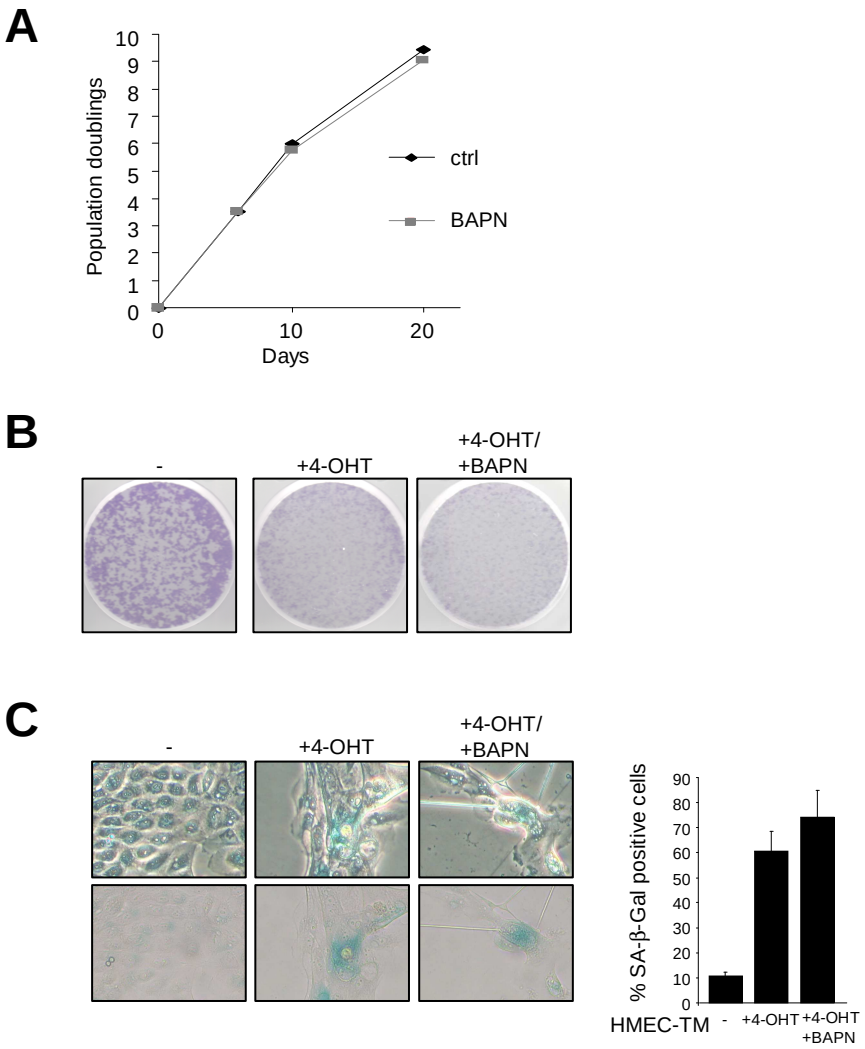
Figure 3: LOX regulates senescence reversal through the integrin-FAK-PI3K pathway. (**A**) HMEC-TM cells were treated for 3 days with 4-OHT, with or without BAPN. BAPN treatment was carried out for 4 days. Cell extracts were prepared and analyzed by immunoblotting with antibodies directed against P-FAK, FAK, and tubulin. (**B**) HMEC-TM cells were treated for 3 days with 4-OHT, with or without FAK inhibitors (d0), FAK inhibitor treatment was sustained for 4 additional days (d4). Cells were fixed and crystal violet stained, or assayed for SA-β-Gal activity (**C**). (**D**) HMEC-TR cells were infected with a retroviral control or FAK-encoding vector and neomycin selected. Selected cells were treated with 4-OHT+BAPN for 3 days (d0) and with BAPN for 4 additional days (d4). Cells were next fixed and crystal violet stained or assayed for SA-β-Gal activity (**E**). (**F**) Diagram illustrating the OIS reversal controlled by LOX through integrin-FAK-PI3K signaling.

Supplemental Figure 1



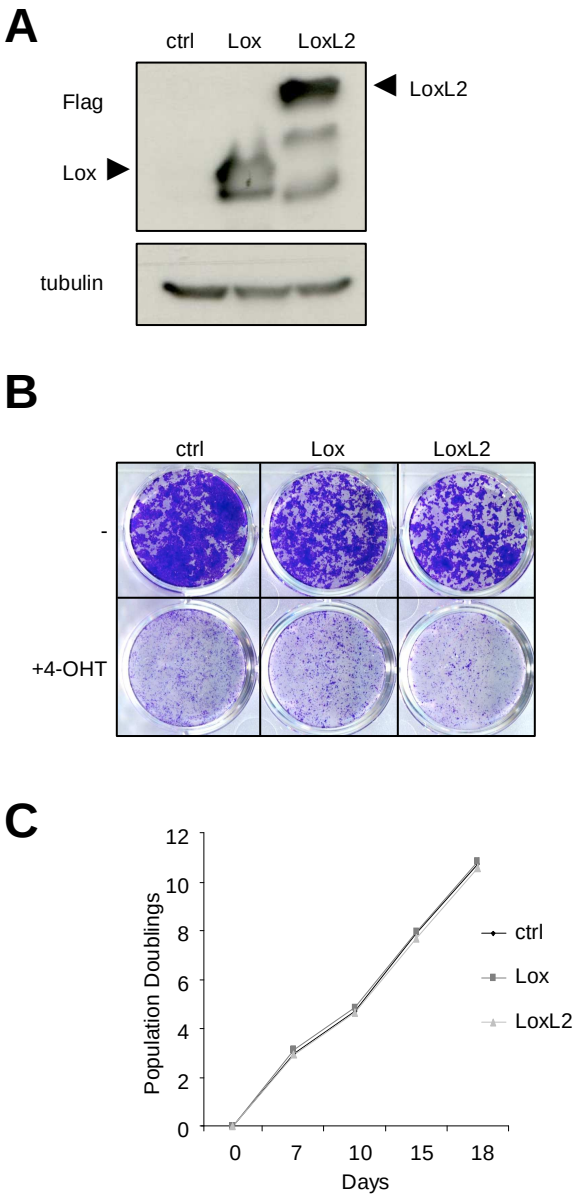
Supplemental Figure 1: HMEC-TM cells enter senescence after MEK activation. HMEC-TM cells were treated for 3 days with 4-OHT to activate MEK. (**A**) Cell extracts were prepared and analyzed by immunoblotting with the indicated antibodies. (**B**) Cells were fixed and crystal violet stained. (**C**) Cells were fixed and assayed for SA-β-Gal activity.

Supplemental Figure 2



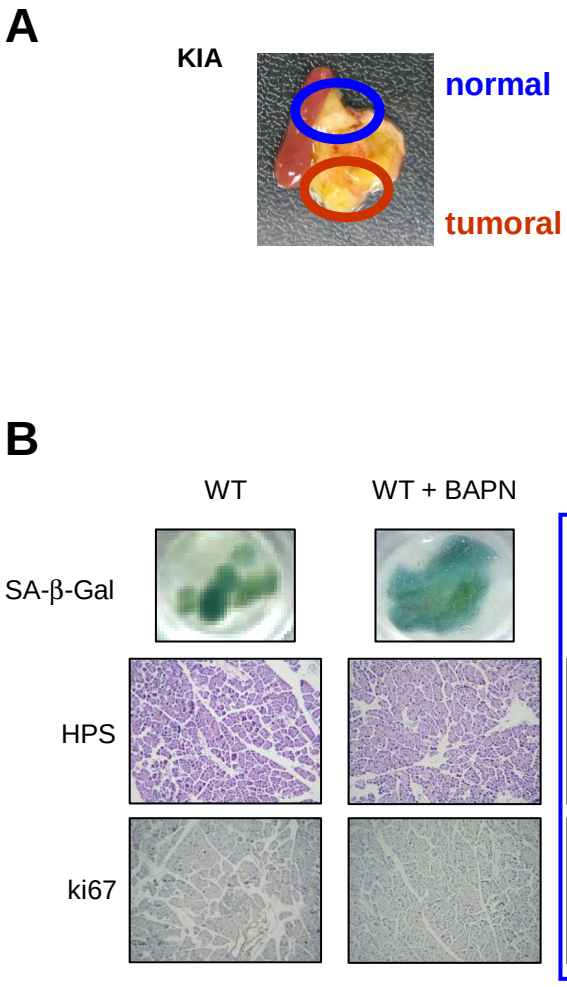
Supplemental Figure 2: LOX inhibition and senescence. (**A**) HMEC-TM cells were treated or not with BAPN every day. After each passage, cells were counted and seeded at the same density. Population doublings are presented. (**B**) HMEC-TM cells were treated or not with 4-OHT or 4-OHT+BAPN for 3 days. Next, they were fixed (d0) and crystal violet stained or assayed for SA-β-Gal activity (**C**).

Supplemental Figure 3



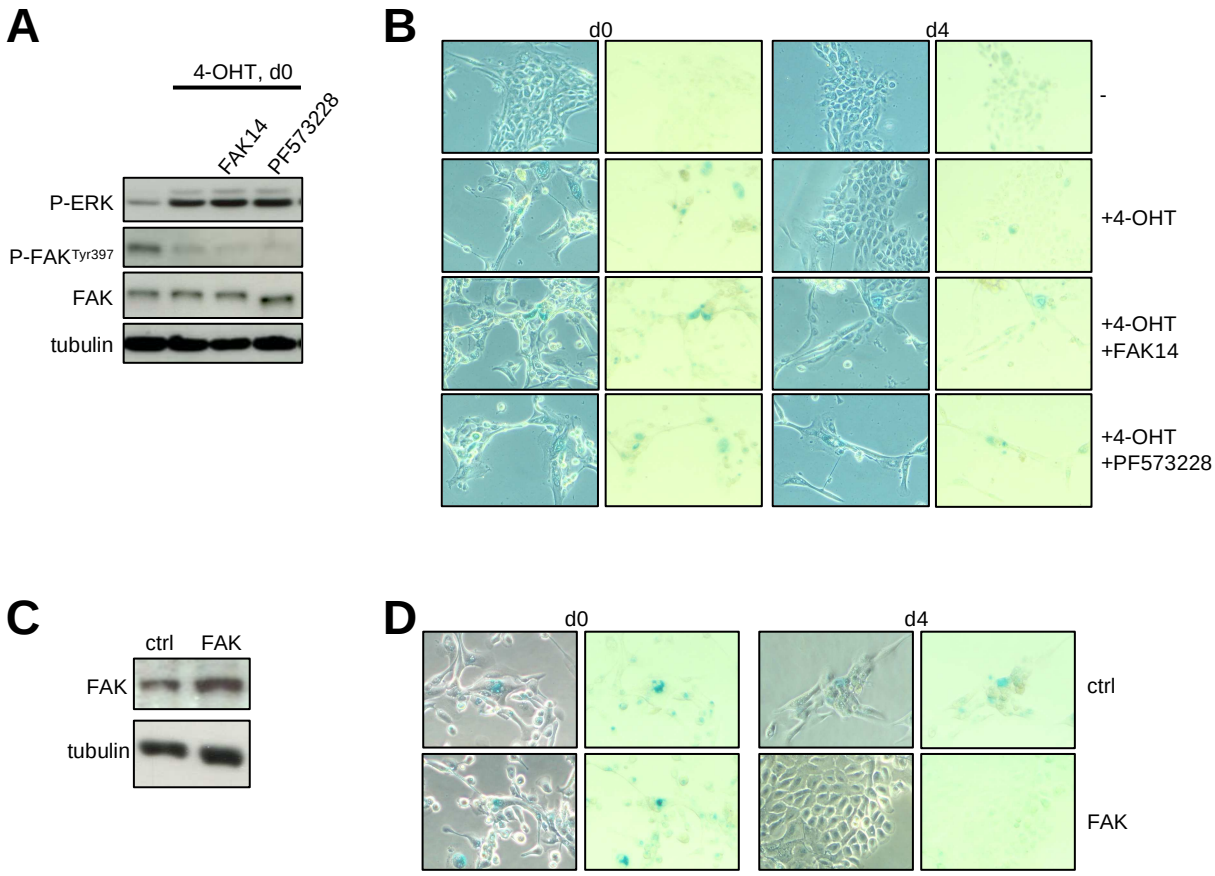
Supplemental Figure 3: LOX constitutive expression and senescence. HMEC-TM cells were infected with a Lox- or LoxL2-expressing or control retroviral vector and puromycin-selected. (**A**) Cell extracts were prepared and analyzed by immunoblotting for Lox or LoxL2 (with an anti-flag antibody) and tubulin. (**B**) HMEC-TM cells were treated for 3 days with 4-OHT, fixed, and stained with crystal violet. (**C**) Growth curves of HMEC-TM cells expressing Lox or not, without oncogenic stress.

Supplemental Figure 4



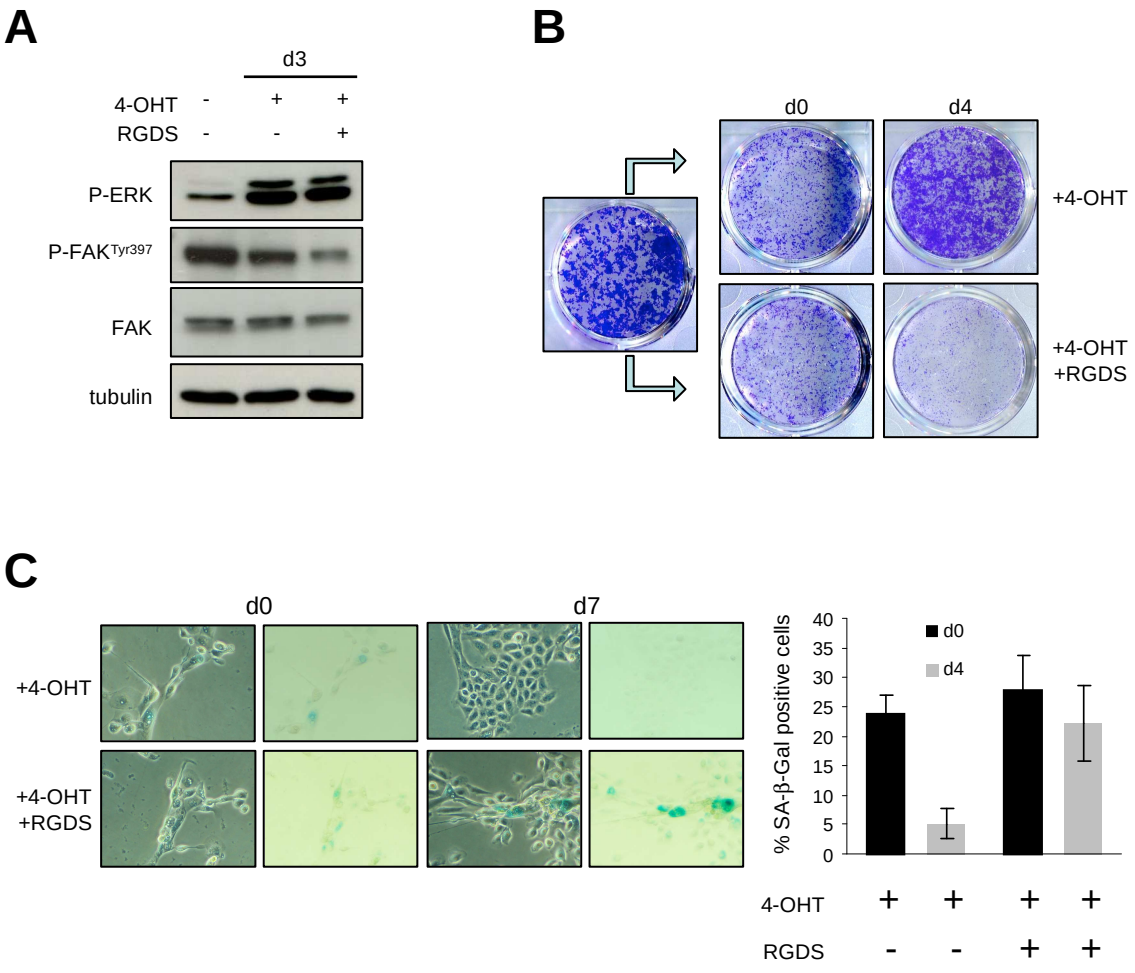
Supplemental Figure 4: A transgenic model of Kras-induced PDAC displays some senescence. Pdx1-Cre; LSL-KrasG12D/+; INK4a/Arflox/lox (KIA) and control Pdx1-Cre; INK4a/Arflox/lox (WT) mice were used. (**A**) KIA mice were sacrificed 45 days after birth and macrodissected. Normal and tumoral samples were extracted. (**B**) Pancreas samples (WT, WT+BAPN, KIA normal, KIA tumoral) were fixed and their normal/tumoral status was histologically determined by HPS staining. An SA-β-Gal activity assay was used to detect senescence and Ki67 staining to determine the proliferative status.

Supplemental Figure 5



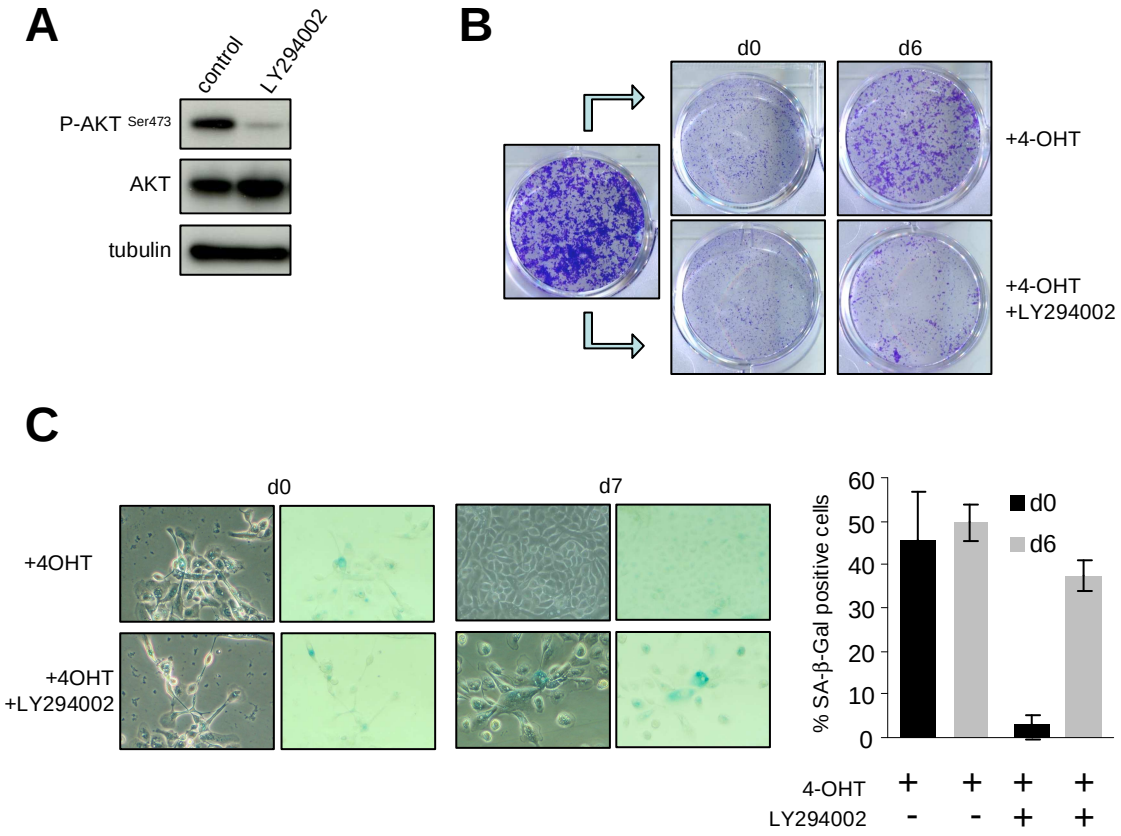
Supplemental Figure 5: FAK signaling and senescence. (**A**) HMEC-TM cells were treated with 4-OHT, with or without FAK inhibitors, for 3 days. Cell extracts were prepared and analyzed (d0) by immunoblotting with antibodies recognizing P-ERK, P-FAK, FAK, and tubulin. (**B**) HMEC-TM cells were treated as in A (d0) and then cultured for an additional 4 days with or without the indicated FAK inhibitors (d4). Cells were fixed and their SA-β-Gal activity assayed on d0 and d4. (**C**) HMEC-TR cells were infected with a FAK-encoding or control retroviral vector and neomycin-selected. Cell extracts were prepared and the FAK level estimated by immunoblotting. (**D**) HMEC-TR cells, control, and additional days (d4). Cells were fixed their SA-β-Gal activity assayed.

Supplemental Figure 6



Supplemental Figure 6: Blocking integrin signaling blocked senescence reversal.(**A**) HMEC-TM cells were treated with 4-OHT, with or without RGDS peptide. Cell extracts were prepared at d3 and analyzed by immunoblotting with antibodies recognizing P-ERK, P-FAK, FAK, and tubulin. (**B**) HMEC-TM cells were treated with 4-OHT with or without the RGDS peptide for 3 days (d0) and then treated only with the RGDS peptide for an additional 4 days (d4). Cells were fixed and crystal violet stained or their SA-β-Gal activity was assayed (**C**).

Supplemental Figure 7



Supplemental Figure 7: Blocking the PI3K pathway blocked senescence reversal. (**A**) HMEC-TM cells were treated with a PI3K inhibitor. Cell extracts were prepared and immunoblotting performed against P-AKT to check the inhibitor efficiency. (**B**) HMEC-TM cells were treated with 4-OHT with or without the PI3K inhibitor for 3 days (d0) and then treated only with the PI3K inhibitor for an additional 7 days (d7). Cells were fixed and crystal violet stained or their SA-β-Gal activity was assayed (**C**).

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Research Perspective

NUAK1 links genomic instability and senescence

David Bernard[1] and Arnaud Augert[1,2]

[1] UMR5238, Apoptose, Cancer et Développement, CNRS/Université de Lyon/Centre Léon Bérard, Lyon, France
[2] UMR8161, Institut de Biologie de Lille, CNRS/Universités de Lille 1 et 2, Lille, France

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E-mail: *BERNARDD@lyon.fnclcc.fr*

NUAK1 and senescence

The AMP-activated protein kinase-related kinase (ARK) family comprises 13 proteins, amongst them NUAK1, that can be classified into five subfamilies: AMP-activated protein kinase (AMPK), salt-induced kinase (SIK), microtubule-affinity-regulating kinase (MARK), brain specific kinase (BRSK), and SNF1-like kinase 1 (NUAK). These proteins regulate biological responses such as metabolism, polarity, cell proliferation or cell death, presumably in a sub-family specific manner [1]. Although the different proteins regulate different responses, their activities are thought to be controlled by the same kinase, LKB1 [2], which phosphorylates a threonine residue in the conserved T-loop of ARK proteins. AMPK proteins are phosphorylated and activated by LKB1 when ATP levels decrease, whereas ARK proteins are phosphorylated and activated independently of intracellular ATP levels [1].

During metabolic stress, ATP levels decrease and LKB1 activates AMPK that, in turn, phosphorylates a subset of proteins. P53 has been identified as one of these proteins, and it is postulated that the phosphorylation and activation of p53 by AMPK leads to cell cycle arrest and senescence [3].

We have recently identified and described the role of NUAK1 in the regulation of replicative senescence. Indeed, the constitutive expression of NUAK1 induces senescence in WI38 normal human fibroblasts whereas its knockdown extends their replicative lifespan. The loss of NUAK1 activation by LKB1 (by using a NUAK1 mutant unresponsive to LKB1 or by inhibiting LKB1 activity in NUAK1 expressing cells) results in a failure of NUAK1 to induce senescence, thus demonstrating the major role of LKB1 in NUAK1-induced senescence. Interestingly, our results support the existence of a p53 independent response, at least in WI38 cells, and emphasize a potential role of aneuploidy in NUAK1-dependent senescence [4].

Aneuploidy and senescence

Aneuploidy or genomic instability due to various factors have been reported to induce senescence [5,6,7]. Interestingly, senescent cells often display elevated aneuploidy, which suggests a putative functional role of aneuploidy in senescence. Nevertheless, it is unclear whether aneuploidy is involved in the establishment of the senescent phenotype and, if prevented, it can impair senescence, at least to some extent. A breakthrough has been achieved with the demonstration that the state of irreversible growth arrest in senescent cells may be due to elevated aneuploidy, putatively through a decrease of LATS1, a kinase involved in mitotic exit [7]. These results suggest that aneuploidy, if not directly involved in the establishment of senescence, can be required for irreversible growth arrest in senescent cells.

Interestingly, aneuploidy was also observed during replicative senescence and during NUAK1-induced premature senescence in our model. More importantly, the replicative lifespan extension due to NUAK1 knockdown correlated with normal ploidy. Altogether, these results suggest that ploidy can be a functional regulator of the senescence program.

We also identified LATS1 as a potential target of NUAK1 and a putative regulator of ploidy in NUAK1-dependent senescence. Altogether, these results suggest that aneuploidy could be part of the endogenous senescence program. Its mis-regulation could therefore induce premature senescence through a process that we chose to term "aneuploidy-induced senescence" (AIS). Our results also suggest that AIS may occur, at least in some settings, without the involvement of the p53 pathway. Interestingly, others have described that the overexpression of Aurora A, a serine threonine kinase tightly associated with the mitotic process, induces senescence in the mammary gland of p53-deficient mice [8]. Hence aneuploidy could be one of the signals triggering senescence and could act, in some settings, independently of p53.

Aneuploidy-induced senescence as a possible safeguard against tumor formation and development

Oncogene activation is one of the hallmarks of cancer cells and a driving force in tumorigenesis [9]. Oncogene-induced senescence (OIS) was described about a decade ago [10], and a long debate has raged about its relevance. With the development of adequate mouse models of cancer susceptibility and new tools to detect senescence *in vivo*, it has become possible do demonstrate its effectiveness in blocking malignant transformation [11].

Aneuploidy, another classical hallmark of cancer cells, is also believed to be involved in tumorigenesis [12]. As mentioned above, induction of aneuploidy can result in premature senescence in various settings [5,6,7]. Together, these observations suggest that AIS, like OIS, could constitute a failsafe mechanism against early tumorigenesis. To validate this hypothesis, it would be interesting to test the presence and the frequency of aneuploidy in benign lesions and to identify the genetic events possibly favoring AIS escape.

The AIS model could resolve the apparent discrepancy about the role of NUAK1 in tumorigenesis. Our recent findings demonstrate an ability of NUAK1 to induce premature senescence in normal human cells whereas others, mainly the team of H. Esumi, have demonstrated a pro tumoral effect of NUAK1 through promoting cell growth and invasion [13,14,15]. However, these last conclusions were based on data obtained in cancer cell lines, in particular in colon cancer cell lines known to be highly aneuploid [16]. Thus, NUAK1 may have no additional effect on genomic stability and instead regulate other targets to confer a growth advantage to the cells. NUAK1 might even add more genomic instability, thus conferring additional growth and invasion advantages to these cancer cells.

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CONFLICT OF INTERESTS STATEMENT

The authors of this manuscript have no conflict of interests to declare.

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Les facteurs sécrétés associés à la sénescence

Des fonctions pro- et anti-tumorales

Arnaud Augert, David Bernard

UMR 8161, Institut de biologie de Lille,
CNRS/Universités de Lille 1 et 2
Institut Pasteur de Lille, IFR 142,
59021 Lille, France.
BERNARDD@lyon.fnclcc.fr

› Il y a près d'un demi-siècle, Hayflick et Moorhead observaient que des cellules primaires mises en culture avaient un potentiel réplicatif limité, et que les cellules adoptaient un comportement particulier, caractérisé par un arrêt irréversible de la prolifération cellulaire qu'accompagnent des changements morphologiques et biochimiques. Ils nommèrent ce processus la sénescence [12]. Au-delà de l'inhibition de l'immortalisation cellulaire, il a été récemment montré que ce processus de sénescence pouvait être activé dans de nombreuses situations de stress : oxydant, génotoxique et oncogénique. Ainsi, on sait maintenant que la sénescence, processus naturel au cours du vieillissement des cellules, peut aussi être induite dans des cellules tumorales. Quels que soient les stimulus, les gènes suppresseurs de tumeur p53 et Rb sont des protagonistes incontournables du processus de sénescence [1].

Le double jeu de la sénescence sur le processus tumoral

Un certain nombre d'études récentes ont montré que la sénescence inhibe le développement tumoral. En effet, qu'elle soit activée par des télomères défectueux, une stimulation oncogénique ou la réactivation d'un gène suppresseur de tumeur, la sénescence inhibe la formation tumorale et, dans certains cas, induit même une régression tumorale, principalement *via* l'activation des gènes suppresseurs p53 et Rb [1].

Cependant, les cellules sénescentes peuvent également avoir une action pro-tumorale sur les cellules voisines, pré-néoplasiques, *via* la sécrétion de facteurs. En effet, les cellules sénescentes présentent un profil de sécrétion particulier appelé le *senescent-associated secreted phenotype* (SASP) ou *senescence messaging secretome* (SMS). Parmi ces facteurs, on retrouve des protéines qui altèrent le micro-environnement tissulaire, telles que des métalloprotéases (MMP) et des cytokines pro-inflammatoires [2, 12, 13]. Des études du groupe de Judith Campisi suggèrent qu'un certain nombre de ces facteurs favorisent la progression tumorale. Ainsi, des cellules épithéliales pré-malignes forment des tumeurs lorsqu'elles sont incubées en présence de fibroblastes sénescents alors qu'elles n'évoluent pas ainsi en présence de fibroblastes normaux ou pré-sénescents [3, 4]. La sécrétion de la MMP-3 par ces fibroblastes sénescents participerait à cet effet paracrine [4]. Dans le même esprit, la sécrétion de cytokines pro-inflammatoires - l'IL-6 et l'IL-8 par exemple - par les cellules sénescentes induirait une transition épithélium-mésenchyme dans des cellules épithéliales pré-malignes, cette transition étant caractéristique de cellules engagées dans un processus métastatique [5].

Jusqu'à récemment, ces deux actions de la sénescence, anti-tumorale *via* l'activation de p53 et/ou de Rb et pro-tumorale via la sécrétion de facteurs, semblaient indépendantes sur le plan moléculaire. Cependant, une série de publications récentes a démontré un lien dans le double jeu de la sénescence sur le processus tumoral puisque certains de ces facteurs secrétés, potentiellement pro-tumoraux, étaient aussi capables d'avoir une action anti-tumorale en activant la sénescence *via* p53 et/ou Rb [1, 6, 8, 9].

Lors d'un criblage génétique fonctionnel, l'équipe de Jésus Gil a mis en évidence le rôle central du récepteur aux chimiokines CXCR2 (récepteur notamment de l'IL8, IL8RB) dans l'induction de la sénescence. En effet, nous avons montré que CXCR2 ainsi que certains de ses ligands, tels que IL-8 et Groα, étaient essentiels à l'induction et au maintien de la sénescence [6]. Dans le même temps, le groupe de Daniel Peeper a montré que l'expression de certaines cytokines augmentait fortement pendant la sénescence. Ils ont ensuite montré que l'IL-6 ainsi produite était essentielle à l'entrée en sénescence des cellules qui la sécrétaient. Dans ces deux cas, l'induction de la sénescence est due à l'activation des gènes suppresseurs de tumeur p53 et/ou Rb. Ainsi, certaines cytokines exprimées par les cellules sénescentes ont un double rôle : un rôle autocrine essentiel dans l'induction de la sénescence, et un rôle paracrine pouvant favoriser la progression tumorale des cellules environnantes contenant des lésions pré-néoplasiques [1, 5].

Phospholipases A2 solubles : nouveaux inducteurs de sénescence

Il reste cependant à définir si cet effet est propre à quelques cytokines ou si d'autres facteurs sécrétés par les cellules sénescentes peuvent exercer cet effet bivalent sur le développement tumoral. Des résultats récents de notre laboratoire suggèrent que tel est le cas : en effet, nous venons de montrer que PLA2R, le récepteur des

phospholipases A2, a un impact important sur la sénescence. Son inhibition retarde l'entrée en sénescence alors que sa surexpression induit une sénescence prématurée des cellules testées. Ce processus de sénescence induit par PLA2R est dépendant de la production de ROS (*reactive oxygen species*), de l'activation des dommages à l'ADN et de p53. PLA2R est le récepteur de différentes phospholipases A2 sécrétées (sPLA2) [7], ce qui pouvait évoquer la responsabilité de certaines sPLA2 dans le phénotype observé. PLA2G2A était un candidat probable car son expression augmente pendant la sénescence, et de fait, la surexpression de PLA2G2A était associée à une entrée prématurée des cellules en sénescence. Ce processus était en partie dépendant de PLA2R puisque des cellules déplétées en PLA2R ne sont pas ou peu sensibles à la surexpression de PLA2G2A [8]. Le groupe de Jae-Ryong Kim a également mis en évidence un rôle des sPLA2 dans la sénescence. Les auteurs ont montré que lors d'un traitement par une autre phospholipase A2 soluble, PLA2GIB, les cellules arrêtent de proliférer et entrent en sénescence. Ils ont ensuite démontré que cet effet n'est pas limité à PLA2GIB puisqu'il peut aussi être induit par PLA2G2A. Leur étude conclut également à l'implication des ROS dans ce processus : leur augmentation induit des dommages à l'ADN et une augmentation de p53, expliquant ainsi le phénotype sénescent des cellules [10].

Les sPLA2 sont des enzymes de 14 à 18 kDa qui hydrolysent la partie sn-2 des phospholipides produisant des acides gras tels que l'acide arachidonique (AA). Ces enzymes jouent un rôle important dans l'inflammation et le contrôle du système immunitaire. Elles peuvent également réguler la production de métalloprotéases et de cytokines pro-inflammatoires et donc potentiellement exercer des effets pro-tumoraux sur les cellules voisines [11]. Néanmoins, l'effet paracrine des sPLA2 sécrétées n'a pas encore été mis en évidence dans un contexte sénescent.

Conclusion

Il est donc possible que de nombreux facteurs décrits dans le SASP soient impliqués à la fois dans l'induction de la sénescence par des effets autocrines et aussi dans une action pro-tumorale par des effets paracrines. À l'heure où la réactivation de la sénescence dans les cellules tumorales est envisagée comme une stratégie thérapeutique, il apparaît important de comprendre et d'évaluer les bénéfices d'une telle stratégie en considérant le lien moléculaire inattendu existant entre les activités pro- et anti-tumorales de la sénescence. ◊

Senescent-associated factors: pro- and anti-tumoral actions

CONFLIT D'INTÉRÊTS

Les auteurs déclarent n'avoir aucun conflit d'intérêts concernant les données publiées dans cet article.

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V.7 Patent 1

David BERNARD, Arnaud AUGERT, Gérard LAMBEAU, Christophe GIRARD and David VINDRIEUX (hereinafter referred to as Assignor); Assignor has discovered:

“PLA2R1 as anti-tumoral compound and as biomarker for the detection of cancer”, (N° 11305885.3 in Europe).

V.8 Patent 2

David BERNARD, Laurent BARTHOLIN, Etienne LELIEVRE and Arnaud AUGERT (hereinafter referred to as Assignor); Assignor has invented certain new and useful improvements in:
“SCREENING METHOD FOR IDENTIFYING COMPOUNDS WHICH BLOCK TUMOUR GROWTH BY INDUCING IRREVERSIBLE SENESCENCE IN TUMOUR CELLS”, set forth in U.S. Application No. 61/298,315, filed January 26, 2010

V.9 Résumé de la Thèse

Le récepteur aux phospholipases A2 (PLA2R1) est un nouveau régulateur inattendu de la sénescence cellulaire et gène suppresseur de tumeurs

Introduction

Durant la tumorigénèse, des mécanismes de sauvegarde tels que la sénescence et l'apoptose sont mis en place. La sénescence a tout d'abord été observée dans des fibroblastes primaires mis en culture qui avaient atteint leur potentiel réplicatif [1]. Plus récemment, il a été montré que la sénescence pouvait être induite de façon prématurée par divers stimuli tels que des stress oncogéniques ou oxydants [2]. La sénescence, caractérisée par un arrêt « irréversible » de la prolifération, s'accompagne de changements morphologiques et biochimiques ainsi que d'une variation de l'expression génique [3]. Les cellules sénescentes sont identifiées entre autre par leur morphologie, l'activité SA-βgal (senescence associated β galactosidase activity) [4] et la formation d'hétérochromatine associée à la sénescence (SAHF) [5].

Il existe différents types de sénescence qui ne diffèrent pas d'un point de vue phénotypique mais varient par la nature de leurs inducteurs. La sénescence réplicative (dépendante de la taille des télomères) limite le potentiel réplicatif. Cet arrêt est levé pendant le processus tumoral lors de l'immortalisation cellulaire. Ainsi, la plupart des tumeurs présentent une augmentation de la télomèrase, l'enzyme impliquée dans le maintien des séquences télomériques [6, 7]. Par ailleurs, la sénescence induite par un stress oncogénique (OIS) bloque la conversion maligne de lésions néoplasiques [8-10]. La sénescence est donc

considérée comme un mécanisme de sauvegarde qui bloque l'initiation et le développement tumoral.

Les mécanismes qui contrôlent l'échappement à la sénescence sont encore peu décrits. Nous avons mis en place un projet ayant pour but d'identifier et de comprendre les événements génétiques impliqués dans l'échappement à la sénescence réplicative et à l'OIS.

Mon travail de thèse a consisté à réaliser un criblage perte de fonction. Le but de ce travail était d'identifier des gènes qui, lorsqu'ils sont inhibés, permettent un échappement à la sénescence réplicative. J'ai donc utilisé une partie de la banque de shRNA (ciblant 2304 gènes)[13] afin d'isoler puis de caractériser de nouveaux régulateurs de la sénescence. Une approche similaire nous avait permis d'isoler des gènes (TOP1 [11] et NUAK1 [12]) qui, quand leurs expressions diminuent, permettent de retarder significativement l'entrée en sénescence réplicative de cellules humaines normales.

Méthodologie

Nous avons utilisé la technique d'interférence à l'ARN. Cette technique permet d'inhiber de manière spécifique l'expression des gènes. Récemment, des banques rétrovirales de shRNA (« short hairpin RNA ») ont été développées et permettent potentiellement d'inhiber de manière stable tous les gènes du génome (Bernards et al., 2006; Silva et al., 2005). Nous avons utilisé une partie de la banque rétrovirale de shRNA (2400 gènes) développée par R. Bernards (Bernards et al., 2006). Cette banque provient du NKI (institut de cancer des Pays-Bas). Cette banque est basée sur l'utilisation de vecteurs rétroviraux (pRetro-Super) contenant des shRNA. La banque développée par R. Bernards (Bernards et al., 2006) permet de cibler 8000 gènes. Pour chaque gène, 3 shRNA ont été construits et clonés dans le

vecteur pRS (Brummelkamp et al., 2002). Le vecteur rétroviral est dérivé du MSCV (« murine stem cell virus »). Une cassette, composée d'un promoteur de type 3 -H1RNA- qui permet la transcription du shRNA et d'un promoteur PGK qui permet l'expression du gène de résistance à la puromycine, a été insérée entre les LTRs. La séquence du shRNA est composée des séquences sens et anti-sens de 19 nucléotides ciblant le gène, d'une boucle (hairpin) de 9 nucléotides et d'une série de poly T pour terminer la transcription.

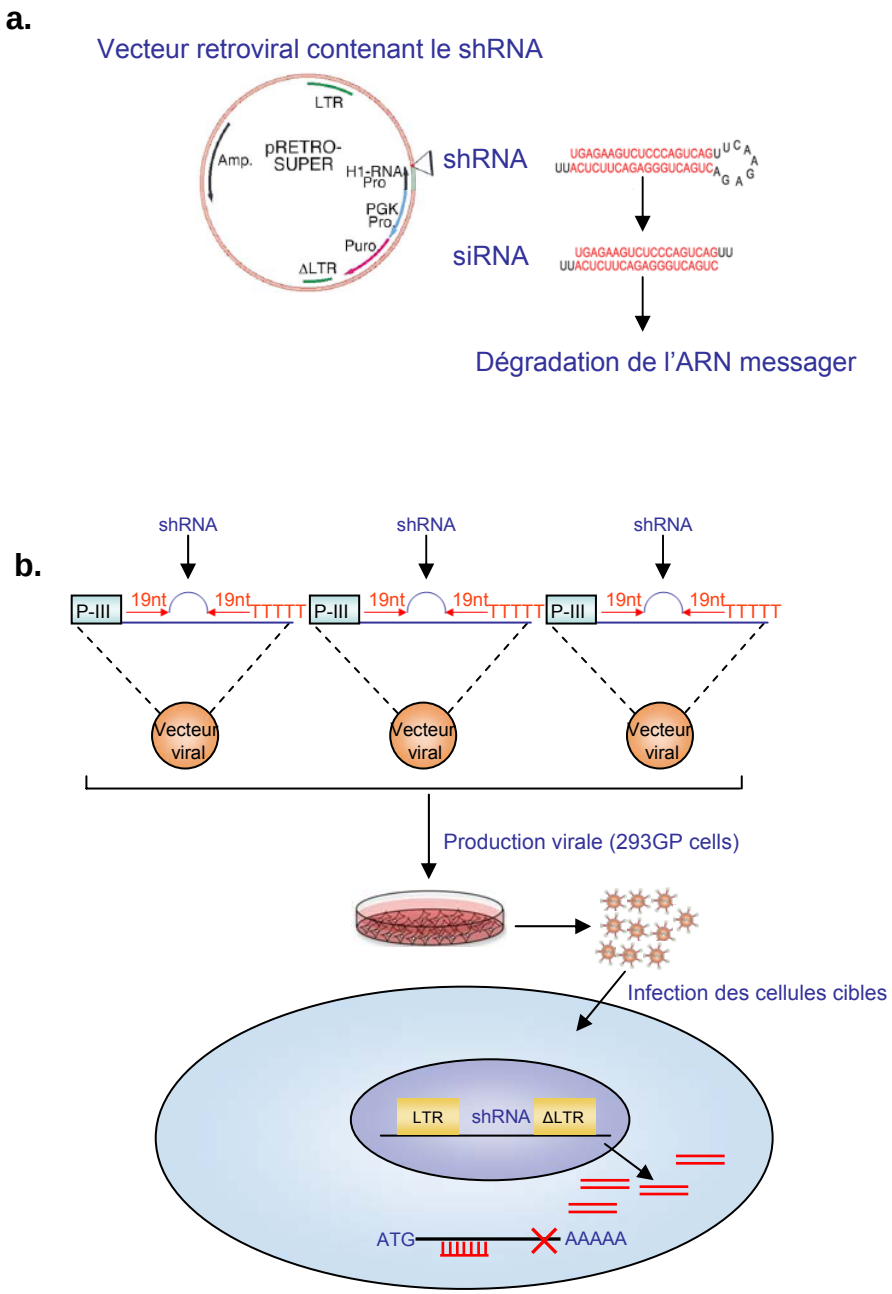


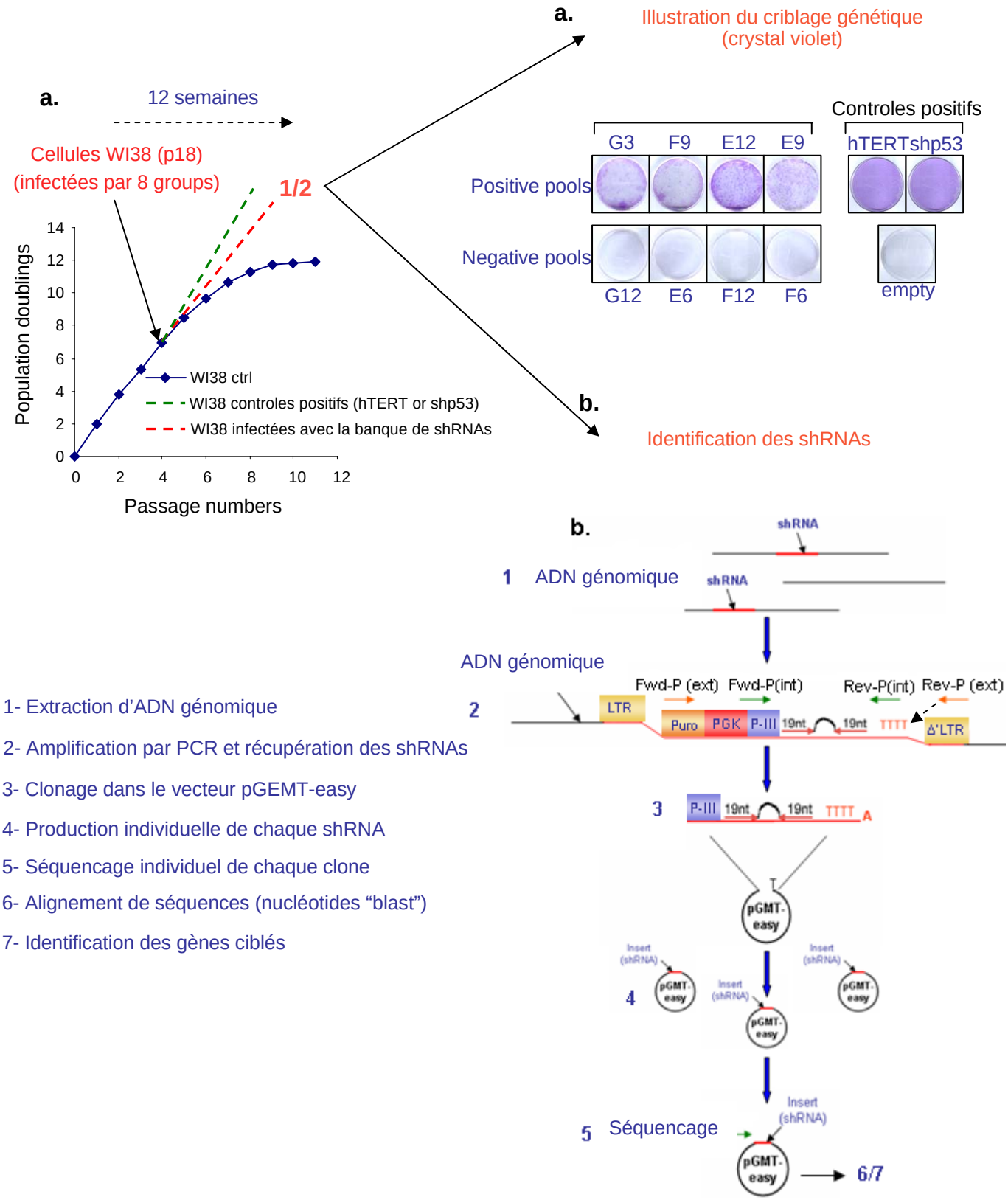
Figure 1 : Représentation schématique de la banque de shRNA utilisée pour le criblage génétique.

Nous avons choisi comme modèle cellulaire des fibroblastes humains normaux (WI38). Ces cellules, lorsqu'elles sont mises en culture, vont arriver à un plateau de sénescence qui est dépendant des voies p53 et/ ou p16/Rb et de la taille des télomères. En effet, l'inhibition de la voie p16/Rb ou de la voie p53 ainsi que l'expression de la sous unité catalytique de la télomèrase (hTERT) permet d'augmenter de manière significative le potentiel réplicatif de ces cellules ou d'immortaliser les cellules dans le cas de l'hTERT (Figure 2).

Le principe de ce criblage est d'infecter les fibroblastes humains normaux (WI38) par la banque de shRNA avant le plateau de sénescence. Une large majorité de cellules va entrer en sénescence (en bleu) (Figure 2). Nous espérons que certaines échapperont à la sénescence grâce à l'inhibition spécifique d'un gène par son shRNA. Ainsi, nous devrions isoler de nouveaux évènements génétiques impliqués dans le contrôle de la sénescence.

Nous avons divisé la banque pRS en 8 sous-groupes, chacun ciblant 288 gènes. Les fibroblastes humains normaux (WI38) ont ainsi été infectés indépendamment par les différents sous-groupes de la banque de shRNA. Après sélection par la puromycine, les cellules ont été passées toutes les semaines pendant 12 semaines (Figure 2). Les cellules infectées par le pRS (C^-) sont entrées en sénescence alors que les cellules infectées par le pRS p53 (C^+) ou l'hTERT ont continué à proliférer. Dans certains cas, les cellules infectées par différents mélanges de la banque ont continué de croître (Figure. 2). Les ADN génomiques de ces cellules ont été extraits et les shRNA potentiellement responsables d'une augmentation du potentiel réplicatif ont été identifiés (Figure. 2).

La figure 2 nous permet de visualiser les mélanges de shRNA qui ont permis aux cellules d'échapper à la sénescence (G3, F9, E12 et E9) et pour lesquelles nous avons préparé l'ADN génomique afin d'identifier les shRNA. Dans les conditions où les cellules n'ont pas formé de clones, nous n'observons quasiment plus de cellules après trois mois de culture. En effet, les cellules entrent en sénescence et meurent ou sont perdues progressivement lors des passages. Les cellules infectées par le contrôle négatif se comportent comme ces dernières.



1- Extraction d'ADN génomique

2- Amplification par PCR et récupération des shRNAs

3- Clonage dans le vecteur pGEMT-easy

4- Production individuelle de chaque shRNA

5- Séquencage individuel de chaque clone

6- Alignement de séquences (nucléotides "blast")

7- Identification des gènes ciblés

Figure 2 : Principe du criblage génétique réalisé dans les fibroblastes humains normaux.

Dix séquences ont été réalisées pour chaque condition où les cellules ont continué à proliférer (Mix G3, F9, E12 et E9). Dans le Tableau 1 sont présentées les séquences qui ont été retrouvées aux moins deux fois dans les dix séquences. Nous avons ensuite réalisé un alignement de séquence sur NCBI afin d'identifier le gène ciblé. Le criblage génétique que j'ai réalisé a permis d'isoler et d'identifier 11 shRNAs dans les colonies ayant échappé à la sénescence réplicative (Figure 3). Mon 1er travail a consisté à cloner et à tester individuellement ces shRNA pour confirmer ou infirmer leur capacité à induire un échappement à la sénescence réplicative. Certains shRNA n'avaient pas cette capacité et ont donc été abandonnés. Les shRNA peuvent exercer un effet aspécifique [14]. Nous avons donc cloné d'autres shRNA ciblant le même gène, car il est admis que 2 shRNA qui ciblent le même gène et qui induisent la même réponse agissent via leur cible spécifique [15]. Etant donné que l'inhibition de ces gènes favorise un échappement à la sénescence, nous avons voulu savoir si l'effet de l'expression constitutive de ces gènes avait l'effet inverse, c'est-à-dire, induisait une sénescence prématurée. Cette stratégie nous a permis de sélectionner 2 candidats (PLA2R et I-2) sur lesquels nous travaillons actuellement.

Liste de potentiels nouveaux régulateurs de la sénescence identifiés grâce au criblage génétique

Gene	Acc No.	Description	Validation
PLA2R1	NM_007366.3	The phospholipase A2 receptor	**validé**
FBXO-32	NM_058229.2	F-box protein 32	-
FKBP-14	NM_017946.2	FK506 binding protein 14	-
PPP1R2	NM_006241.4	protein phosphatase 1 regulatory subunit 2	**validé**
MED12	NM_005120.1	mediator of RNA polymerase II transcription	-
USP3	NM_006537.2	ubiquitin specific peptidase 3	**“aspécifique”**
TGM1	NM_000359.1	transglutaminase 1	-
ADRBK2	NM_005160.2	adrenergic beta receptor kinase 2	-
HSPA4L	NM_014278.2	heat shock 70kDa protein 4-like	-
FGF19	NM_005117.2	fibroblast growth factor 19	-
HCLS	NM_000411.4	holocarboxylase synthetase	-

Figure 3 : Liste de potentiels nouveaux régulateurs de la sénescence identifiés lors du criblage génétique.

Le travail sur I-2 étant trop préliminaire, j'ai fait le choix de ne présenter que le travail sur le récepteur aux phospholipases A2 (PLA2R1).

PLA2R1, un nouveau régulateur de la sénescence

Suite au criblage génétique et à l'identification de PLA2R1 comme un potentiel régulateur de la sénescence, nous avons démontré que l'inhibition du récepteur favorise un échappement à la sénescence réplicative ainsi qu'à la sénescence induite prématurément par un stress oxydant. Nous avons ensuite observé que l'expression de PLA2R1 augmente dans les cellules sénescentes et que son expression constitutive dans les cellules jeunes induit une sénescence prématurée. Les analyses biochimiques ont démontré que PLA2R1 régule les espèces réactives d'oxygènes (ERO) et la sénescence en activant la voie des dommages à l'ADN et p53 (Figure 4). Ce premier travail identifie PLA2R1 comme un potentiel gène suppresseur de tumeur crucial dans l'induction de la sénescence et dans l'activation de p53, qui est lui-même un gène suppresseur de tumeur fréquemment retrouvé muté ou inhibé dans les cancers [16]. Ce travail à été publié en 2009 dans « EMBO reports ». Pour plus d'informations, se référer à l'article qui est inclus dans le manuscrit.

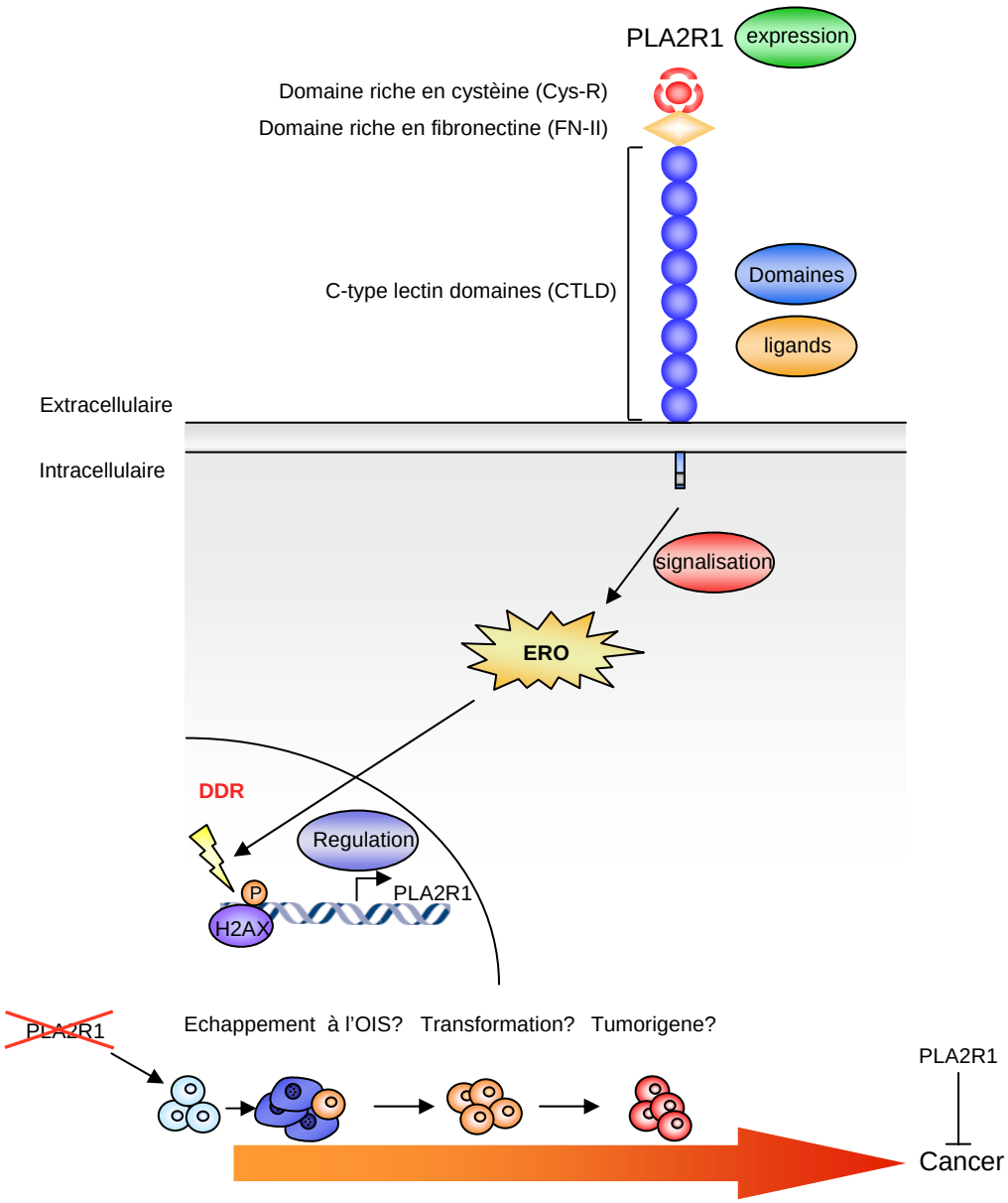


Figure 4 : Récapitulatif des découvertes que nous avons pu réaliser dans l'article scientifique que nous avons publié en 2009 dans la revue EMBO reports. Cette figure contient également des hypothèses qui ont été faites suite à ces premiers résultats.

PLA2R1, un nouveau gène suppresseur de tumeurs

Dans la première étude, nous avons aussi observé, que la diminution de l'expression de PLA2R1, en plus de pouvoir réguler la sénescence de fibroblastes (HDF), était aussi capable de réguler la sénescence réplicative dans des cellules épithéliales humaines mammaires (HMEC) [17].

D'après la banque de données Oncomine (version 3), nous avons pu observer que l'expression de PLA2R1 diminue dans les tissus tumoraux comparés aux tissus sains. Cette diminution d'expression se retrouve principalement dans les tumeurs du rénal et mammaires. Nous avons voulu valider expérimentalement ces données. Pour ceci nous avons eu accès à des tissus tumoraux et normaux de rein de même patient pour lesquels nous avons pu analyser l'expression de PLA2R1. De manière intéressante, nous avons pu observer que l'expression de PLA2R1 diminuait dans les tissus tumoraux (pour plus d'informations se référer a l'article en cours de préparation présenté dans le manuscrit).

Nous avons ensuite voulu savoir quel pouvait être le mécanisme associé avec la diminution d'expression de PLA2R1dans les tumeurs. Une analyse bioinformatique du promoteur de PLA2R a révélé la présence d'un ilot CpG de 1400 paires de bases. Les gènes suppresseurs de tumeurs sont souvent réprimés par hyperméthylation [20]. Nous avons donc étudié la méthylation du promoteur dans les tumeurs mammaires. De manière intéressante la méthylation du promoteur corrèle à l'expression de PLA2R1. Une inhibition chimique des ADN méthyltransferases (DNMT) par la 5 aza-deoxycytidine (5-AZA) induit une réactivation de l'expression de PLA2R1 dans les cellules ou PLA2R1 est méthylé suggérant que la

méthyaltion est fonctionnellement impliquée dans la diminution d'expression de PLA2R1 observée dans les tumeurs.

L'OIS est décrit comme un mécanisme de sauvegarde qui bloque la conversion maligne de lésions bénignes [8-10]. Nous avons donc voulu examiner si la perte d'expression de PLA2R1 pouvait favoriser l'échappement à l'OIS (induite par Her-2, Ras ou MEK) dans les HMEC. De manière intéressante, l'inhibition de PLA2R1, tout comme celle du gène suppresseur de tumeur p53 favorise un échappement à l'OIS induite par différents oncogènes.

Le processus tumoral est composé de différentes étapes. Nous avons voulu analyser si la diminution d'expression de PLA2R1 pouvait favoriser la transformation cellulaire et la tumorigénicité des cellules. De manière intéressante, l'inhibition de PLA2R1 par ARN interférence permet aux cellules de pousser en conditions d'agar mou et forment des tumeurs lorsqu'on les injecte dans des souris immunodéprimées. Ces ensembles de données nous ont amené à penser que PLA2R1 pouvait être un nouveau gène suppresseur de tumeurs.

Nous sommes allés plus loin dans l'étude. En effet, nous avons démontré que lorsque PLA2R1 était ré exprimé dans les tumeurs, il induisait une mort cellulaire (apoptose) pouvant ainsi induire la mort de cellules cancéreuses. Ces découvertes sont importantes pour plusieurs raisons. Nous avons démontré une fonction pour un gène qui jusqu'à présent n'était pas beaucoup étudiée. De plus, nous avons démontré que ce gène était un nouveau gène suppresseur de tumeurs. Il est possible que PLA2R1 puisse être utilisé comme bio-marqueur. De plus nous avons récemment démontré que cette protéine pourrait avoir un potentiel à visée thérapeutique étant donné son potentiel d'action sur les cellules tumorales.

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Résumé de la Thèse

Titre de la Thèse: Le récepteur aux phospholipases A2 (PLA2R1) est un nouveau régulateur inattendu de la sénescence cellulaire et un gène suppresseur de tumeurs.

Activée dans les stades précoces du développement tumoral, la sénescence empêche la progression tumorale en induisant un arrêt de prolifération cellulaire. Ainsi, tout comme l'apoptose, ce mécanisme doit être altéré pour qu'une cellule devienne tumorale. Malgré ce rôle crucial de protection contre la progression tumorale, nous connaissons peu les mécanismes moléculaires qui régulent l'échappement à la sénescence. A l'aide d'une banque de shRNA ciblant 8000 gènes, nous avons réalisé un criblage génétique dans le but d'isoler de nouveaux régulateurs, qui lors qu'ils sont inhibés, favorisent un échappement à la sénescence. Ce criblage nous a ainsi permis d'isoler PLA2R1 comme un nouveau régulateur de la sénescence. Nous avons montré que ce gène régulait la sénescence par l'activation du gène suppresseur de tumeurs p53. Un deuxième travail nous a ensuite permis d'identifier PLA2R1 comme un nouveau gène suppresseur de tumeurs. Dans un futur proche PLA2R1 pourra, peut-être, être utilisé comme bio-marqueur. De plus nous avons récemment démontré que cette protéine pourrait avoir un potentiel à visée thérapeutique étant donné son potentiel d'action sur les cellules tumorales. L'ensemble de ces travaux nous ont donc permis d'isoler PLA2R1 comme un nouveau régulateur de la sénescence et gène suppresseur de tumeurs.

Key words: PLA2R1, Senescence Cellulaire, Cancer, Espèces réactives d'oxygène (ERO).

Thesis Resume

Thesis title: An unanticipated role for the Phospholipase A2 receptor (PLA2R1) as a novel cellular senescence regulator and tumour suppressor gene.

Activated in early stages of tumorigenesis, senescence, by blocking proliferation, inhibits tumour growth. Therefore, just like other fails safe mechanisms such as apoptosis, its escape is a property that cancer cell acquire. Although senescence plays a crucial role in tumour suppression and blockage, there is still much to learn about the mechanisms regulating this phenomenon. Using a shRNA library targeting 8000 human genes, we performed a loss of function genetic screen in order to identify genes that when down-regulated, would allow a senescence escape. Using this strategy, we were able to identify PLA2R1 as a novel regulator of cellular senescence by modulating the activation of the p53 tumour suppressor gene. In a second work, we demonstrated that PLA2R1 is a candidate tumour suppressor gene. In the future, PLA2R1 might be used as a biomarker. Finally, we have demonstrated that PLA2R1 could have therapeutic potential as it induces apoptosis in a myriad of cancer cell lines. Altogether, the work performed during my thesis as enabled us to identify PLA2R1 as a novel cellular senescence regulator and a putative new tumour suppressor gene with therapeutic potential.

Key words: PLA2R1, Cellular Senescence, Cancer, Reactive oxygen species (ROS).