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Roles of the multifunctional protein E4F1 in cellular senescence

Rôles de la protéine multifonctionnelle E4F1 au cours de la senescence.

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For my Grandmother Jagusia

"Every man has his own destiny: the only imperative is to follow it, to accept it, no matter where it leads him". **Henry Miller**

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Foreword

Evolutionary biologists regard aging as an aftermath of the declining force of natural selection with age. Some theories say that aging in complex multicellular organisms is partially provoked by the antagonistically pleiotropic effects of tumour-suppressive mechanism – mechanisms that evolved to prevent the development of cancer in young organisms.

As aging is considered to be a consequence of the declining force of natural selection with age, age-related diseases can be considered as phenotypes, which have escaped the force of natural selection. Age-related diseases are usually believed to be degenerative in nature, a result of the overall decline in tissue structure and function, which is a hallmark of aging. There are, however, exceptions – and the most eminent case is cancer. One can define cancer as a 'gain-of-function disease': cells must attain functions such as hyperproliferation, resistance to apoptosis or migratory and invasive properties in order to give rise to malignant tumours. Cancer is not a universal feature of aging as there are organisms, which do not develop cancer (*Drosophila melanogaster, Caenorhabditis elegans*). This brings one to ask the question: what is the difference between organisms that develop cancer from those that do not develop it? One obvious reason is the presence in adult organisms of renewable somatic tissues, which consist of dividing or division-competent cells. These tissue capacities seem to put the organism at risk for developing cancer which is not surprising because DNA replication puts cells in jeopardy of acquiring and fixing mutations and somatic mutations are the major cause of cancer.

Why would evolution select for organisms with renewable tissues given the danger of developing cancer? The answer might be that renewable tissues have the ability to regenerate or repair tissues damaged by injury or endogenous degenerative processes and this might have outweighed their risk, in conjunction with the co-evolution of mechanisms to suppress cancer.

Considering that cell division can lead to mutations and therefore to cancer, organisms with renewable tissues had to evolve strategies to prevent cancer. These strategies are defined as tumour-suppressor mechanisms. Those

mechanisms are classified into two categories – caretakers and gatekeepers. Caretakers act on the genome by preventing or repairing DNA damage, whereas gatekeepers act on cells, causing them to die (apoptosis) or permanently arrest proliferation (senescence). Consequently, gatekeepers restrain the development of cancer by eliminating or preventing the growth of potential cancer cells. It has been proposed that gatekeeper tumour suppressors have both beneficial (anti-cancer) and deleterious (pro-aging) effects depending on the age of the organism. This is an example of so-called evolutionary antagonistic pleiotropy which explains in part why organisms age.

Antagonistic pleiotropy simply holds that what is good for an organism at a young age can be bad for it when it is old.

How might the gatekeeper tumour suppressor genes – those controlling apoptosis and cellular senescence – be antagonistically pleiotropic? Apoptosis prevents cancer by eliminating cells that are damaged or otherwise potentially oncogenic. On the other hand, apoptosis can eventually deplete tissues of their constituent cells and/or deplete the stem cell pools that reside in renewable tissues. Thus, with increasing age, apoptosis might cause a global loss of tissue structure and function, which is a visible sign of aging. Cellular senescence prevents cancer by arresting the growth of potentially oncogenic cells. However, with increasing age, some senescent cells accumulate in organisms and this accumulation can lead to an important loss of both tissue structure and function.

Mechanisms that protect complex organisms from cancer (gatekeeper tumour suppressors) do so in balance against the mechanisms, which promote longevity (Fig. 1). These cancer protection mechanisms involve apoptosis and cellular senescence, which were mentioned above. Both apoptosis and senescence are crucial for suppressing malignant transformation, yet both have the potential to contribute to aging and age-related pathologies.

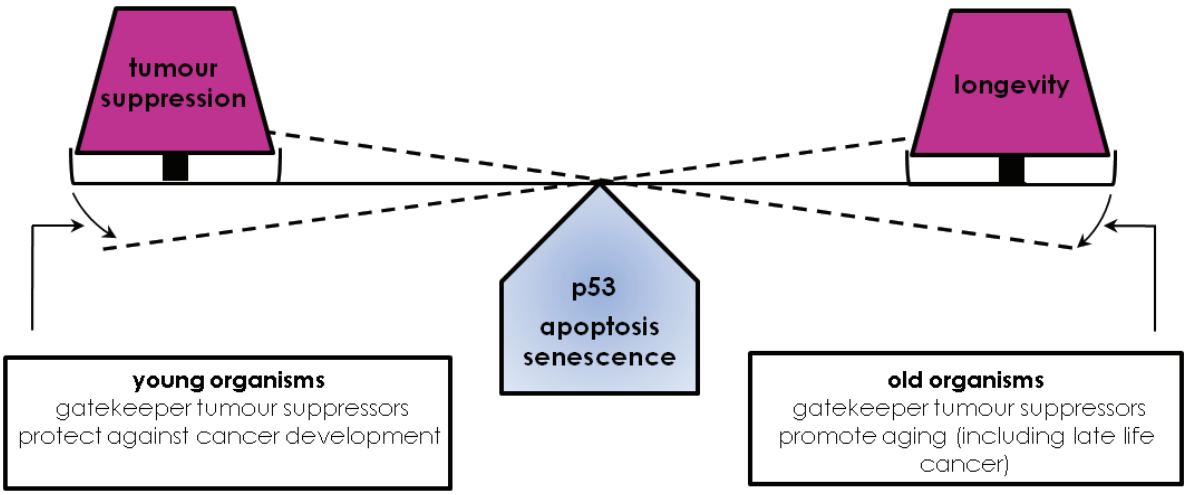

Fig. 1 | **Tumour suppression and longevity are balanced**. Mechanisms that protect against cancer are balanced against those that ensure longevity. In particular, the gatekeeper tumour suppressors such as p53, which regulate apoptosis and senescence of dividing cells are antagonistically pleiotropic – inhibiting cancer in young organisms but favouring aging in old organisms (after Campisi, 2005, modified).

* * *

My PhD project involves the study of cellular senescence in the context of its tumour-suppressive role and the implication of E4F1 protein in this process. The choice of this particular project was made upon encouraging preliminary results obtained in the laboratory and also upon the availability of biological models, e.g. mouse primary cells derived from E4F1 conditional knockout and human primary cell lines. The inducible conditional knockout system, which enables controlling E4F1's expression in time and space, gave me a great opportunity to use various experimental approaches and eventually realise the objective of my PhD work which was to address the significance of E4F1 mediated activities in cellular senescence.

The Introduction to my PhD results is divided into the following two parts: senescence and E4F1. In the first part of the Introduction I decided to develop several aspects concerning our present knowledge of senescence, to characterize well this, sometimes called "artifact of cell culture", process. All this was in order to understand my experimental work results better, with the intention of preparing the reader for perspectives. The second part of the introduction is dedicated to E4F1 and its various roles in crucial cellular

processes. This organisation of the Introduction already enables the reader to spot the possible link between senescence and E4F1.

Research Questions and Experimental Model, and Material and Methods follow the Introduction. Then I present the results obtained during the three years of my PhD. Most of the results concern the primary cell mouse model; the rest represents recent findings from the primary cell line human model.

Finally, the last part of this manuscript is devoted to perspectives in both the near and distant future and to a general discussion of my results and E4F1's involvement in the phenomenon of senescence and its possible roles in organism aging.

Introduction

1. Senescence

1.1. Definition

Cellular senescence was formally described more than four decades ago when Hayflick first suggested that "serially cultured human diploid cells have a finite lifetime *in vitro*" (Hayflick, 1965). These classic experiments showed that human fibroblasts initially underwent robust proliferation in cell culture conditions. However, gradually – over many population doublings – cell division capacity declined. Eventually, all the cells in culture ceased their growth. The non-dividing cells remained viable, metabolically active for many weeks but failed to proliferate despite the fact that they were not confluent (an experimental situation that induces transient cell cycle arrest) and the availability of sufficient nutrients and growth factors in the medium.

The word *senescence* is derived from the Latin word *senex*, meaning *old man* or *old age*. One can define this biological process as an irreversible growth arrest mainly in the G1 phase of the cell cycle of primary (not-transformed) cells in response to various stresses (Serrano and Blasco, 2001).

1.2. Causes and types of cellular senescence

Nowadays, senescence is considered a cellular stress response. Compelling evidence obtained in recent years demonstrates that DNA damage is a common mediator for both *replicative senescence*, which is triggered by telomere shortening, and *premature cellular senescence* induced by various stressors such as oncogenic stress or oxidative stress (Fig. 2).

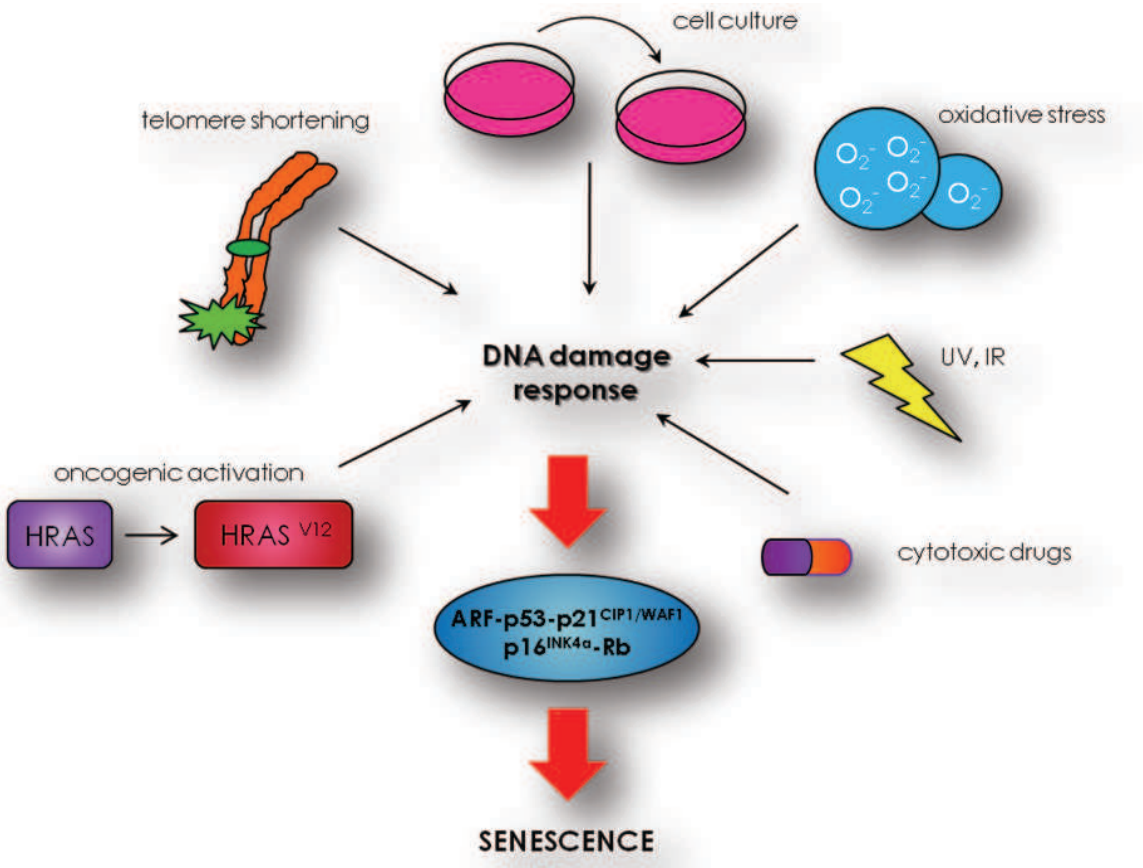

Fig. 2 | **Many roads lead to senescence: DNA damage response is a central mediator in triggering cellular senescence.** Various stresses can contribute to the establishment of cellular senescence, which functions as a self-defence mechanism to prevent the proliferation of damaged cells. Telomere shortening resulting from end-replication problems or precise loss, and various other stressors such as acute oxidative stress treatment or over-expression of oncogenes all trigger a DNA damage response during induction of cellular senescence. The signalling pathways activated by DNA damage response converge on the p53 and Rb proteins (after Collado and Serrano, 2006; Chen *et al.*, 2007; modified).

1.2.1. Senescence caused by DNA damage

Many cell types undergo senescence in response to severe DNA damage that can occur anywhere in the genome (DiLeonardo *et al.*, 1994). Thanks to the DNA damage response (DDR), cells *sense* damaged DNA, particularly double-strand breaks (DSBs). Then the cell cycle progression is arrested, allowing cells to repair the damage or enter either senescence or apoptosis (in the case of persisting DNA damage). Many proteins participate in DDR, including protein kinases (such as ATM or CHK2), adaptor proteins (i.e. 53BP1) and chromatin modifiers, such as γ-H2AX. H2AX becomes phosphorylated on serine 139, then called γ-H2AX, as a reaction to DNA

double-strand breaks (DSB) (Fig. 3). Many proteins implicated in DNA damage response localise to the DNA-damage foci that are detected in senescent cells.

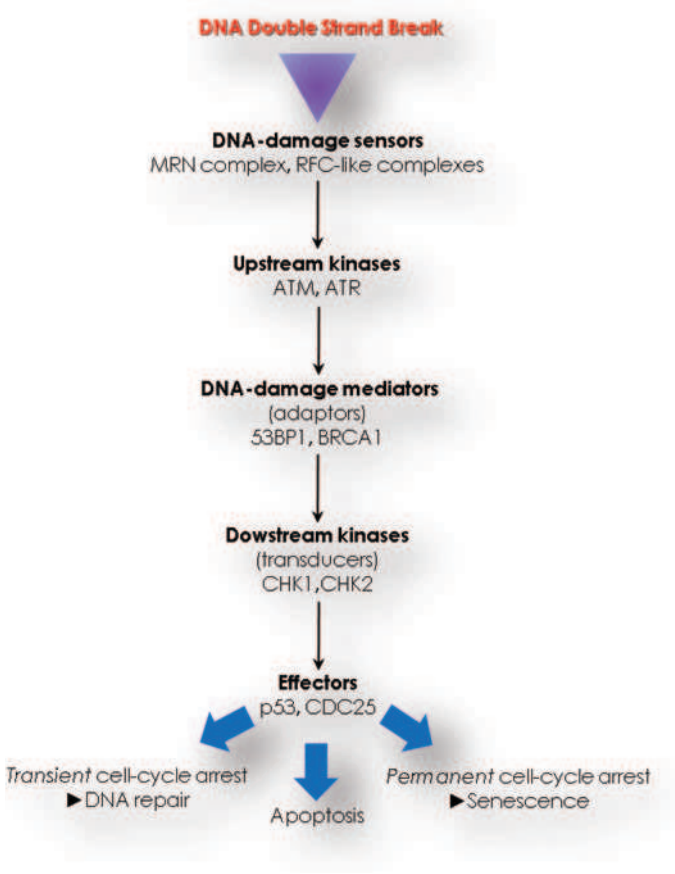

Fig. 3 | **The DNA-damage response.** DNA damage in the form of DNA double-strand breaks and other DNA discontinuities is thought to be *sensed* by a host of factors such as replication factor C (RFC)-like complexes (which contain the cell-cycle-checkpoint protein RAD17) or by the MRN protein complex (MRE11-RAD50-NBS1). Detection of DNA damage then leads to the activation of upstream protein kinases such as ataxia telangiectasia mutated (ATM) and ATM and Rad-3 related (ATR), which trigger immediate events such as phosphorylation of the histone variant H2AX. The modified chromatin recruits multiple proteins. Some of these proteins augment signalling by the upstream kinases, participate in transducing the damage signal and optimize repair activities by other proteins, the recruitment of which depends on the nature of the damage and position in the cell cycle. Several adaptor proteins, including 53BP1 and BRCA1, orchestrate the orderly recruitment of DNA-damage response proteins, as well as the function of downstream kinases such as checkpoint-1 (CHK1) and CHK2, which propagate the damage signal to effector molecules such as CDC25 and the tumour suppressor p53. The effector molecules halt cell-cycle progression, either transiently – enabling DNA repair if the damage was not severe – or permanently (senescence), or else trigger cell death (apoptosis) (after Campisi and d'Adda di Fagagna, 2007, modified).

Damage-initiated senescence strongly depends on p53 protein and is usually accompanied by increased expression of its direct target gene product $p21^{CIP1/WAF1}$ (encoded by *CDKN1A*) (DiLeonardo *et al.*, 1994). Another protein that is often induced is $p16^{INK4a}$ (encoded by *CDKN2A* or *INK4a*), albeit

with delayed kinetics. Activation of $p16^{INK4a}$ provides a second barrier to cease cell proliferation (Beausejour *et al.*, 2003). The importance of the ARF-p53-$p21^{CIP1/WAF1}$ and $p16^{INK4a}$-Rb pathways will be discussed further in the Introduction.

Many chemotherapeutic drugs cause DNA damage. As expected, those drugs, administered at low concentrations (high concentrations activate apoptotic death response), induce senescence in normally proliferating untransformed cells. Surprisingly they also induce senescence in some tumour cells, both in culture and *in vivo* (Roninson, 2003).

1.2.1.1. Telomere-dependent (replicative) senescence

Telomere shortening provided the first molecular evidence of growth arrest of human cells in culture (Harley *et al.*, 1990; Bodnar *et al.*, 1998). Dysfunctional telomeres trigger senescence through the p53 pathway (Herbig *et al.*, 2004). This response is termed telomere-dependent cellular senescence. This type of senescence occurs only in human fibroblasts, which enter senescence before they actually acquire critically short telomeres, therefore avoiding genomic instability leading to cancer.

Telomeres are specialised functional complexes that protect the ends of eukaryotic chromosomes. Those stretches of repetitive DNA (5' – TTAGGG – 3' in vertebrates) and associated proteins that cap the ends of linear chromosomes protect them from degradation or fusion by DNA-repair process (McClintock, 1941; Blackburn, 2001; de Lange 2002). Their name is derived from the Greek nouns *telos* meaning *end* and *meros* meaning *part*. Telomeres can be thought of as the aglet of a shoelace, which is the little plastic bit on the end to protect it from fraying, just as the telomere regions prevent DNA loss at chromosome ends (Elizabeth Blackburn on telomeres). The exact telomeric structure remains unknown, but mammalian telomeres are believed to end in a large circular structure, termed a t – loop (Griffith *et al.*, 1999). They are maintained by telomerase, a specialised ribonucleoprotein complex that includes an RNA template (TERC) and a reverse transcriptase catalytic subunit (TERT).

Linear DNA molecules such as those of eukaryotic chromosomes require mechanisms besides the conventional DNA polymerases to complete the replication of their ends. It was predicted that without such a mechanism, terminal attrition of chromosomal DNA would eventually lead to loss of genetic information and prevent cells from multiplying. In most eukaryotes, this problem is solved by having the ribonucleoprotein enzyme telomerase, which lengthens terminal regions of telomeric DNA by RNA-templated addition of tandemly repeated, often G-rich, telomeric sequences (Greider and Blackburn, 1985; Yu *et al.*, 1990). Although telomeric sequences vary between species, fundamentally, telomeres and telomerase function very similarly throughout the eukaryotes. Because standard DNA polymerases cannot completely replicate DNA ends, cells lose 50-200 base pairs of telomeric DNA during each S phase of the cell cycle (Fig. 4) (Harley *et al.*, 1990). Dysfunctional telomeres trigger a classical DNA damage response (DDR): biochemical analyses revealed that human diploid fibroblasts (HDFs), which entered replicative senescence, display molecular markers characteristic of cells bearing DNA DSBs (d'Adda di Fagagna *et al.*, 2003; Takai *et al.*, 2003, Herbig *et al.*, 2004). These markers of the DDR include phosphorylated γ-H2AX, p53-binding protein 1 (TP53BP1), NBS1, MDC1 and CHK2. Many of these proteins localise directly to dysfunctional telomeres to form dysfunctional telomere-induced foci, and their inactivation in senescent cells restores cell cycle progression into S-phase (Takai *et al.*, 2003). These results suggest that dysfunctional telomeres are indeed recognized as DSBs that impinge on p53 and/or RB tumour suppressor pathways to induce expression of their regulators $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ to initiate replicative senescence (Sage *et al.*, 2003).

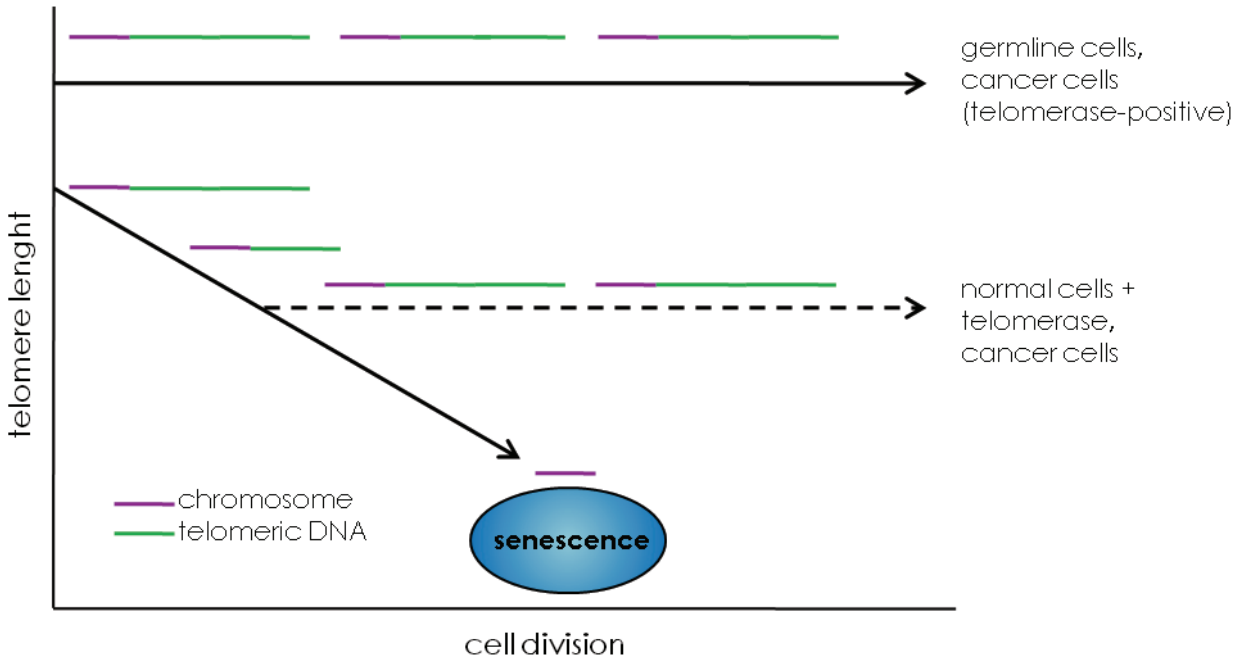

Fig. 4 | **Telomere-initiated cellular senescence.** Telomeres are regions of repetitive DNA and associated proteins at the end of a chromosome. Telomere lengths are maintained by the enzyme telomerase, which is expressed by cells comprising the germline as well as many cancer cells. Most normal human somatic cells do not express telomerase. In such cells, telomere lengths decline with each cell division cycle. Finally, one or a few telomeres become sufficiently short to trigger a DDR, to which cells respond by undergoing senescence (after Campisi and d'Adda di Fagagna, 2007).

Functional inactivation of p53 and Rb by antisense oligonucleotides (Hara *et al.*, 1991) or by viral oncoproteins (Shay *et al.*, 1991) can extend replicative potential in HDFs, driving further telomere erosion and culminating in a period of massive cell death and rampant genomic instability that is termed crisis. Crisis serves as a second, p53-independent checkpoint in tumour suppression and is characterised by multiple chromosomal fusions (Counter *et al.*, 1992). Virally transformed human cells that eliminate p53 and/or Rb function can escape crisis at low frequencies, and invariably adopt telomere maintenance programmes: 80-90% of human tumours have some telomerase activity, and the remainder maintain telomeres by a recombination-mediated process termed ALT (alternative lengthening of telomeres). Indeed, the end-replication problem can be circumvented by telomerase. Most normal somatic cells do not express TERT, or express it at levels which are too low to prevent telomere shortening (Masutomi *et al.*, 2003). A thorough analysis of primary human samples has showed modest levels of telomerase activity in proliferative tissues with high renewal potential

such as the bone marrow, skin, gastrointestinal tract and testis as well as in activated lymphocytes (Yui *et al.*, 1998, Liu *et al.*, 1999; Forsyth *et al.*, 2002). On the other hand, a robust telomerase expression is observed in human embryonic stem cells (Takahashi *et al.*, 2007) and cancer cells (Hahn *et al.*, 1999; Remes *et al.*, 2000). Ectopic expression of TERT in human fibroblasts prevents telomere shortening and consequently circumvents replicative senescence (Bodnar *et al.*, 1998). It is worth mentioning that telomerase does not prevent senescence caused by DNA damage or other senescence-inducing factors (Chen *et al.*, 2001). This point is illustrated by the behaviour of many mouse cells. Contrary to human cells, cells from laboratory mice possess long telomeres (>20 kb). Nevertheless, many mouse cells enter senescence after only a few doublings under standard culture conditions. This arrest is caused by the supraphysiological oxygen level (20%) used in standard culture protocols. Mouse cells are much more sensitive to the classical cell culture conditions (20% oxygen) than human cells, a concentration that is sufficient to cause oxidative stress damages that trigger efficient senescence. Consistent with this notion, it was shown that culturing primary murine cells in low oxygen conditions increases very significantly the lifespan of such cells (see below).

1.2.1.2. Senescence caused by oxidative stress

Reactive oxygen species (ROS) have many cellular functions such as second messenger, anti-bacterial agent, mutagen, aging accelerant and growth stimulant. With regard to neoplasia, the view has generally been that ROS cause cancer through a number of mechanisms, including the induction of DNA damage and alteration of intracellular signaling (Finkel, 2003). Since the introduction of the 'Free Radical Theory of Aging' by Harman over 50 years ago, oxidants have been inextricably linked to aging. Senescence, which many, but not all, view as an *in vitro* recapitulation of cellular premature aging, appears to represent an interesting and testable model for the role of ROS in the aging process. In many ways experiments performed over the last several decades have validated the link between oxidants and senescence. Increasing intracellular oxidants by altering ambient oxygen

concentrations or lowering antioxidant levels, accelerates the onset of senescence (Fig. 5), while lowering ambient oxygen concentration or increasing ROS scavenging appears to delay senescence.

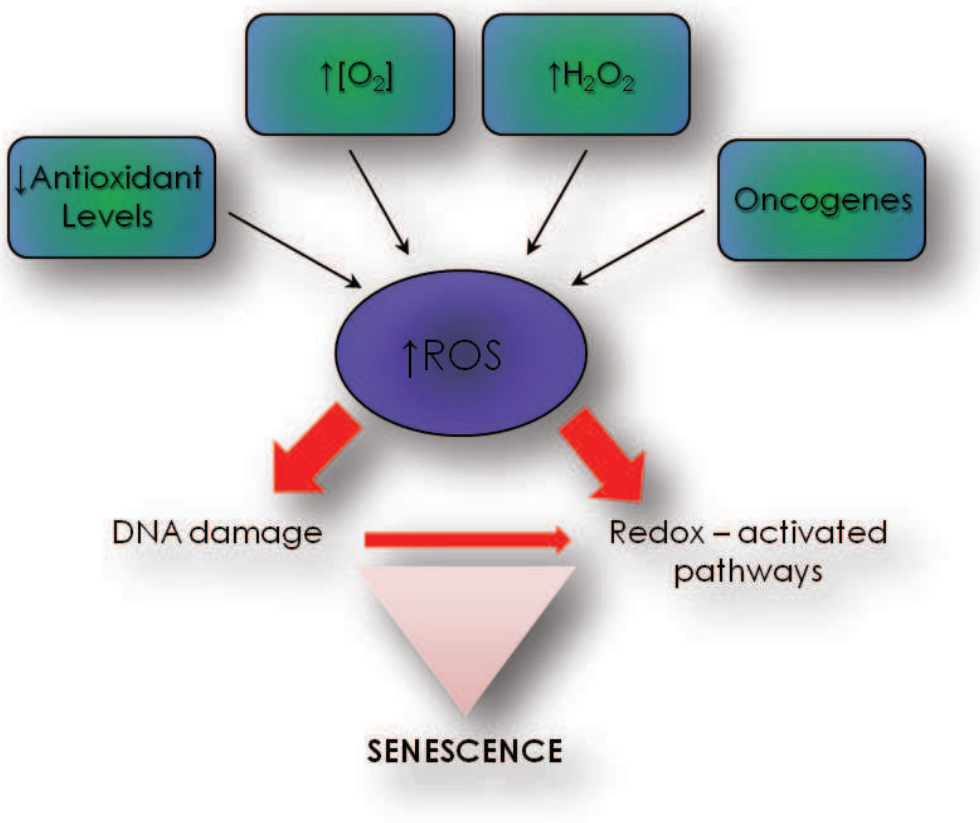

Fig. 5 | **ROS as a common mediator of cellular senescence.** Various intrinsic or extrinsic signals, such as changing the intracellular antioxidant status, ambient oxygen concentration, cell exposure to hydrogen peroxide or the expression of certain oncogenes, produce a rise in intracellular ROS levels. Increase in ROS within cells follows and triggers senescence entry. Oxidants may directly activate certain redox-sensitive pathways linked to senescence. Alternatively, oxidants might non-specifically induce a spectrum of damage to cellular components (such as DNA) that directly leads to senescence or this damage might induce senescence by secondarily activating important intracellular pathways, such as DDR (after Lu and Finkel, 2008).

Most cells in our body are exposed to oxygen concentration closer to 5% rather than the 20% of ambient air. Using this knowledge, Packer and Fuehr provided the first systematic evaluation of the effect of ambient oxygen on senescence (Packer and Fuehr, 1977). Using the human diploid fibroblast strain WI-38, Packer and Fuehr demonstrated that the cumulative number of population doublings was not a fixed number but rather was influenced by the amount of ambient oxygen. Compared to cells grown in the standard tissue culture environment of 20%, cells grown in 10% oxygen had

considerably more population doublings (on average 20–30% more) whereas cells grown in a 50% oxygen environment had a similarly reduced *in vitro* lifespan. These results suggested that oxidative damage might be limiting for cell growth in culture. Nowadays, there has been visible refinement in our ability to define and understand the molecular events occurring with oxygen exposure. Culture of primary mouse cells at 20% versus 3% oxygen has revealed that cells cultured at higher oxygen concentration entered senescence, while those maintained at 3% did not (Busuttil *et al.*, 2003; Parinello *et al.*, 2003). Those experiments also demonstrated that MEFs grown in the standard 20% oxygen exhibited 3-4-fold higher levels of oxidatively damaged DNA than cells grown at 3% (Parinello *et al.*, 2003). Furthermore, mouse cells grown at 20% oxygen exhibit more damage than human cells, which are cultured at this same oxygen concentration, suggesting important species differences in oxygen sensitivity. Clearly, another important difference between mouse and human cells is the length of their telomeres. While telomere attrition is not felt to be limiting for the growth of primary mouse cells, the loss of telomere length is felt to be the predominant mechanism for human replicative cell senescence. In this regard it is interesting to note that telomere shortening is also dependent on ambient oxygen concentration. For instance, evidence suggests that under normoxic conditions, telomeres shorten by roughly 90 base pairs per population doubling, while the same cells grown in 40% oxygen reduce their telomeres at a rate of nearly 500 base pairs per division (von Zglinicki *et al.*, 1995). With the above examples one can conclude that non-physiological oxygen concentration limits cellular lifespan *in vitro*. Exposure of cells to direct oxidative stress, such as sub-lethal hydrogen peroxide concentrations, produce a senescent-like state wherein the treated cells remain viable but become unresponsive to growth factors (Chen and Ames, 1994). Another link between intracellular ROS levels and cellular senescence came from observations where the levels of intracellular antioxidants varied. For instance, the addition of lipophillic spin trapping chemical antioxidant α-phenyl-t-butyl nitrone (PBN) extended the lifespan of human cells in culture (Chen *et al.*, 1995). Other experiments demonstrated that human fibroblasts engineered to have increased expression of

extracellular superoxide dismutase (SOD) showed an extension of lifespan under both normoxic and hyperoxia conditions (Serra *et al.*, 2003). This extension appeared to be predominant through a reduction in the rate of telomere attrition. The converse experiments have also been performed. For instance, RNAi mediated knockdown of SOD1 (the copper–zinc cytosolic form of the enzyme) in primary human fibroblasts induced a rapid senescent arrest (Blander *et al.*, 2003).

Another factor, which contributes to overproduction of ROS and therefore onset and establishment of senescence, is the constitutive expression of oncogenes such as RAS. It was demonstrated that in primary cells, increased RAS expression results in an increase in intracellular ROS levels (Lee *et al.*, 1999). This rise in ROS appears to be important for inducing cellular senescence because culturing RAS-expressing cells in either low oxygen or treating these cells with a hydrogen peroxide scavenging agent such as N-acetylcysteine (NAC) delayed RAS-induced senescence (Lee *et al.*, 1999). It remains unclear how ROS exactly function in the case of RAS-induced senescence, as well as how RAS expression triggers a rise in ROS levels within cells.

1.2.1.3. Oncogene-induced senescence (OIS)

Oncogene-induced senescence is a mechanism that restricts tumour progression during the early steps of cellular transformation. Oncogenes are genes which, when mutated or constitutively expressed at high levels, have a potential to transform cells in conjunction with additional mutations (Land *et al.*, 1983). The prototypic oncogene is H-RAS that was first identified in 1982 as a human oncogene in human bladder and lung carcinoma cell lines. Activated RAS proteins were shown to be capable of transforming immortalized rodent cells (Der *et al.*, 1982; Parada *et al.*, 1982). However, even at that time, additional cooperating genetic alterations (such as the c-Myc, or the viral oncoproteins E1A or the SV40 large T antigen) were shown to be required for RAS to transform primary cells (Land *et al.*, 1983). Studies described in 1997 provided an explanation for this finding, when it was shown

that in wild-type cells, activated RAS actually triggered an initial wave of proliferation, followed by an irreversible growth arrest known as oncogene-induced senescence (OIS) (Serrano *et al.*, 1997). Initially, this phenomenon was first observed when an oncogenic form of H-*RAS* (harbouring the constitutively activating mutation on Valine 12) was expressed in rat embryo fibroblasts (Hirakawa and Ruley, 1988). Subsequently, other members of the RAS signalling pathway (such as RAF or MEK) as well as pro-proliferative nuclear proteins (for example E2F1) were shown to cause senescence when over-expressed or expressed as oncogenic versions (Zhu *et al.*, 1998; Lin *et al.*, 1998; Dimri *et al.*, 2000).

Because oncogenes that induce senescence stimulate rapid cell proliferation (which even accompanies loss of contact growth inhibition, a hallmark of cancer), the senescence response seems to counteract excessive mitogen stimulation that puts cells at risk of oncogenic transformation (Fig. 6). This initial burst of proliferation is followed by the activation of two tumour suppressor pathways, the p53 and Rb pathways (Fig. 7). Accordingly, RAS-induced senescence can be bypassed by functional inactivation of the Rb and p53 pathways, suggesting that this response may have evolved as a mechanism of tumour suppression. Consistent with that notion, RAS-expressing murine cells that harbour mutation of the p53 tumour suppressor are fully transformed, as shown by their ability to develop tumours *in vivo* upon injection in nude mice (Lin and Lowe, 2001).

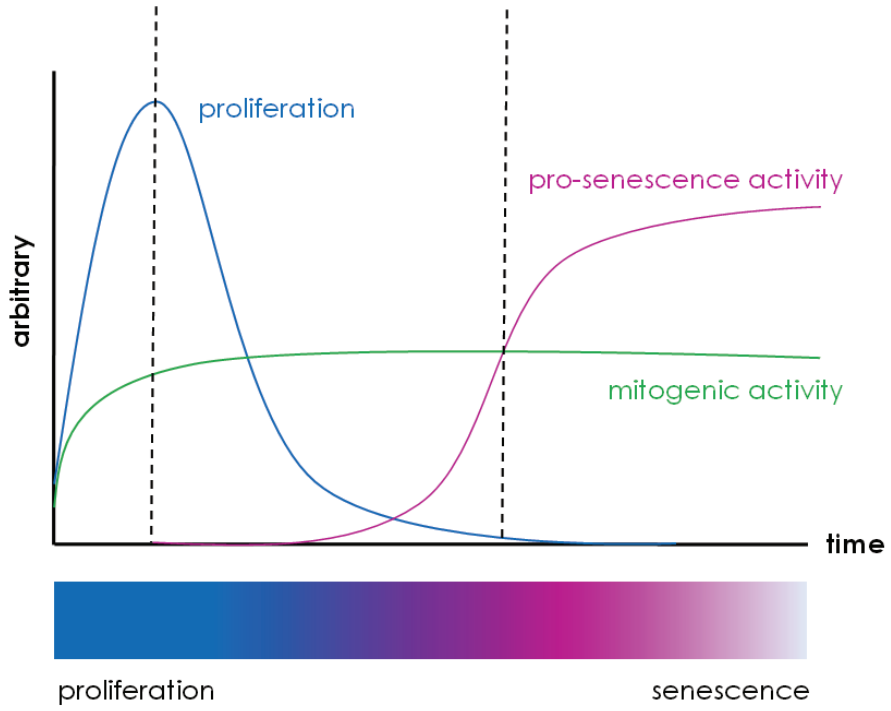

Fig. 6 | **Oncogene-induced senescence.** Constitutively active mitogenic stimuli induce rapid cell proliferation, but the senescence machinery is triggered and eventually overcomes the mitogenic activity (after Narita, 2007; modified).

Oncogene-induced senescence does not entail telomere shortening but many oncogenes induce a robust DDR owing to the aberrant DNA replication and/or by an oncogene-driven accumulation of reactive oxygen species (ROS) (Lee *et al.*, 1999) (Fig. 7). Di Micco *et al.* (2006) have shown that senescent cells stall in S phase, have an augmented number of active replicons and exhibit defects in DNA replication fork progression, resulting in an activation of ATR and ultimately ATM. Oncogene-induced senescence has also been proposed to be triggered by the accumulation of ROS. Consistent with this hypothesis, OIS can be bypassed in murine cells by growing cells in low oxygen (Lee *et al.*, 1999; Parrinello *et al.*, 2003; MacLaren *et al.*, 2004). Notably, ROS scavengers can suppress senescence in some systems, and inactivation of the *seladin-1* gene, which has been proposed to be a sensor of ROS, is also sufficient to bypass RAS-induced senescence in BJ fibroblasts (Lee *et al.*, 1999; Wu *et al.*, 2004). Despite these findings, not all oncogenes increase ROS (for example Myc; Ray *et al.*, 2006), and it is still unknown whether a wild-type activated RAS allele is capable of inducing sufficiently high levels of ROS.

It was further discovered that cells exposed to an oncogenic stress evoke a global negative feedback response that potently suppresses RAS and/or its effectors suggesting that cells might be attempting to neutralise the aberrant signal (Courtois-Cox *et al.*, 2006). This suppression appeared to be primarily regulated by post-translational events, such as phosphorylation and inhibition of RAS activators.

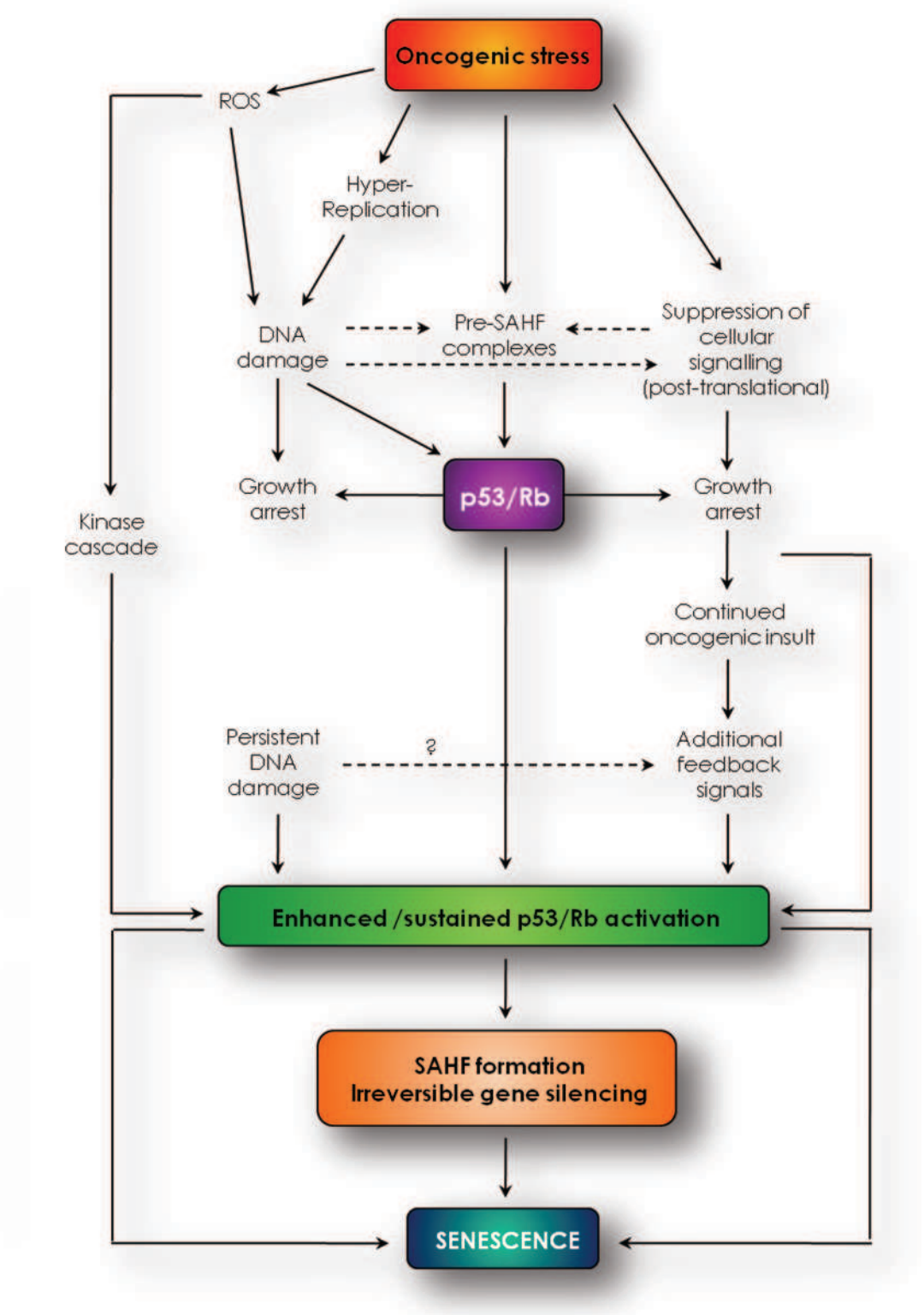

Fig. 7 | **An integrated model of oncogene-induced senescence.** Replicative stress, ROS, suppression of intracellular signalling pathways and chromatin remodelling via SAHF (senescence-associated heterochromatic foci) formation have all been proposed to regulate the senescence response. The scheme illustrates how these signals may cooperate to do so. All senescence triggers can activate Rb and p53 and multiple pathways may be required to cooperate to achieve a threshold and/or sustained activation of Rb and p53. Once this occurs, chromatin remodeling may 'lock in' the growth arrest.

1.3. Senescent phenotype

Multicellular organisms contain both mitotic (dividing) and post-mitotic (non-dividing, differentiated) cells. Mitotic cells have the capacity to proliferate (divide) and they include epithelial, stromal (fibroblastic) and endothelial cells that constitute essential renewable tissues and organs, such as skin, intestines and liver. Dividing cells are also present as major components of haematopoietic systems and cells such as glia, and include undifferentiated stem and progenitor cells that provide many tissues with the differentiated cells. As mitotic cells are prone to transformation into cancer cells they are also predisposed to undergo cellular senescence when challenged by stimuli that have a tumorigenic potential. Albeit mitotic cells are capable of proliferation, they can also spend long intervals in a reversible state of cell cycle arrest termed *quiescence* or G0. Such cells can be stimulated to re-enter cell cycle in response to physiological signals.

Table 1 | **Senescence, differentiation and quiescence – comparison.**

	SENESCENCE	DIFFERENTIATION	QUIESCENCE
Stability	permanent*	permanent	reversible
Induced by:	telomere shortening, severe DNA damage, oncogene activation, oxidative stress	many different stimuli depending on the cell type and tissue origin	serum starvation, growth factor deprivation, culture at high confluency, mild DNA damage
SABG activity	present	Absent**	absent***
SAHFs	present in senescent human cells	absent, but presence of facultative heterochromatin	absent
Cell cycle inhibitors	p53, $p21^{CIP1/WAF1}$, ARF, Rb, $p16^{INK4a}$	$p21^{CIP1/WAF1}$, $p27^{KIP1}$, $p57^{KIP2}$	$p21^{CIP1/WAF1}$, Rb, $p27^{KIP1}$
Molecular markers	uniducible c-fos expression to serum stimulation	uniducible c-fos expression to serum stimulation	inducible c-fos expression to serum stimulation

* only when ARF-p53-$p21^{CIP1/WAF1}$ and $p16^{INK4a}$-Rb pathways are functional and intact
** TGF-β increases SABG staining, SABG used as a marker of differentiation of human prostate epithelial cells.
*** In the case of high confluency cells stain positive for SABG staining

Post-mitotic cells such as differentiated neurons and some muscle cells (i.e. cells that constitute skeletal muscles, excluding satellite cells) are incapable of proliferation. Because they have already lost the ability to divide, post-mitotic cells do not undergo cellular senescence as currently defined. Differentiated and senescent cells are irreversibly blocked from re-entering cell cycle divisions contrary to cells which are in the state of quiescence (Table 1). The senescent phenotype is represented by a few changes that are neither *universal* nor *exclusive* to the senescent state. Apart from the permanent cell-cycle arrest discussed above, senescent cells often become resistant to cell-death signals (Wang, 1995; Seluanov *et al.*, 2001; Yeo *et al.*, 2000; Rochette and Brash, 2008), they acquire widespread changes in cell morphology and metabolism, gene expression and chromatin organisation (Fig. 8).

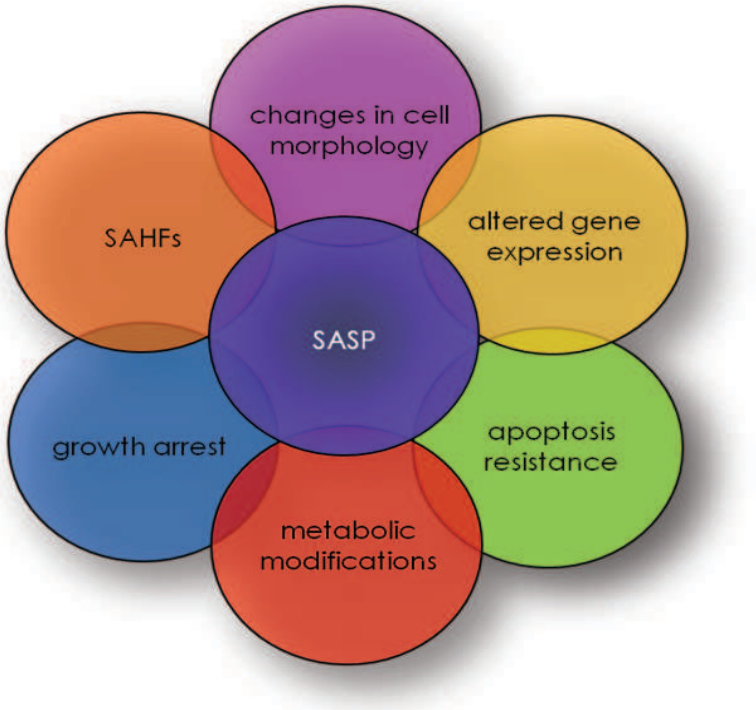

Fig. 8 | **The senescent phenotype.** Initially mitotic cells respond to various stress stimuli by undergoing cellular senescence. The senescence response causes striking changes in the cellular phenotype: permanent arrest of proliferation, modifications of cell morphology, resistance to apoptosis (in some senescent cells), altered pattern of gene expression, expression of a SABG – a proof of metabolic changes that occurred in the cell, and SAHFs and existence of SASP (senescence-associated secretory phenotype) (from Campisi and d'Adda di Fagagna, 2007; modified).

1.3.1. Growth arrest, cell morphology and metabolism

The hallmark of cellular senescence is the permanent, irreversible (in the absence of experimental manipulation) cell cycle arrest, usually with DNA content typical of G1 phase (DiLeonardo *et al.*, 1994; Serrano *et al.*, 1997). This replication failure is primarily caused by the expression of dominant cell cycle inhibitors (for more details on those inhibitors see following chapters).

The features and stringency of the senescence growth arrest vary depending on the species and genetic background of the cell. For example, most mouse fibroblasts senesce with G1 DNA content, however a defect in the stress-signalling kinase MKK7 primary induces G2-M arrest (Wada *et al.*, 2004). Similarly, some oncogenes cause a fraction of cells to senesce with a G2-phase content (Olsen *et al.*, 2002; Di Micco *et al.*, 2006). Additionally, some tumour cells, those which retain the ability to undergo senescence, tend to arrest in G2- or S-phase of the cell cycle (Oliva *et al.*, 2008). Finally, human and rodent cells differ strikingly in the stringency of the senescence growth arrest (Itahana *et al.*, 2004) (discussed in a separate chapter).

Senescent cells undergo distinctive changes in morphology to become enlarged, flattened and granular but remain viable and metabolically active for long periods of time in culture (Matsumura *et al.*, 1979; Goldstein, 1990). Those metabolic changes are illustrated by the increase in senescence-associated β-galactosidase (SABG) activity (Dimri *et al.*, 1995), one of the most widely used senescence markers.

1.3.2. Apoptosis resistance

Apoptosis is one of the processes that triggers programmed cell death (PCD) in multicellular organisms (Ellis *et al.*, 1991). Similarly to senescence, apoptosis is an extreme response to cellular stress and is a powerful tumour-suppressive mechanism. While senescence blocks the growth of damaged or stressed cells, apoptosis literally eliminates them. Some human cell types acquire resistance to certain apoptotic signals when they become senescent. For instance, senescent human fibroblasts resist ceramid-induced

apoptosis but endothelial cells do not (Hempel *et al.* 2004). Senescent human fibroblasts also evade apoptosis induced by growth factor deprivation and oxidative stress, but remain sensitive to apoptosis induced by the engagement of the Fas death receptor (Tepper *et al.*, 2000). The resistance of senescent cells to apoptotic cell death explains their overall stability in cell culture. This feature might also explain senescent cell accumulation in organisms with age although it is not an exclusive factor that contributes to this phenomenon.

Although most cells are capable of both responses, it is not clear what determines whether cells undergo senescence or apoptosis. One determinant is cell type, for example, damaged fibroblasts and epithelial cells tend to senesce whereas damaged lymphocytes are more likely to undergo apoptosis. The nature and intensity of stress might also contribute to senescent cell fate (Rebbaa *et al.*, 2003; Seluanov, 2001). Furthermore, manipulation of pro- and anti-apoptotic proteins can cause a change in cells' original destiny (Rebbaa *et al.*, 2003; Crescenzi *et al.*, 2003).

In conclusion, it is likely that both mechanisms – apoptosis and senescence – *communicate*. One common regulator of these two processes is the p53 tumour-suppressor protein. The mechanism of apoptosis resistance of senescent cells remains poorly understood. In some cells, this resistance might be due to expression changes in proteins that inhibit, promote or execute apoptotic cell death (Marcotte *et al.*, 2004; Murata *et al.*, 2006). In other cases, p53 might act as a *router* of cellular response and preferentially transactivate genes that arrest proliferation rather than those that promote apoptosis (Jackson and Pereira-Smith, 2006).

1.3.3. Altered gene expression

Cellular senescence is accompanied by many changes in gene expression (Linskens *et al.*, 1995; Shelton *et al.*, 1999; Zhang *et al.*, 2003; Mason *et al.*, 2004; Yoon *et al.*, 2004; Jackson and Pereira-Smith, 2006; Troukagos *et al.*, 2006), some of which cause permanent growth arrest but also

characteristic alterations in cellular morphology and function. These changes tend to be highly cell-type-specific.

p53 and Rb tumour-suppressor pathways play essential roles in mediating the growth arrest in senescence (reviewed in Campisi, 2003). CDK inhibitors, which act on the Rb pathways, are often up-regulated in senescent cells. DNA microarray-based profiling of senescent cells demonstrated that the transcripts of the cell cycle inhibitors $p16^{INK4a}$, $p15^{INK4b}$ and $p21^{CIP1/WAF1}$ were all significantly up-regulated in oncogenic RAS-induced senescent human fibroblasts (IMR90) (Mason *et al.*, 2004). Accumulation of *p16*^{INK4a} mRNA was also observed in senescent TIG-1 and TIG-2 human fibroblast strains (Hara *et al.*, 1996) and HDFs from newborn foreskin, NHF1, MRC-5 and WI-38 strains (Alcorta *et al.*, 1996). No up-regulation of *p16*^{INK4a} was observed in BJ human fibroblasts, HUVEC endothelial cells and RP340 strain (Shelton *et al.*, 1999). In senescent chicken embryonic fibroblasts (CEFs) an increase in $p15^{INK4b}$ mRNA levels was observed contrary to ARF RNA levels (Kim *et al.*, 2006).

Moreover, the expression of genes encoding for $p18^{INK4c}$, $p27^{KIP1}$ and $p57^{KIP2}$ are often decreased (Mason *et al.*, 2004; Alcorta *et al.*, 1996). *E2F1* gene and many of the E2F target genes, including *cyclin E*, *PCNA* and *MCM6*, are also down-regulated in senescent IMR90 cells (Mason *et al.*, 2004). In addition, gene expression analysis revealed that *cdc2*, *cyclin B1*, *cdc25c* and *PLK-1* are among several G2/M genes that are significantly down-regulated in RAS-induced human fibroblasts. Additionally, mitotic genes such as *DDX11* (*CHL-1like*), *BUB1B, CENPA, CENPF* and topoisomerase IIa are also dramatically down-regulated.

Other DNA microarray analyses showed that such genes as *DcR2* (decoy death receptor 2), *DEC1* (differentiated embryo-chondrocyte) were up-regulated in premalignant neoplastic lesions of the skin, lung and pancreas but not in malignant tumours (Collado *et al.*, 2005). These markers were tested in several mouse models of OIS and they are specific for this type of senescence. The high levels of these markers correlated well with the presence of SABG activity and SAHFs. The functional involvement in senescence of *DcR2* (Nagane *et al.*, 2001) and of *DEC1* (Giatromanolaki *et*

al., 2003) is currently unknown. *DcR2* is a p53-induced gene and is one of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) decoy receptors. It suppresses TRAIL-induced apoptosis and has been shown to regulate chemosensitivity (Liu *et al.*, 2005). *DEC1* is a member of basic helix-loop-helix family of transcription factors and it has been shown to be involved in the control of circadian rhythms, but it also seems to be involved in many other signalling pathways (Yamada *et al.*, 2005).

Plasminogen activator inhibitor 1 (PAI-1), an inhibitor of the activity of the urokinase-type plasminogen activator protease, uPA, was also demonstrated to be up-regulated during replicative senescence (Mu *et al.*, 1995; Kortlever *et al.*, 2006) and during RAS-induced senescence (Serrano *et al.*, 1997). *PAI-1* is also a p53 target gene and *PAI-1* knockdown results in escape from replicative senescence both in primary mouse embryo fibroblasts and primary human BJ fibroblasts (Kortlever *et al.*, 2006).

Investigation of the signalling pathways leading to OIS has revealed several important players that, similar to $p16^{INK4a}$ or ARF, are involved in the initiation of senescence, such as promyelotic leukaemia protein (PML) or the transcription factors ID1, ETS1 and ETS2, JUN and DMP1. However, the value of these molecules as markers of senescence is limited because downstream alterations can cancel their effects on senescence.

1.3.4. Senescence-associated secretory phenotype (SASP)

Senescent human (Coppé *et al.*, 2008) and mouse (Coppé *et al.*, 2010) cells secrete many biologically active proteins, a phenotype referred to as the senescence-associated secretory phenotype (SASP) (Coppé *et al.*, 2008; Coppé *et al.*, 2010). This phenotype has also been termed the senescence messaging secretome (SMS) (Kuilman and Peeper, 2009). The SASP represents one of the darkest sides of the senescence response, since senescent cells that develop altered secretory activities may have either beneficial or deleterious effects, depending on the cell type and the age of the organism (Campisi, 2005). On the one hand, SASP can provoke changes in the tissue microenvironment, thereby relaxing its control over cell behaviour and

promoting tumorigenesis (Krtolica *et al.*, 2001; Green, 2008; Kuilman *et al.*, 2008). It was shown that some secretory molecules, such as HGF/SF, FGF7/KGF, IGFBPs, AREG, IL-1α, IL-1β, MCP-1 and some matrix metalloproteinases, can promote malignant phenotypes in nearby premalignant cells (Krtolica *et al.*, 2001; Bavik *et al.*, 2006; Liu and Hornsby, 2007). On the other hand, some SASP factors might be beneficial. For example, a number of them reinforce the senescence growth arrest in an autocrine manner (Acosta *et al.*, 2008; Kortlever *et al.*, 2006; Wajapeyee *et al.*, 2008). Others may allow damaged cells to communicate their compromised state (Rodier *et al.*, 2009) in order to stimulate tissue repair or limit pathology (Krizhanovsky *et al.*, 2008).

The potential existence of the SASP was already suggested by large-scale comparative gene (mRNA) expression studies performed on fibroblasts from different-aged donors and different tissues of origin. Among the cells that have been shown to senesce and secrete biologically active molecules are liver stellate cells (Schnabl *et al.*, 2003), endothelial cells (Chen *et al.*, 2002), and epithelial cells of the retinal pigment, mammary gland, lung and prostate (Shelton *et al.*, 1999; Schwarze *et al.*, 2002; Zhang *et al.*, 2003). Senescence-associated changes in gene expression are specific and mostly conserved within individual cell types. Most differences between the molecular signatures of presenescent and senescent cells entail cell-cycle and metabolism-related genes, as well as genes encoding the secretory proteins that constitute the SASP. The SASP includes several families of soluble and insoluble factors, some of which are described in Table 2.

Table 2 | **The senescence-associated secretory phenotype (SASP) factors. Model systems, properties and involvement in senescence.**

SASP factor(s)	Cell system	*In vitro*	links	*In vivo*
IGF1	Murine skeletal muscle satellite	IGF1 transgene allows lifespan extension	PI3K activation p27 inactivation	ND
IGF2 and IGF2R	Murine fibroblast (MEF)	IGF2R KO and IGF2 transgenic parthenogenetic MEF show increased long-term proliferation IGF2 KO biparental MEF show decreased long-term proliferation	Possible link to CDK inhibitors	ND
IGFBP3	Human endothelial (HUVEC)	IGFBP3: induced in REP senescence, sufficient and required for senescence	Bypass of REP senescence due to $IGFBP3^{KD}$ associated with lower p53 and p21, and ↑ FOXO3A levels	IGFBP3 expression increases with age
IGFBP5	Human endothelial (HUVEC)	IGFBP5: upregulated in REP senescence, sufficient and required for senescence	Bypass REP due to $IGFBP5^{KD}$ associated with lower p53 and p21, and ↑ FOXO3A levels	ND
IGFBP7	Human melanocyte Human fibroblast (BJ)	IGFBP7: up-regulated in OIS ($BRAF^{E600}$), sufficient and required for senescence	Bypass OIS due to $IGFBP7^{KD}$ associated with ↓ INK4a and p-ERK and ↑ Ac-H3K9 levels	*IGFBP7* promoter methylation in $BRAF^{E600}$ melanoma ↑ expression of IGFBP7 in nevi
PAI1	Murine fibroblast (MEF) Human fibroblast (BJ)	PAI1 up-regulated and sufficient for senescence PAI1 KO and KD and uPA expression + manipulation of PI3K, AKt, GSK3β and cyclin D1 allows to bypass senescence	REP senescence-associated with lower PI3K, Akt and nuclear cyclin D1, ↑GSK3β activity and levels p53-independent	ND
TGFβ	Murine keratinocytes expressing v-ras	TGFβ up-regulated in replicative senescence TGFβ KO, neutralizing ab or DN-	Bypass of REP senescence due to absence of TGFβ	*Tgfb1*$^{-/-}$ v-ras keratinocyte xenografts often

		TβR allows to bypass senescence TGFβ exposure increases SABG activity	associated with INK4b and INK4a down-regulation; ARF and p53 unaffected	progress to malignant carcinoma
WNT2	Human fibroblasts (WI-38) Human epithelial (RPE)	Repression of WNT2 in REP senescence and OIS ($HRAS^{V12}$) WNT2 KD increases amount of SAHF and HIRA foci WNT3A exposure inhibits the formation of SAHF and HIRA and increases proliferation	WNT2 repression occurs independently of INK4a and p53	ND
IL-6 and IL-8	Human fibroblast (Tig3 and IMR90)	IL-6 and IL-8 upregulated in OIS ($BRAF^{E600}$) IL-6, IL-6R, IL-8 and C/EBPβ KDs allow bypass of OIS	OIS bypass associated with collapse of inflammatory transcriptome and SAHF, and lower INK4b levels C/EBPβ is an essential regulator of IL-6	IL-8 expression increased $INK4a^{+}$ non-proliferating human colon adenoma cell stretches
CXCR2	Human fibroblast (BJ, WI-38 and IMR90)	CXCR2 cytokines up-regulated in REP senescence and OIS ($HRAS^{V12}$/MEK) CXCR2, IL-8 and GRO1 sufficient and required for senescence	NF-κB regulator of CRCX2 cytokines	Senescence markers and CXCR2 and its ligands increase (papillomas) Expression of CXCR2 increases (PIN)

Ab, antibody; Ac-H3K9 lysine 9 acetylation on histone H3; AS, antisense; REP, replicative; KO, knockout; KD, knockdown; ND, not determined; DN, dominant-negative;

1.3.5. Senescence-associated heterochromatin focis (SAHFs)

In most cases, the senescence phenotype progressively accumulates over multiple cell cycles and senescent cells display a specific gene expression profile. These observations suggest an active involvement of epigenetic gene regulation. Consistent with this view, certain types of human cells exhibit senescent arrest that is accompanied by senescence-associated heterochromatin focis (SAHFs), a new type of facultative heterochromatin (Narita *et al.*, 2003). Remarkably, in a senescent cell each senescence-

associated heterochromatin focus results from condensation of an individual chromosome (Zhang *et al.*, 2007).

SAHFs are enriched for markers of heterochromatin, such as heterochromatin protein 1 (HP1) and lysine 9 trimethylated of histone H3 (H3K9me3) and exclude euchromatin markers (Narita *et al.*, 2003) (Fig. 9). Interestingly, SAHF formation is largely dependent on the $p16^{INK4a}$-Rb pathway in Ras-induced senescence; the impact of p53 on SAHF is marginal (Narita *et al.*, 2003). The $p16^{INK4a}$-Rb pathway links SAHF formation and cell cycle gene silencing as heterochromatin markers are accumulated on the promoters of E2F-target genes during senescence (Narita *et al.*, 2003; Rastogi *et al.*, 2006).

Deeper characterisation of SAHFs showed that macroH2A is enriched in senescence-associated heterochromatin foci (Zhang *et al.*, 2005). MacroH2A is a transcriptionally repressive variant of histone H2A which, in mammals, is a marker of inactive X chromosome, a form of facultative heterochromatin (Constanzi and Pehrson, 1998). More recently, some experiments revealed that high-mobility group A (HMGA) proteins are senescence-associated chromatin-binding proteins and that HMGA proteins are essential structural components of SAHFs (Narita *et al.*, 2006).

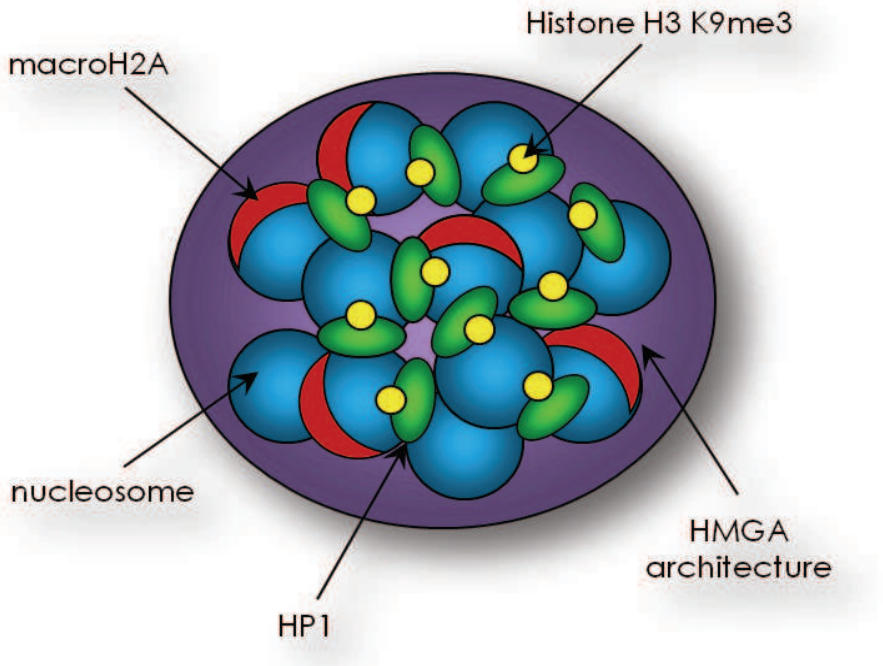

Fig. 9 | **Senescence-associated heterochromatin foci structure.** SAHF contains a variety of chromatin proteins, such as K9-methylated histone H3 and HP1, macroH2A (histone variant) and HMGA proteins (architectural proteins) (after Narita, 2007; modified).

The HMGA1 and HMGA2 proteins are non-histone architectural chromatin proteins and have three 'AT-hook' domains that are responsible for binding DNA (Reeves, 2001). Those proteins have been linked to cellular proliferation and are described as oncogenes (Mantovani *et al.*, 1998; Reeves, 2001); expression of HMGA proteins is induced by growth factors or serum stimulation (Lanahan *et al.*, 1992). Both *HMGA* genes are highly expressed in proliferating cells of the embryo, in many tumours, and are down-regulated upon differentiation (Zhou *et al.*, 1995; Reeves, 2001). It was demonstrated that HMGA proteins can promote tumorigenesis both *in vitro* (Takaha *et al.*, 2004) and *in vivo* (Fedele *et al.*, 2005, 2006) and gene amplifications and translocations occur in many human cancers (Reeves, 2001). Therefore, accumulation of HMGA proteins on chromatin during senescence and their functional association with the senescent phenotype are quite puzzling. Strikingly, knockdown of HMGA1 completely abolishes SAHF architecture, whereas HMGA2 has a lesser impact.

SAHF form through a cascade of temporally and mechanistically separable events; in other words, formation of SAHF is a multi-step process. The earliest detectable event to date is chromosome condensation to form a SAHF focus that is detectable by DAPI staining of DNA. Two chromatin regulators, HIRA and ASF1a, drive chromosome condensation during SAHF assembly in human cells (Zhang *et al.*, 2005). HIRA and ASF1a are the human orthologs of proteins known to create transcriptionally silent heterochromatin in yeast, flies and plants (Kaufman *et al.*, 1998; Moshkin *et al.*, 2002; Phelps-Durr *et al.*, 2005; Sharp *et al.*, 2002; Singer *et al.*, 1998). After HIRA/ASF1a-driven chromosome condensation, histone H3 is methylated on lysine 9, creating a binding site for HP1 proteins and the histone variant macroH2A is incorporated (Zhang *et al.*, 2005). The factors responsible for incorporation of macroH2A are unknown, but are likely to include an ATP-dependent remodeling factor as a histone H2A exchange factor (Bruno *et al.*, 2003; Kobor *et al.*, 2004).

HMGA proteins and histone H1 bind to similar sites on chromatin and can compete with each other for chromatin binding (Catez *et al.*, 2002, 2004). Consequently, Funayama *et al.* (2006) proposed that HMGA proteins

displace histone H1 from SAHF. The time when this occurs in relation to chromosome condensation is not clear, since a comparative kinetic analysis has not been reported. Nevertheless, displacement of histone H1 and deposition of HMGA proteins seems to be a rather early event in the formation of SAHF, perhaps occurring at the same time as, or prior to, chromosome condensation. If so, the mechanistic relationship between HIRA/ASF1a-mediated nucleosome deposition and HMGA recruitment will need to be clarified.

1.4. Cellular senescence and the cell-cycle

Senescence depends on a number of pathways that together result in a permanent and irreversible cell-cycle arrest. Cells harbouring mutations in key regulatory components of these pathways delay or bypass senescence and can proliferate beyond their normal limits (are not limited by Hayflick limit), that is they become immortal.

One of those pathways involves the molecular machinery that controls cell-cycle progression, which relies on the sequential activation of a family of protein kinases, the cyclin-dependent kinases (CDKs) (reviewed by Sherr and Roberts, 1999). When quiescent cells are stimulated to proliferate the first CDKs to be activated are CDK4 and CDK6 (CDK4/6) by association with cyclin D. The activity of the CDK4/6-cyclin D is followed by subsequent activation of the CDK2-cyclin E and CDK2-cyclin A kinases which trigger DNA replication. Upon termination of DNA synthesis, CDK1-cyclin B complex is activated and the cell enters mitosis (cyclin A is degraded when a cell enters early prophase). The activity of CDK-cyclin complexes is under tight regulation. One level of control involves the CDK inhibitors (CKIs), which are of special relevance to senescence: they participate in the establishment and stable maintenance of the cell-cycle arrest, which is characteristic of senescent cells. CKIs belong to two families: INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$) and CIP/KIP family ($p21^{CIP1/WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$). The INK4-type CKIs are responsible for inhibiting CDK4/6 kinases while CIP/KIP proteins effectively block CDK4/6-cyclin D and CDK2-cyclinE/A complexes.

1.4.1. The p16^{INK4a} – Rb pathway

1.4.1.1. p16^{INK4a} and Rb

The retinoblastoma protein (Rb) is one of the main substrates of the CDK4/6 kinases. In its unphosphorylated (or hypophosphorylated) form, Rb associates with several transcription factors in order to silence their transactivation functions. Of great relevance among the Rb-regulated proteins is the E2F multigene family of transcription factors (E2Fs), which activate the expression of important cell cycle proteins such as cyclin E and A at the G1/S transition. Upon phosphorylation by CDK/cyclin complexes, Rb-mediated repression is released, allowing E2Fs to activate DNA replication (reviewed by Kaelin, 1999). By inhibiting CDK4/6 kinases the INK4 CKIs participate in maintaining Rb in its unphosphorylated, growth-suppressive state (Fig. 10).

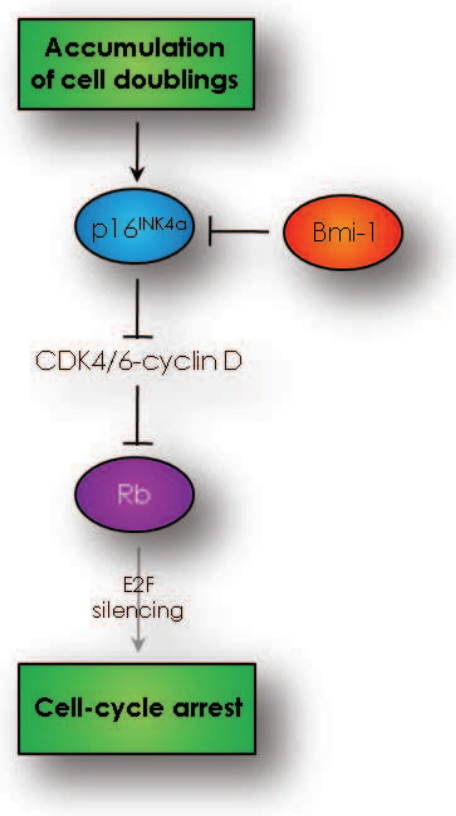

Fig. 10 | **The p16^{INK4a}-Rb pathway (after Bringold and Serrano, 2000).**

Among the four members of the INK4 family, $p16^{INK4a}$ is an important tumour suppressor that is inactivated in a large proportion (>30%) of human tumours (reviewed by Ruas and Peters, 1998). Interestingly, $p16^{INK4a}$ and Rb exhibit a reciprocal pattern of alterations: these tumours that have lost $p16^{INK4a}$ expression retain Rb, whereas tumours that have lost Rb retain $p16^{INK4a}$. There are, of course, tumours that retain both Rb and $p16^{INK4a}$, and in these cases it is frequent to find CDK4 or cyclin D over-expression (Ruas and Peters, 1998).

The critical property that distinguishes $p16^{INK4a}$ from the other members of the INK4 family seems to be its capacity to be transcriptionally up-regulated in response to senescence and oncogenic stress. The *p16^{INK4a}* gene is progressively up-regulated in response to the accumulation of population doublings (Palmero *et al.*, 1997; Zindy *et al.*, 1997). Consistent with the role of $p16^{INK4a}$ in senescence, inactivation of the $p16^{INK4a}$–Rb pathway results in lifespan extension or immortalisation of human cells (Jarrard *et al.*, 1999; Maurelli *et al.*, 2006). This is manifested by the elevated frequency of $p16^{INK4a}$ gene inactivation in immortal cell lines (Loughran *et al.*, 1996; Brenner *et al.*, 1998). Similarly, viral oncoproteins such as SV40 large T antigen, adenoviral E1A protein or herpes virus E7 protein inactivate the growth suppression functions of Rb and facilitate immortalisation (reviewed in Jansen-Dürr, 1996).

1.4.1.2. Bmi-1

The Bmi-1 protein was first identified as an oncogene, which cooperates with c-myc in the generation of mouse B-cell lymphomas (Haupt *et al.* 1991; van Lohuizen *et al.* 1991a). Bmi-1 belongs to the Polycomb group of genes (van Lohuizen *et al.*, 1991b); in general, this protein family modifies the structure of large chromatin domains, leading to their repression. Abnormal activation of Bmi-1 has been reported in human tumours (Beà *et al.*, 2001; Kim *et al.*, 2004) and there is ample evidence that over-expression of Bmi-1 in mice results in leukaemia (Haupt *et al.*, 1993). The immortalising potential of Bmi-1 has been linked to its ability to down-regulate the *INK4a/ARF* locus expression (Jacobs *et al.*, 1999). Through specific promoters and distinct open

reading frames the *INK4a/ARF* locus encodes $p16^{INK4a}$, and a completely unrelated protein, known as ARF (Alternative Reading Frame). The two products participate in major tumour-suppressor networks that are disabled in human cancer and influence key physiological processes such as replicative senescence, apoptosis and stem-cell self-renewal. Although the Polycomb complexes in which Bmi-1 is active presumably regulate expression of many genes, several pieces of evidence point to $p16^{INK4a}$ and ARF as key targets for Bmi-1 (Jacobs *et al.*, 1999; Lessard and Sauvageau 2003; Molofsky *et al.* 2003; Bruggeman *et al.* 2005). Mouse embryonic fibroblasts isolated from genetically Bmi-1 deficient mice have elevated $p16^{INK4a}$ and ARF levels and enter senescence prematurely (Jacobs *et al.*, 1999). Conversely, over-expression of Bmi-1 promotes proliferation, and allows MEF immortalisation by repression of *INK4a/ARF* locus and, in combination with H-RAS, leads to neoplastic transformation (Jacobs *et al.*, 1999). In addition, *Bmi-1*$^{-/-}$ *INK4a/ARF*$^{-/-}$ MEFs do not show premature senescence (Jacobs *et al.*, 1999). These results clearly indicate that the *INK4a/ARF* locus is a critical target for Bmi-1; they connect transcriptional repression by Polycomb-group proteins with cell-cycle control and senescence (Fig. 11).

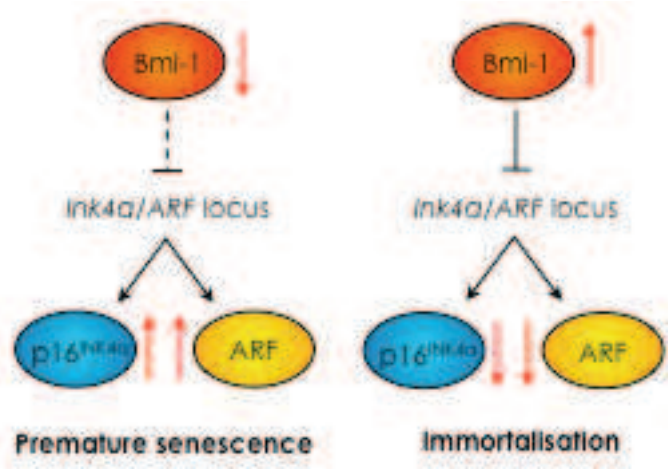

Fig. 11 | **Bmi-1 regulates *INK4a/ARF* locus.**

Decrease in Bmi-1 expression was also observed during replicative senescence in human fibroblasts and Bmi-1 over-expression extends the replicative life span of human fibroblasts by suppressing the $p16^{INK4a}$ –

dependent senescence pathway (Itahana *et al.*, 2003). In human cells, Bmi-1 regulates proliferation and senescence mainly by inhibiting the $p16^{INK4a}$ -Rb pathway (Itahana *et al.*, 2003) (contrary to mouse cells where the ARF-p53 pathway seems to play a more important role); however, the mechanism of this inhibition is still poorly explored (Dellino *et al.*, 2004).

Besides the regulation by Bmi-1 INK4a/ARF locus acts independently of Bmi-1 as a sensor of oncogenic stress. The locus is activated in response to the oncogenic stress. In particular, the presence of oncogenic Ras in a normal cell can activate the expression of both $p16^{INK4a}$ and ARF resulting in cell-cycle arrest (Serrano *et al.,* 1995; Serrano *et al.,* 1997; Palmero *et al.,* 1998). Also, other oncogenes, such as Myc, E2F1 and the adenoviral oncoprotein E1a, activate the expression of ARF (but not of $p16^{INK4a}$) (Zindy *et al.,* 1998; de Stachina *et al.,* 1998; Bates *et al.,* 1998). When expressed in normal cells, Myc, E2F1 and E1a, elicit a strong apoptotic response mediated primarily through activation of p53. It is now well established that the activation of p53 by Myc and E1a is mediated by the upregularion of ARF (Zindy *et al.,* 1998 ; de Stachina *et al.,* 1998). In summary, the INK4a/ARF locus is activated in response to oncogenic stresses, thus preventing the propagation of cells carrying activated oncogenes (Sherr, 1998; Weinberg, 1998).

1.4.2. The ARF-p53-$p21^{CIP1/WAF1}$ pathway

1.4.2.1. p53

The tumour-suppressor protein p53 is inactivated in approximately half of human cancers. In normal cells, p53 is expressed as a latent transcription factor with a very short half-life (minutes) (McVean *et al.*, 2002). DNA damage is the most widely studied stimulus that activates p53. Activation of p53 involves complex post-translational modifications that include phosphorylation, acetylation, ubiquitylation, SUMOylation, neddylation etc. That results in an increased half-life (hours) and in conformation changes that increase p53 competence for DNA binding and transactivation (reviewed in Giaccia and Kastan, 1998). Several targets of p53 have been identified,

which are implicated in a variety of processes, such as cell-cycle arrest, senescence and apoptosis, cellular metabolism, as well as stem cell self-renewal. p53 levels are determined mainly by the rate of p53 ubiquitylation and subsequent proteolytic degradation. The main ubiquitin ligase E3-type enzyme for p53 is the MDM2 oncogene (also known as *HDM2* when referring specifically to the human gene) (Fig. 12), although several other E3 ligases have been suggested to participate in p53 regulation (ARF-BP/Mule, Cop1, etc.).

MDM2 is an oncogene frequently amplified or over-expressed in sarcomas and some other human cancers (reviewed by Momand *et al.*, 1998). Concurrent with the role of MDM2 as a repressor of p53 function, most tumours over-expressing MDM2 do not have detectable p53 levels and do not carry mutations in p53 gene (Momand *et al.*, 1998).

The transcriptional activity of p53 is progressively activated with the accumulation of cell doublings (Afshari *et al.*, 1993; Atadja *et al.*, 1995) (Fig. 12). Since senescence is characterised by a permanent cell-cycle block, significant emphasis has been placed on the p53 targets that mediate cell-cycle arrest and especially on the CDKI – $p21^{CIP1/WAF1}$.

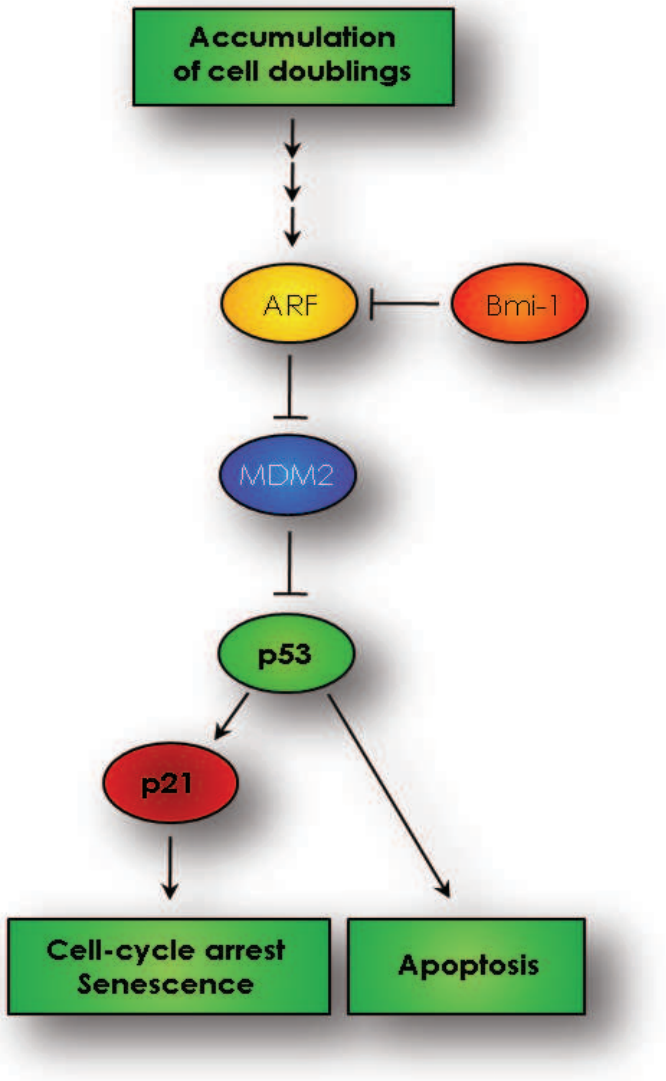

Fig. 12 | **The ARF-p53-p21$^{CIP1/WAF1}$ pathway (after Bringold and Serrano, 2000).**

1.4.2.1.1. p53 SUMOylation

Small Ubiquitin-like Modifier or SUMO proteins are a family of small proteins that are covalently attached to and detached from other proteins in cells to modify their function. SUMOylation is a post-translational modification involved in various cellular processes, such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, senescence, protein stability, response to stress, and progression through the cell cycle. SUMO proteins are similar to ubiquitin; and SUMOylation is directed by an enzymatic cascade analogous to that involved in ubiquitination; it engages E1, E2 and E3 enzymes. (Melchior *et al.*, 2003 ; Seeler and Dejean, 2003). There are three confirmed SUMO

isoforms in humans; SUMO-1, SUMO-2 and SUMO-3. SUMO-2/3 show a high degree of similarity to each other and are distinct from SUMO-1. First, SUMO is matured by a C- terminal hydrolase exposing a Gly-Gly motif, thus allowing subsequent conjugation steps to take place. The initial ATP-driven activation step is performed by the heterodimeric E1 enzyme, SAE1/SAE2, followed by a conjugation step executed by the E2 enzyme, hUbc9, and is concluded by an E3 ligase facilitating transfer to the substrate. To date, four types of SUMO E3 ligases have been identified, the PIAS (protein inhibitor of activated STAT) family of proteins including PIAS 1, PIAS 3, PIAS γ, PIAS xα and PIAS xβ, the PIAS-like proteins Nse2 and ZIMP10, RanBP2 and the polycomb protein Pc2 (Hay, 2005). Proteins conjugated to SUMO are highly prone to deconjugation. Several deconjugating isopeptidases including SENP1, SENP2, SENP3 and SENP6 have been described (Hay, 2005). It is the dynamic process of conjugation and deconjugation that generally results in only a small portion of target proteins being modified, thus making detection and functional analysis of SUMOylated proteins a challenge.

Recently, it was reported that SUMOylation is linked to the execution of cellular senescence. First it was demonstrated that over-expression of the E3 SUMO ligase PIASy in normal human fibroblasts recruits the p53 and Rb tumour-suppressor pathways to provoke a senescence arrest (Bischof *et al*., 2006). Induction of senescence requires PIASy E3 activity and is specific for this member of the PIAS ligase family. PIASy stimulates SUMOylation and transcriptional activity of p53 and increases Rb-dependent co-repression through recruitment to E2F-responsive promoters. In addition, fibroblasts lacking PIASy exhibit a highly reduced capacity to undergo senescence in response to a pro-senescent signal.

Then it was shown that cells over-expressing SUMO-2/3 exhibited a phenotype of premature senescence, which was described by cellular morphology and SABG activity (Li *et al.,* 2006). $p21^{CIP1/WAF1}$ was up-regulated in cells over-expressing SUMO-2/3 and both p53 and Rb were found to be modified by SUMO-2/3.

1.4.2.2. p21$^{CIP1/WAF1}$

p21$^{CIP1/WAF1}$ establishes p53-dependent G1 growth arrest (Deng *et al.*, 1995; Brugarolas *et al.*, 1995). Many studies support the view that p21$^{CIP1/WAF1}$ suppresses tumours by promoting cell cycle arrest in response to various stimuli.

p21$^{CIP1/WAF1}$ mediates its various biological activities primarily by binding to and inhibiting the kinase activity of the cyclin-dependent kinases (CDKs) CDK4/6, CDK2 and CDK1 leading to growth arrest at specific stages in the cell cycle. In addition, by binding to proliferating cell nuclear antigen (PCNA), p21$^{CIP1/WAF1}$ interferes with PCNA-dependent DNA polymerase activity, thereby inhibiting DNA replication and modulating various PCNA-dependent DNA repair processes.

p21$^{CIP1/WAF1}$ levels are elevated during senescence in several cellular systems (Tahara *et al.*, 1995) and indeed p21$^{CIP1/WAF1}$ gene was found in the screening of senescence-inducing genes (Noda *et al.*, 1994). Nevertheless, the question of whether p21$^{CIP1/WAF1}$ is essential or not for the establishment of senescence has yet to be unambiguously answered (Brown *et al.*, 1997; Pantoja and Serrano, 1999). For example, mouse embryonic fibroblasts lacking p21$^{CIP1/WAF1}$ undergo senescence (Pantoja and Serrano, 1999); they enter senescence and have a lifespan similar to wild-type cells. Moreover, p21$^{CIP1/WAF1}$ is not essential for the establishment of RAS-induced senescence in MEFs (Castro *et al.*, 2004), therefore, it may be concluded that in MEFs p21$^{CIP1/WAF1}$ is not essential either for senescence or for preventing neoplastic transformation by oncogenic RAS.

1.4.2.3. ARF

One of the most unexpected discoveries of recent years was that the *CDKN2A* (also referred to as *INK4a/ARF*) locus encoded for another protein: ARF (called p19^{ARF} in mouse cells, p14^{ARF} in humans). The ARF protein turned out to be a potent inhibitor of the p53-degrading activity of MDM2 (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998). Over-expression of ARF results in

p53 stabilisation and activation of its downstream targets. It is likely that $p16^{INK4a}$-Rb and ARF-p53-$p21^{CIP1/WAF1}$ pathways are used in a species-type specific manner during the establishment of senescence. For instance, *ARF* gene is transcriptionally up-regulated during senescence in mouse fibroblasts (Kamijo *et al.*, 1997, Jacobs *et al.*, 1999) but not in human keratinocytes (Munro *et al.*, 1997). MEFs genetically deficient in *ARF* but not $p16^{INK4a}$ are immortal, indicating that *ARF* is essential for senescence in those cells (Kamijo *et al.*, 1997).

Over-expression of Bmi-1 represses both expression of $p16^{INK4a}$ and *ARF* and, therefore, the immortalising properties of Bmi-1 are probably due to the combined effects on the entire locus.

1.4.3. p53-independent senescence

It was recently shown that RASSF1A (RAS association domain family 1 isoform A) is implicated in senescence. RASSF1A is a putative tumour suppressor and the expression of the RASSF1A gene product is frequently lost in cancers of the lung and other organs (Dammann *et al.,* 2000). Mechanistically, RASSF1A affects several cellular functions, such as microtubule dynamics, migration, proliferation, and apoptosis; however, its tumour-suppressive mechanism is incompletely understood. Thaler and colleagues (2009) led a study about the functional consequences of RASSF1A expression in RASSF1A-deficient human lung cancer cell lines. They observed that RASSF1A induces cell cycle arrest in G1 phase and senescence *in vitro* and also in tumours established in immunodeficient mice. RASSF1A-mediated growth inhibition was accompanied by the up-regulation of $p21^{CIP1/WAF1}$ and proceeded independently of p53, ARF, and $p16^{INK4a}$ (Thaler *et al.*, 2009).

Another example of p53-independent senescence was provided by Lin and collegues (2010). They found that a combination of Skp2 inactivation and ectopic expression of oncogenic RAS leads to senescence that does not depend on the ARF–p53 pathway and DNA damage response but instead depends on Atf4, p27 and $p21^{CIP1/WAF1}$. Skp2 is a critical component of the Skp2–SCF complex which acts as an E3 ligase to target p27 and other

substrates for ubiquitylation and degradation (Bloom *et al*., 2003 ; Nakayama and Nakayama, 2005). Recent studies suggest that Skp2 may have oncogenic activity (Gstaiger *et al.,* 2001; Shim *et al*., 2003) Notably, Skp2 over-expression is frequently observed in human cancer (Chiarle *et al.,* 2002), strongly suggesting that Skp2 over-expression may contribute to tumorigenesis.

p63 differs from its homologue p53 in that its role as a tumour suppressor is controversial, an issue complicated as *p63* gene encodes six different proteins. Isoforms containing or lacking an amino-terminal p53-like transactivation domain are referred to as TA and ΔN isoforms, respectively, and they have distinct, even opposing, functions (Yang *et al*., 1998). ΔNp63 isoforms enhance proliferation (Patturajan *et al*., 2002) and inhibit apoptosis (Rocco *et al*., 2006), whereas TAp63 isoforms induce apoptosis (Gressner *et al*., 2005) and inhibit cell-cycle progression (Gallegos *et al*., 2008). It was demonstrated that TAp63 isoforms are robust mediators of senescence, which inhibit tumorigenesis *in vivo* (Guo *et al.,* 2009). Whereas a gain of TAp63 induces senescence, loss of p63 enhances sarcoma development in mice lacking p53. Using a TAp63-specific conditional mouse model, it was shown that TAp63 isoforms are essential for RAS-induced senescence, and that TAp63 deficiency increases proliferation and enhances RAS-mediated oncogenesis in the context of p53 deficiency.

In vivo TAp63 induces senescence independently of p53, ARF and $p16^{INK4a}$, but requires $p21^{CIP1/WAF1}$ and Rb. TAp63-mediated senescence overrides RAS-induced transformation of *p53* null cells, preventing tumour initiation, and over-expression of TAp63 activates $p21^{CIP1/WAF1}$, induces senescence and inhibits progression of established tumours *in vivo*. Therefore TAp63 isoforms function as tumour suppressors by regulating senescence through p53-independent pathways.

Recent findings show that RAS oncogene-induced senescence can be mediated by the haplo-insufficient tumour suppressor apoptosis-stimulating protein of p53(ASPP)2 through a novel and p53/ARF/$p21^{CIP1/WAF1}$-independent pathway (Wang *et al.,* 2010). ASPP2 suppresses RAS-induced small ubiquitin-like modifier (SUMO)-modified nuclear cyclin D1 and inhibits retinoblastoma

protein (Rb) phosphorylation. The lysine residue, K33, of cyclin D1 was found to be the key site for this newly identified regulation. In agreement with the fact that its nuclear localisation is required for its oncogenic activity, it was demonstrated that nuclear cyclin D1 is far more potent than wild-type (WT) cyclin D1 in bypassing RAS-induced senescence. Therefore, the study identified SUMO modification as a positive regulator of nuclear cyclin D1, and revealed a new way by which cell cycle entry and senescence are regulated.

1.4.4. RAS, an oncogene that induces senescence

RAS is mutated in many human tumours and its prevalence reaches approximately 20% of all tumours, thus RAS is the most frequently activated oncogene overall (reviewed by Bos, 1989). The RAS protein family consists of H-RAS, K-RAS and N-RAS. By 1984, mutant K-RAS alleles were found in lung carcinoma specimens, indicating that these mutations did not arise as a consequence of culturing cells *in vitro* (Santos *et al.*, 1984). Moreover, specific associations were found between the various RAS oncogenes and particular types of human cancer (Table 3). K-RAS mutations were frequent in pancreatic (Hirai *et al.*, 1985) and colon carcinomas (Hand *et al.*, 1984). H-RAS mutations were predominantly found in bladder carcinomas (Fujita *et al.*, 1984), and N-RAS mutations were linked primarily to lymphoid malignancies (Gambke *et al.*, 1984; Gambke *et al.*, 1985; Bos *et al.*, 1985) and melanomas (Padua *et al.*, 1985).

Oncogenic RAS has been shown to cause senescence in primary cells through the activation of the ARF-p53-$p21^{CIP1/WAF1}$ and/or $p16^{INK4a}$-Rb tumour suppressor pathways (Serrano *et al.*, 1997; Voorhoeve and Agami, 2003). These observations suggested that the normal response of "uninitiated, naive" cells to hyperactive RAS signalling is to undergo cell-cycle arrest and/or senescence rather than unlimited proliferation and/or transformation. RAS is a point of convergence of most mitogenic extracellular stimuli. Its activation occurs by recruitment to the membrane through coupling proteins such as Shc, which in turn recognizes specific tyrosine phosphorylated sites in growth

factor receptors. As extracellular signals converge on RAS, a number of intracellular signals are sprayed from RAS. In this manner, RAS activates signalling routes that have been implicated in cellular proliferation, survival, generation of oxidative stress and cytoskeletal changes. In particular, the MAPK cascade, which includes the kinases RAF/MEK/ERK, is essential for proliferation (Fig. 13). The MAPK pathway is a chain of proteins in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. The signal starts when a growth factor binds to the receptor on the cell surface and ends when the DNA in the nucleus expresses a protein and produces some change in the cell, such as cell division. The pathway includes many proteins which communicate by adding phosphate groups to a neighbouring protein, which acts as an "on" or "off" switch. Receptor-linked tyrosine kinase such as the epidermal growth factor receptor (EGFR) are activated by extracellular ligands. Binding of epidermal growth factor (EGF) to the EGFR activates the tyrosine kinase activity of the cytoplasmic domain of the receptor. The EGFR becomes phosporylated on tyrosine residues. Then the cascade of protein phosphorylation is turned on and successive proteins become activated, among them so-called docking proteins (SOS, Shc) which activate RAS. Activated Ras stimulates the protein kinase activity of RAF kinase. RAF kinase phosphorylates and activates MEK. MEK phosphorylates and activates mitogen-activated protein kinase (MAPK) ERK1/2. When one of the proteins in the pathway is mutated, it can be stuck in the "on" or "off" position which is a necessary step in the development of many cancers. Oncogenic RAS causes a continous activation of the MAPK pathway. Following ectopic expression of oncogenic RAS, a brief period of increased proliferation ensues prior to the induction of senescence. This period of proliferation puts stress on the cell, leading to activation of a stress checkpoint. Di Micco and colleagues found robust activation of a DNA damage response (DDR) following expression of H-RAS^{V12} (Di Micco *et al.*, 2006). Interestingly, inactivation of components of the DDR could block RAS-induced senescence and allow for transformation. Additionally, H-RAS^{V12} expression increased the number of active replicons during DNA replication, but led to replication fork instability or stalling. Therefore, activation of a DNA

damage response induces senescence in response to overexpression of oncogenic RAS.

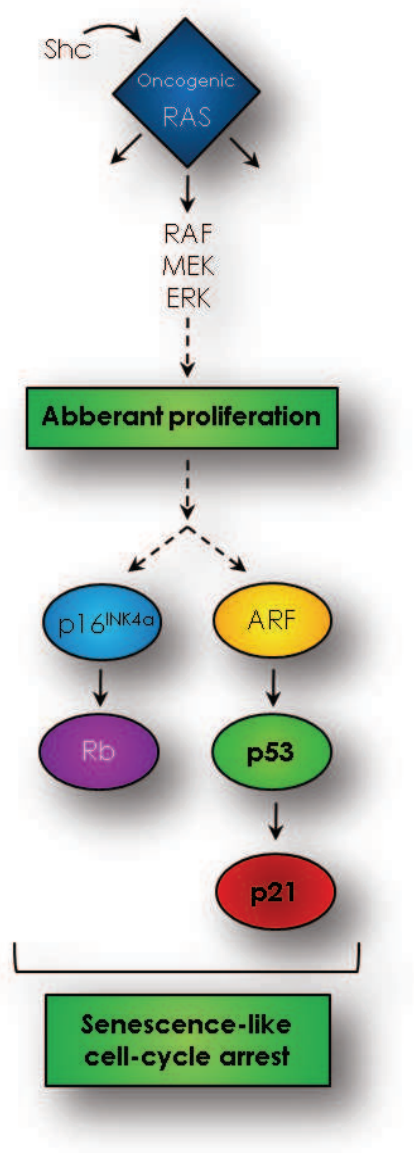

Fig. 13 | **The Ras oncogene and its effects on senescence (after Bringold and Serrano, 2000).**

Table 3 | **Ras mutations in some human cancers (after Karnoub and Weinberg, 2008)**

Tissue	H-Ras [%]	K-Ras [%]	N-Ras [%]
Adrenal gland	1	0	5
Biliary tract	0	32	1
Breast	1	5	1
Cervix	9	8	1
Endometrium	1	14	0
Eye	0	4	1
Gastro-intestinal tract (site indeterminate)	0	19	0
Haematopoietic and lymphoid tissue	0	5	12
Large intestine	0	32	3
Liver	0	7	4
Lung	1	17	1
Ovary	0	15	4
Pancreas	0	60	2
Pituitary	2	0	0
Prostate	6	8	1
Salivary gland	16	4	0
Skin	5	2	19
Small intestine	0	20	25
Stomach	4	6	2
Testis	0	5	4
Thymus	0	15	0
Urinary tract	12	4	3

Data derived from the catalogue of somatic mutations in cancer (COSMIC) of the Wellcome Trust Sanger Institute, Cambridge, UK. ND = not determined.

As mentioned above, the initial effect of oncogenic RAS in normal cells is to trigger proliferation. This "aberrant" proliferation is not sustained and after a few doublings, cells enter into a permanent cell-cycle arrest, senescence. This RAS-induced senescence is accompanied by over-expression of both

p16^{INK4a} and ARF, and the consequent activation of Rb and p53 (Serrano *et al.*, 1997; Palmero *et al.*, 1998; Ferbeyre *et al.*, 2002). In some systems, RAS or RAF can also induce p21$^{CIP1/WAF1}$ independently of p53 (Sewing *et al.*, 1997; Woods *et al.*, 1997). Neoplastic transformation by oncogenic RAS is only manifested when it is not balanced by the above-mentioned senescence response. More specifically, primary mouse fibroblasts genetically deficient in *p16^{INK4a}/ARF*, *ARF* alone or *p53*, are directly transformed by RAS, with no apparent need for additional genetic alterations (Serrano *et al.*, 1997; Kamijo *et al.*, 1997; Ferbeyre *et al.*, 2002). It was also demonstrated that RAS (H-RAS more specifically) cooperates with elevated expression of Bmi-1 to transform human mammary epithelial cells (Datta *et al.*, 2007) and to promote increased proliferation, invasion and resistance to apoptosis *in vitro* with an increased rate of spontaneous metastases in a–mammary xenograft model (Hoenerhoff *et al.*, 2009).

Contrary, cells genetically deficient in p21$^{CIP1/WAF1}$ or p27^{KIP1} cannot be transformed directly by RAS, thus behaving like wild-type cells (Pantoja and Serrano, 1999; Groth *et al.*, 2000). Together, these observations demonstrate that not all the components of the senescence-inducing pathways are equally important for Ras-induced senescence.

One of the key issues raised by the mutational activation of RAS oncogenes in human tumours was whether they have initiating – or at least a causative – role in tumour development, or whether they are merely activated as a consequence of the genetic instability of malignant cells. In the early eighties of the twentieth century, mutated RAS oncogenes were identified in experimental tumours of rodents that were exposed to chemical or physical carcinogens (Balmain and Pragnall, 1983; Sukumar *et al.*, 1983). Evidence that RAS can be involved in oncogenesis was obtained in 1985 when Zarb *et al.* showed that H-RAS oncogenes, which were activated in chemically induced mammary carcinomas of rats, were activated by the type of mutation that is known to be caused by the initiating carcinogen (similarly in carcinogen-induced skin papillomas - 90% harbour H-RAS mutation). The generation of tumours in mice harbouring RAS transgenes has lent further proof to the oncogenic properties of these genes *in vivo* (Table 4).

Direct proof for the requirement of continuous RAS expression for tumour maintenance has been obtained recently using mice carrying inducible K-RAS transgenes (Fisher *et al.*, 2001; Tuveson *et al*, 2004; Guerra *et al.*, 2003).

Table 4 | **Some mouse models to study RAS function (after Malumbres and Barbacid, 2003).**

Model	Description	Features
MMTV-v-H-RAS	Oncogenic H-RAS expression under the MMTV promoter	Mammary tumours, hyperplasia of the hardenian gland and other malignancies
Elastase-I-H-RAS	Oncogenic H-RAS expression under the elastase-I-regulating elements	Rapid development (two days) of neoplasia in the foetal exocrine pancreas
Tyr (Tet)–RAS	Oncogenic H-RAS under the control of the tyrosinase promoter and tetracycline regulatory elements	Melanoma development; tumour regression after removal of activator
CCSP (Tet)–K-RAS G12D	Tet-regulated K-RAS oncogene driven by the Clara cell-secretory protein promoter	Adenocarcinomas of the lung and apoptotic regression after removal of inductor
K- RAS$^{-/-}$	K-RAS knockout	Embryonic lethality (E12-E14); foetal liver defects, anaemia
N- RAS$^{-/-}$	N-RAS knockout	Viable; defective immune response and T-cell function
H- RAS$^{-/-}$	H-RAS knockout	No obvious phenotype; decreased tumour formation after carcinogenic treatment
K-RAS^{LA}	Oncogenic K-RAS G12D after spontaneous recombination	'spontaneous' lung tumours, thymic lymphomas and skin papillomas
LSL-K-RASG12D	Cre-inducible form of an oncogenic K-RAS knock-in	Tumours after activation of RAS oncogenic form with Cre recombinase

1.5. Senescence in mouse and human cells - mice are not small people

Senescence mechanisms clearly differ in mouse and human cells. Both human and mouse cells have been used to study this particular type of growth arrest, although each system has its peculiarities. Primary human fibroblasts, for example, have a more extended proliferative potential than their mouse counterparts in culture, yet rarely become spontaneously adapted to continuous growth as immortalised cell lines. They are more resistant than rodent cells to oncogene-mediated transformation and tend to be more genetically stable. Despite these obvious differences, it has been very tempting to link conclusions drawn from studies in these different cell systems, equating senescence in mouse and human cells. When wild-type mouse embryonic fibroblasts (MEFs) are grown in standard culture conditions, they experience a senescence-like phenotype, apparently triggered by oxidative stress (Parinello *et al.*, 2003), as there is no conclusive evidence that the replicative arrest experienced by primary mouse fibroblasts in culture depends upon a biological clock that measures the generation number. Instead, the progressive loss in proliferative capacity may result from cumulative trauma imposed by our routine of *in vitro* cell culture conditions.

Non-physiological *in vitro* culture conditions, including disruption of cell-cell contacts, lack of heterotypic interactions between different cell types, the medium-to-cell ratio, persistent RAS activation by high mitogen concentration, absence of appropriate survival factors, hyperoxia and plating on plastic are very likely to induce stress responses that influence the establishment of senescence.

Telomere-dependent, replicative – *intrinsic* – senescence occurs in human cells, as telomere shortening is inevitable due to the lack of active telomerase. Mouse embryonic fibroblasts (MEFs) have also a finite replicative capacity and show a senescent phenotype when replicatively inactive. However, it is believed that the replicative senescence of MEFs is not induced *intrinsically* but is rather induced by *extrinsic* factors (the above-mentioned cell culture conditions). As mentioned earlier in the Introduction, laboratory mice have very long telomeres compared to human cells and, therefore, it is

rather unlikely that replicative senescence of mouse cells is due to telomere shortening (Sherr and DePinho, 2000; Wright and Shay, 2000). However, the study of telomere erosion in mouse cells lacking the telomerase RNA component (mTR) (Blasco *et al.*, 1997) demonstrated that due to long telomeres of *Mus musculus*, the effects of telomere shortening become noticeable following extensive passages of *mTR* null cells and after successive generations of breeding (the first generation of *mTR*$^{-/-}$ mice did not show any distinguishing phenotypes compared to their wild type littermates). Telomere shortening leads to genetic instability in the late-generation of *mTR* null mice and results in testicular germ cell depletion, uterine and intestinal villus atrophy, diminished haematopoietic stem cell function, impaired mitogenic responses of lymphocytes and dermatitis. As during the DNA damage response, p53 activation in this setting can trigger either cell cycle arrest or apoptosis depending on cell type and additional signals. In early passage MEFs derived from late-generation *mTR* null animals, the levels of p53 and p53-responsive p21$^{CIP1/WAF1}$ were significantly higher than those detected in normal MEFs at an equivalent passage number, and fewer cells were in the cycle. Therefore, telomere attrition in MEFs can lead to a p53 response and, as might be expected, the proliferative block is attenuated in cells derived from generationally matched animals lacking both *mTR* and *Trp53*. The latter cells have an extended survival but exhibit a greater number of end-to-end chromosome fusions in comparison to *mTR* null cells that retained p53 function (Chin *et al.*, 1999). Hence, when mouse telomeres become dysfunctional, exposed chromosomal termini and/or resulting fusion–bridge–breakage events generate DNA damage signals that induce p53-dependent cell cycle arrest or apoptosis. This behaviour of cultured late-generation *mTR*-deficient mouse cells more closely resembles the response of primary human cells entering crisis.

The introduction of an oncogene such as *RAS* also triggers a senescence-like response in both mouse and human primary cells (Serrano *et al.*, 1997). Although provoked by distinct physiological signals, these forms of senescence use the same cellular control machinery, involving the p53 and Rb pathways, to elicit this response. However, the relative contribution of

these pathways to senescence differs in the cells of the two species (Fig. 14). The p53 pathway seems to have a predominant role in regulating the senescence of murine fibroblasts, whereas the Rb pathway seems to have a principal role in human cells. In response to culture- or *RAS*-induced senescence, wild-type MEFs show an increase in ARF and p53 levels (Serrano *et al.*, 1997; Zindy *et al.*, 1998). Consistent with this, MEFs from *ARF* null or *Trp53* null mice escape senescence (Kamijo *et al.*, 1997; Harvey *et al.*, 1993) (Fig. 13). However, *CDKN1A* (*p21*) null MEFs fail to escape senescence, indicating that a p53 target other than p21 is the relevant downstream effector of senescence in murine cells (Pantoja *et al.*, 1999).

Senescent MEFs also show an increase in the levels of $p16^{INK4a}$; however, the need for disruption of the $p16^{INK4a}$-Rb pathway in bypassing senescence in MEFs remains a subject of some debate. Mouse fibroblasts lacking *Rb* still undergo *RAS*-induced senescence (Peeper *et al.*, 2001). However, it is more difficult to gauge the contribution of $p16^{INK4a}$ loss. A complication is that both $p16^{INK4a}$ and ARF are encoded by the same locus, in part through overlapping reading frames. In parallel, but using two different strategies, two teams developed 'pure' $p16^{INK4a}$ – with unaffected levels of ARF – knockout mice (Sharpless *et al.*, 2001; Krimpenfort *et al.*, 2001). MEFs lacking $p16^{INK4a}$ and expressing *ARF* have none of the properties conferred by *ARF* loss alone. More specifically, $p16^{INK4a}$ null MEFs proliferate normally in culture, exhibit unchanged cell cycle profiles and cell size, arrest properly in response to serum deprivation or ionizing radiation, and do not have any advantage in forming colonies when plated at low density. The $p16^{INK4a}$ null MEFs become senescent when passaged on a 3T3 or 3T9 protocol, and cannot be transformed by oncogenic RAS alone. Comparative characteristics of $p16^{INK4a}$*-ARF*, *ARF* or $p16^{INK4a}$ null MEFs are shown in Table 5.

Table 5 | **Mouse embryonic fibroblasts lacking *Ink4a* and/or *ARF* (after Sherr, 2001, modified)**

MEFs Lacking p16*Ink4a* and/or *ARF*			
***Ink4a/ARF* Δ exons 2,3** **$p16^{INK4a}$-*ARF* null**	***Arf* Δ exon 1β** ***ARF* null**	***Ink4a* mutant** **$p16^{INK4a}$ null**	***Ink4a* Δ exon 1α** **$p16^{INK4a}$ null**
Unusually rapid MEF proliferation rate	Unusually rapid MEF proliferation rate	Normal MEF proliferation rate	Normal MEF proliferation rate
MEFs are immortal and susceptible to transformation by oncogenic RAS	MEFs are immortal and susceptible to transformation by oncogenic RAS.	MEFs undergo senescence and resist transformation by oncogenic RAS	MEFs undergo senescence and resist transformation by oncogenic RAS. Spontaneous immortalisation occurs at an accelerated rate
Serrano *et al.*, 1996	**Kamijo *et al.*, 1997**	**Krimpenfort *et al.*, 2001**	**Sharpless *et al.*, 2001.**

Taken together, these observations indicate that the ARF-p53-$p21^{CIP1/WAF1}$ pathway is the principal determinant of senescence in murine cells. Interestingly, *Rb/p107* double-knockout and *Rb/p107/p130* triple knockout MEFs have been shown to escape *RAS*-induced senescence (Sage *et al.*, 2000; Dannenberg *et al.*, 2000; Peeper *et al.*, 2001), indicating that the Rb pathway might after all contribute to murine cellular senescence, albeit in a more minor way than in human cells. A contrasting response is seen in human cells. As human fibroblasts reach replicative senescence (or, alternatively, experience an active RAS oncoprotein), the levels of $p16^{INK4a}$, increase significantly and $p16^{INK4a}$ upregulation do not depend on the ARF-p53-$p21^{CIP1/WAF1}$ pathway (Wei *et al.*, 2001).

Also, cultured human mammary epithelial cells and keratinocytes show up-regulation of $p16^{INK4a}$ and associated growth arrest early on; however, they spontaneously lose $p16^{INK4a}$ expression, frequently *via* promoter methylation, and thereby gain a proliferative advantage.

Subsequent introduction of telomerase reverse transcriptase (TERT; the catalytic subunit of human telomerase) into these cells enables them to undergo immortalisation (Dickson *et al.*, 2000; Stampfer *et al.*, 2001). More recently, it has been shown that dermal fibroblasts that were derived from a human patient with a mutation in both copies of the *CDKN2A* locus (encoding $p16^{INK4a}$), which renders the resulting $p16^{INK4a}$ protein non-functional, are resistant to oncogenic RAS-induced senescence (Huot *et al.*, 2002). Observations such as these indicate that $p16^{INK4a}$ is the key governor of senescence in human cells. However, *TP53* or *CDKN1A* (*p21*) no human fibroblasts have been shown to escape replicative senescence (Rogan *et al.*, 1995; Brown *et al.*, 1997); nevertheless, they undergo *RAS*-induced senescence, indicating that the contribution of the p53 pathway to human cell senescence is minor compared with its central role in murine cells.

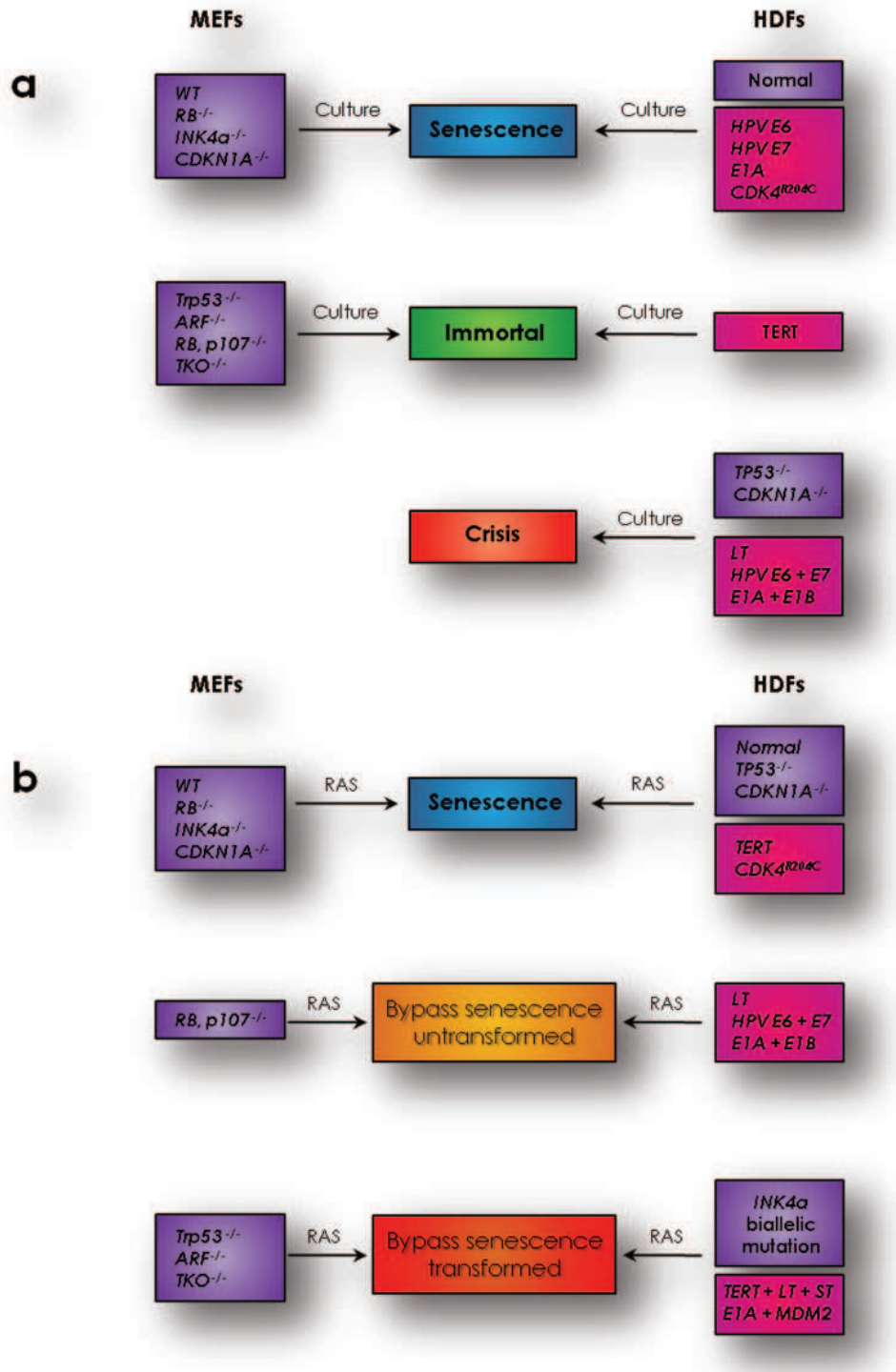

Fig. 14 | **Escape from culture- and oncogenic *RAS*-induced senescence.** Effects of various genetic backgrounds (violet boxes) and introduced genes (pink boxes) on senescence. **a** | Effects on bypassing culture-stress-induced senescence in mouse embryonic fibroblasts (MEFs) (left) and on bypassing replicative senescence in human diploid fibroblasts (HDFs)(right) **b** | Effects on bypassing Ras-induced senescence in MEFs (left) and HDFs (right) and subsequent transformation. *CDK4R24C*, INK4A-insensitive mutant of cyclin-dependent kinase 4; *CDKN1A*, encodes $p21^{CIP1/WAF1}$; HPV, human papillomavirus; LT, simian virus 40 large T; *Rb*, retinoblastoma; ST, simian virus 40 small T; TERT, telomerase reverse transcriptase; TKO, triple knockout of Rb, p107 and p130; WT, wild type. (after Rangarajan and Weinberg, 2003)

1.6. Senescence in tumours: a barrier to tumorigenesis?

Melanocyte senescence associated with the presence of oncogenic $BRAF^{V600}$ in skin *nevi* was part of the initial reports describing cellular senescence *in vivo* (Michaloglou *et al.*, 2005). Similarly, human PIN (prostatic intraepithelial neoplasia) lesions, as well as their murine counterparts, express markers of senescence (Chen *et al.*, 2005; Acosta *et al.*, 2008).

NF1 is the gene implicated in senescence in humans (Courtois-Cox *et al.*, 2006). Loss-of-function mutation of *NF1* underlies the familial cancer syndrome known as neurofibromatosis type 1. *NF1* encodes an RAS GTPase-activating protein (GAP), a negative regulator of RAS activity. The absence of NF1 results in hyperactivated RAS signalling and the formation of neoplastic lesions, known as neurofibromas, which were shown to express markers of senescence (Courtois-Cox *et al.*, 2006). Finally, premalignant human colon adenomas also exhibit features of senescence, which are associated with the presence of senescence markers (Bartkova *et al.*, 2006; Kuilman *et al.*, 2008).

All these examples confirm the presence of cellular senescence *in vivo*, in human as well as in murine tissues. Of note, these lesions correspond to benign lesions, suggesting that cellular senescence is a self-defence mechanism that constrains tumour growth during the early stages of tumorigenesis. Consistent with that notion, these authors have also shown that senescence markers disappear as cells progress to more transformed stages during tumour progression, as illustrated during the adenoma to carcinoma transition in skin tumours. Importantly, the original identification of senescent tumour cells was obtained from lung adenomas, pancreatic intraductal neoplasias, PIN lesions and *nevi,* which are all pre-malignant tumours (Table 6). By contrast, senescent phenotype is absent in the corresponding malignant stages, which are lung adenocarcinomas, pancreatic ductal adenocarcinomas, prostate adenocarcinomas and melanomas, respectively.

Those observations led to the conclusion that progression to malignancy requires evading senescence, and senescence itself is a tumour-suppressive mechanism. Evidence for the tumour suppressor role of

senescence was first obtained with mouse models of cancer based on the ablation of the tumour suppressor *PTEN* in prostate (Chen *et al.*, 2005) or the expression of oncogenic *N-RAS* in haematopoietic system (Braig *et al.*, 2005). In both cases, the initiating oncogenic event led to the development of senescent pre-malignant lesions with little evidence of apoptosis. Interestingly, loss of senescence markers and full-blown malignancy occurred when the oncogenic event was combined with simultaneous deletion of mediators of senescence response, such as the tumour suppressor p53 (Chen *et al.*, 2005) or the histone methyltransferase SUV39H1 implicated in methylation of histone 3 on lysine 9 (Braig *et al.*, 2005). The association between senescence and tumour suppression has subsequently been supported in other mouse models of cancer, such as $BRAF^{V600}$-induced melanomas (Goel *et al.*, 2009) and $H\text{-}Ras^{G12V}$-induced mammary tumours (Sarkisian *et al.*, 2007). In these cases, deletion of *CDKN2A* or *Trp53* abrogated senescence and allowed progression to malignant stages, providing a compelling case for a causal link between tumour suppression and the induction of senescence by $p16^{INK4a}$, ARF and p53. However, not all the senescent pre-malignant stages are strictly dependent on $p16^{INK4a}$, ARF and p53. In this regard, senescent pre-lymphomagenic thymocytes produced by enforced expression of β-catenin showed a stable senescent response even in the absence of p53, although loss of p53 allowed some thymocytes to progress to lymphoma (Xu *et al.*, 2008). Similarly, the absence of $p16^{INK4a}$ favoured the development of $BRAF^{V600}$-induced melanomas, but *nevi* with detectable senescence were still produced (Dhomen et al., 2009). This supports the data in human *nevi* indicating that not all senescent cells are positive for $p16^{INK4a}$. It also supports the fact that individuals from a Dutch family with hereditary melanoma still developed *nevi* despite carrying inactivating mutations in both copies of the *CDKN2A* locus (Michaloglou *et al.*, 2005). Finally, deletion of Vhl in mouse kidney results in senescence with an associated increase of $p27^{KIP1}$, but not $p16^{INK4a}$, ARF and p53. This suggests that other cell cycle regulators are also engaged to implement senescence (Young *et al.*, 2008). Similarly, transgenic AKT1-driven PIN lesions also show increased $p27^{KIP1}$ expression, which is associated with senescence (Majumder *et al.*, 2008) – when AKT1 was

expressed in combination with genetic deletion of *CDKN1B* (encoding $p27^{KIP1}$) senescence was absent and mice developed invasive prostate cancer (Majumder *et al.*, 2008). For a summary of mouse models of tumour cell senescence see Table 7.

Table 6 | **Human tumours showing cell senescence**

Associated oncogenic event	tumour	Evidence of senescence*
***NF1* inactivation**	Dermal neurofibromas	SABG and INK4a
***Braf*^{V600E} mutation**	Nevi	SABG, INK4a and low Ki67
Not determined	Prostate intraepithelial neoplasia	SABG and CXCR2
Not determined	Colon adenomas	SABG, INK4a and IL-8

*Evidence of senescence based on staining and immunohistochemistry (after Collado and Serrano, 2010)

Table 7 | **Some mouse models of tumour cell senescence**

Gene	Tissue or tumour	Evidence of senescence*
Oncogene activation BCR-Abl induced induction of INK4a/ARF in ALL model (Sherr lab) is another example for induction of senescence in the haematopoietic compartment		
***Hras*^{G12V}**	Mammary tumours, bladder tumours, and DMBA and TPA-induced papillomas	SABG, γH2AX, p53, INK4a, p21, ARF and low Ki67
***Kras*^{G12V}**	Lung adenomas and pancreatic intraductal neoplasias	SABG, INK4a, DEC1, DCR2, INK4b, HP1γ and low KI67
***Nras*^{G12D}**	Lymphoproliferative disorders	SABG
***Braf*^{V600E}**	Nevi and lung adenomas	SABG, DEC1, ARF and low Ki67
Rheb	Prostate intraepithelial neoplasia	SABG and low Ki67
E2f3	Pituitary hyperplasia	SABG, INK4a and ARF; presence of SAHF; low BrdU
Akt1	Prostate intraepithelial neoplasia	SABG, p27, HP1α, HP1γ and low BrdU

Oncogene inactivation		
Myc	Lymphoma, osteosarcoma, liver carcinoma and lung carcinoma	SABG, INK4b, p21 and presence of H3K9me3
Tumour suppressor inactivation		
PTEN	Prostate intraepithelial neoplasia	SABG, ARF, p53, p21 and low Ki67
Vhl	Kidney	SABG, p27 and DCR2
Tumour suppressor activation		
Trp53	Sarcomas and liver carcinomas	SABG, INK4b, INK4a and DCR2; low histone H3 phosphorylation, low Ki67

*Evidence of senescence based on staining and immunohistochemistry (after Collado and Serrano, 2010)

1.7. Senescence and aging

The link between cellular senescence and organismal aging is very tentative (reviewed by Campisi, 2005), although establishing a direct causative relationship is challenging. To start with, the factors that trigger senescence are *not exclusive* for senescence and also trigger apoptosis and quiescence, which makes it difficult to dissect the contribution of each of these responses to aging. An additional complexity is that senescence may have an impact on aging through two nonexclusive and possibly concomitant mechanisms (Table 8). Firstly, accumulation of senescent cells in tissues may reach a point that compromises tissue integrity and functionality. Secondly, senescent cells may limit the regenerative potential of adult stem cells (this limitation may be produced by apoptosis or quiescence too). In one extreme, aging of an organism could be provoked by the accumulation of senescent cells; in the other extreme, accumulation of senescent cells *per se* could be simply harmless, and aging could result from the exhaustion of the regenerative potential of stem cells.

Table 8 | **Senescence of stem and committed cells (after Collado *et al.*, 2007)**

	Newborn	**Adult**	**Old**
characteristics	Long telomeres Low *INK4a* expression Low DNA damage	Medium telomeres Modest *INK4a* expressior Modest DNA damage	Short telomeres High *INK4a* expression High DNA damage
Tissue renewal	optimal	efficient	inefficient
Differentiated tissue	No senescence	Low senescence	High senescence
	Tumour initiation		
Pre-malignant tumour	Most tumour cells undergo senescence (or apoptosis) A few may escape and give rise to carcinoma		

Nevertheless, it was demonstrated that a number of senescent cells increase with age and senescent cells are found at sites of age-related pathologies. Recent findings implicate $p16^{INK4a}$ -dependent senescence in three hallmarks of aging: decrements in neurogenesis, haematopoiesis and pancreatic function (Janzen *et al.*, 2006; Krishnamurthy *et al.*, 2006; Molofsky *et al.*, 2006). $p16^{INK4a}$ expression increases with age in the stem and progenitor cells of the mouse brain, bone marrow and pancreas, where it suppresses stem cell proliferation and tissue regeneration. However, it is not yet known whether the $p16^{INK4a}$-positive stem and progenitor cells are in fact senescent. It is possible that both the tumour-suppressive and pro-ageing activities of $p16^{INK4a}$ are partly due to growth suppression without senescence. Nonetheless, these findings suggest that the tumour-suppressive activity of $p16^{INK4a}$ might be inextricably linked to its pro-aging effects.

The age-related decline in stem-cell growth and tissue regeneration is substantially retarded in mice lacking $p16^{INK4a}$ (Krimpenfort *et al.*, 2001; Sharpless *et al.*, 2001). However, as expected, those mice die prematurely (in late middle-age) of cancer. On the other hand, mice with increased activity for the $p16^{INK4a}$ and ARF tumour suppressors (so-called "super *INK4a/ARF*" mouse strain carrying one or several additional copies of the entire *INK4a/ARF*

locus; Matheu *et al.*, 2004) manifest higher cancer protection compared to normal, non-transgenic mice and present normal aging and lifespan. Interestingly, mice carrying two additional copies of *INK4a/ARF* locus have further increased cancer protection compared to "super *INK4a/ARF*" and surprisingly lower scores in aging markers and age-related pathologies, and in extended median longevity (Matheu *et al.*, 2009). At first glance, the increased longevity of those mice could be due to a much lower incidence of cancer. However, experiments performed on mice with a cancer-free genetic background did not confirm this hypothesis (Matheu *et al.*, 2009). This suggests that *INK4a/ARF* locus possesses a potent anti-aging activity, which is separable from its anti-cancer effect.

The literature on p53 since it was first described (DeLeo *et al.*, 1979) is enormous (more than 44,000 entries in PubMed), but only a small proportion of the most recent articles deals with the possibility that the activity of p53 might be linked to aging and longevity. A phenotype of accelerated aging and reduced lifespan was reported in 2002 in association with a mutant allele of *p53* ("m"; deletion mutation of the first six exons of the *p53* gene) that confers phenotypes consistent with activated p53 rather than inactivated p53 (Tyner *et al.*, 2002). Mutant $p53^{+/m}$ (with one "m" allele and one normal allele of p53) exhibited enhanced resistance to spontaneous tumours compared with wild-type ($p53^{+/+}$) littermates. As $p53^{+/m}$ mice aged, they displayed an early onset of phenotypes associated with premature aging, which included reduced longevity, osteoporosis, generalised organ atrophy and a diminished stress tolerance. Tyner *et al.* proposed that the early aging phenotype of $p53^{+/m}$ mice was in part a result of enhanced activity of wild-type p53 in some tissues and faster reduction in the proliferation of stem cells (stem cells undergo senescence sooner) compared to their $p53^{+/+}$ counterparts. Another laboratory has shown that a short isoform of p53 called p44 has a role in the regulation of size and life span in the mouse. Over-expression of p44 in mice with the normal complement of p53 alleles (p44-tg mice) causes premature aging phenotype similar to that of $p53^{+/m}$ mice (Maier *et al.*, 2004). A pro-aging activity of p53 has also been unveiled by studying mice with constitutively high levels of endogenous damage, such as those deficient in

BRCA1 or those with a defect in the nuclear envelope that recapitulates the human progeroid Hutchinson-Gilford syndrome (Cao *et al.*, 2003; Varela *et al.*, 2005). No pro-aging phenotype was obtained in mice with decreased levels of Mdm2 (therefore with high activity of endogenous p53) although those animals were resistant to tumour formation (Mendrysa *et al.*, 2006).

Other investigators have generated genetically manipulated mice with a modest increase in normally regulated p53. "Super p53" mice (carrying p53-tg allele in addition to two endogenous alleles; Garcia-Cao *et al.*, 2002) exhibit an enhanced response to DNA damage. Those mice are significantly protected from cancer when compared with normal mice. "Super p53" mice do not show any indication of premature aging, probably reflecting the fact that p53 is under normal regulatory control. Moreover, mice possessing two additional copies of the p53 transgene (p53-tg/tg) had the same longevity profile as "super p53" and WT mice (Matheu *et al.*, 2009), indicating that increasing the p53 "dose" is not sufficient to have an impact on longevity.

Other hypermorphic models generated by the Serrano laboratory include "super ARF-p53" mice, which contain an additional copy of *p53* gene together with an additional copy of *INK4a-ARF* gene (Matheu *et al.*, 2007), or mice which possess additional copies of *p53, INK4a-ARF* and *telomerase* genes (Tomas-Loba *et al.*, 2008). Genetically manipulated mice with increased, but otherwise normally regulated levels of ARF and p53, present strong cancer resistance and have decreased levels of aging-associated damage. It was observed that these mice presented decreased oxidative damage (decreased ROS and increased expression of antioxidant genes), which had a visible impact on prolongation of the lifespan of the animal. These observations extend the protective role of the ARF/p53 pathway as it confers protection against aging-associated oxidative damage.

Additional copies of *p53, INK4a-ARF* and *telomerase* genes further increase the lifespan of transgenic animals compared to "super p53-ARF" mice (26%) and to "super p53" mice (40.2%). A list of mouse models of accelerated or delayed aging is represented in Table 9.

Table 9 | **Some mouse models of accelerated and delayed aging.**

Model or mutation	phenotype
$p53^{+/m}$	The C-terminal end of p53 causes constitutive p53 wild-type activation, cancer resistance and premature aging
p44-tg	TG expression of p44; premature aging
BRCA1Δ11/ Δ11 p53+/-	Early tumour incidence; premature aging
Zmpste24-/-	Accelerated aging, persisting p53 activation; phenotype partially reversed in Zmpste24-/-; p53-/- mice
***mdm2 puro*/Δ7–12**	Increased Cancer resistance; no effects on aging
super INK4a-ARF (INK4a/ARF-tg)	Increased Cancer resistance; no effects on aging
INK4a/ARF-tg/tg	Further increased cancer resistance (compared with INK4a/ARF-tg mice), extended longevity
super p53 mice (p53-tg)	Increased cancer resistance; no effects on aging
p53-tg/tg	Cancer resistance similar to p53-tg mice; no effects on aging
SARF-Sp53 mice (super INK4a-ARF; super p53)	Strong cancer resistance; decreased oxidative damage, prolongation of the animals' lifespan
Sp53/Sp16/SArf/TgTert (super p53 ; super INK4a-ARF, TERT-tg)	Visible delay in aging

2. E4F1

2.1. Discovery

The exploration of protein networks deregulated by viral oncoproteins led to the discovery in the late eighties of several essential regulators of cell division and cell survival. One of the many purposes of adenoviruses is to inactivate cell apoptotic response and reactivate the cell cycle in order to modify the machinery of their host for the transcription and replication of their own genome (adenoviruses are entirely dependent on the host cell's DNA and RNA synthesising machinery, and RNA processing machinery). Based on that knowledge, scientists attempted to identify the cellular targets of viral oncoproteins, such as adenoviral E1A or E1B. Among those E4F1 was originally discovered as a ubiquitously expressed cellular transcription factor regulated by the viral oncoprotein E1A13S (Lee and Green, 1987). While most other cellular targets of E1A discovered at the same time (E2F/pRB, CBP/p300, PCAF, CtBP, ATF/Creb etc.) have been extensively studied and recognized as central regulators of cell proliferation and survival, E4F1 functions remained, inexplicably, poorly investigated until recently.

2.2. E4F1 and the viral cycle

The products of the adenovirus early region 1A (*E1A*) gene are potent oncoproteins. E1A was shown to inhibit cell proliferation in part by targeting essential cell cycle regulators such as the retinoblastoma tumour-suppressor protein (Rb) (Fig. 15). This event leads to the release of the cellular transcription factors of the E2F family, which, in turn, stimulate transcription of the adenoviral *E2* gene and of cellular genes that are critical for the G1 to S-phase progression (Ren *et al.*, 2002; Wells *et al.*, 2002). In addition to the E2F transactivators, the E1A oncoprotein regulates the activity of the E4F1 transcription factor that was shown to be required for transcription of the adenoviral E4 gene (Nevins *et al.*, 1988; Raychaudhuri *et al.*, 1987).

The *E4F1* gene encodes a 120-kDa protein, p120E4F1. It was proposed that upon E1A expression, p120E4F1 is proteolytically cleaved, yielding a 50-kDa protein, p50E4F, which is believed to represent a transcriptionally active form (Fernandes and Rooney, 1997; Nevins, 1990; Nevins *et al.*, 1988; Raychaudhuri *et al.*, 1987). Although both p50E4F1 and p120E4F1 can recognise the same DNA sequence *in vitro* (Fernandes and Rooney, 1997), they seem to regulate gene expression differentially *in vivo*. Therefore, while p50E4F1 is believed to act as a transcriptional activator, over-expression of p120E4F1 was shown to repress transcription of the viral E4 and E1A genes (Fig. 15) (Fognani *et al.*, 1993). This mechanism of regulation is rather controversial, as the existence of the p50E4F1 isoform, in the absence of E1A overexpression, has not been confirmed. Precise characterisation of the E4F1-cleaved site and identification of the protease involved in the cleavage remains to be determined.

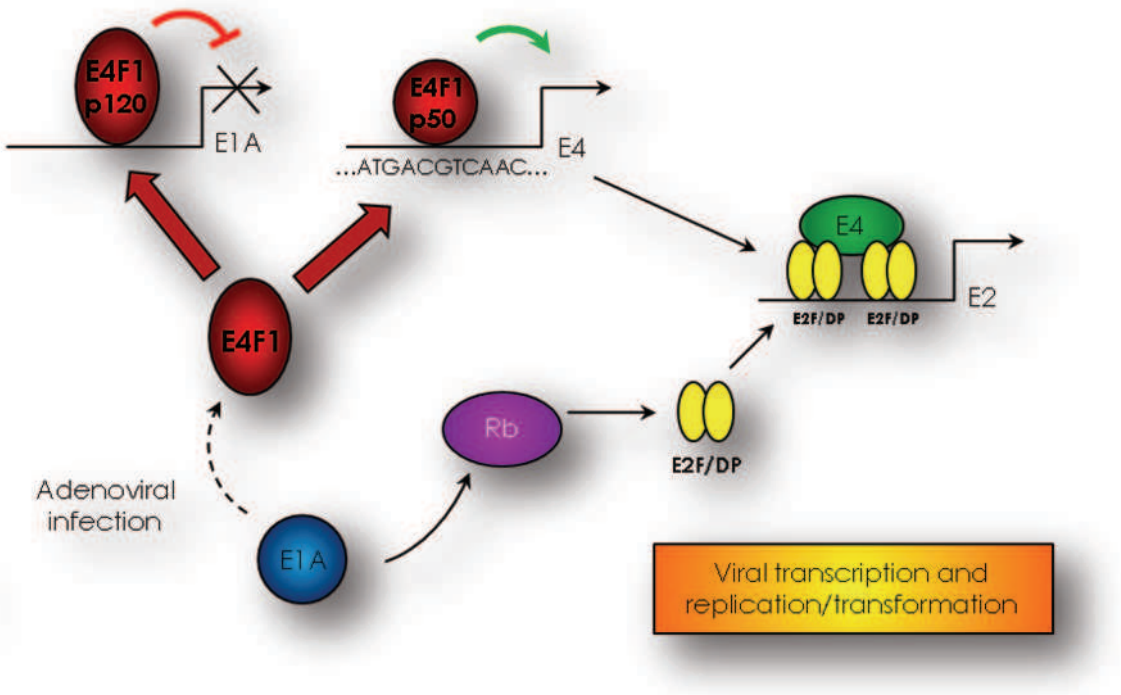

Fig. 15 | **E4F1 and the viral cycle – the protein discovery.** E4F1 was initially discovered as a target of E1A oncoprotein among other cellular factors.

2.3. E4F1 – a target of other viral oncoproteins

Two other DNA viral oncoproteins are also capable of targeting E4F1 protein: an avian adenovirus named CELO and the hepatitis B virus.

Gam1 was identified in an anti-apoptosis screen (Chiocca *et al.*, 1997; Glotzer *et al.*, 2000). Gam1 induces transcription from many eukaryotic promoters, notably by inhibiting the enzymatic activity of histone deacetylase 1 (HDAC1) (Chiocca, 2002). This leads to the activation of specific genes implicated in the regulation of the cell cycle, control of proliferation and development. Gam1 plays an important role in the regulation of protein sumoylation too as it interferes with the activity of SUMO-E1 ligases, therefore inhibiting protein post-translational modifications (Boggio, 2004). It was previously demonstrated that E4F1 and HDAC1 interact both *in vitro* and *in vivo*, and that E4F1-mediated transcriptional repression of the viral E4 promoter is suppressed in the presence of HDAC inhibitor, trichostatin A (TSA) or by over-expression of Gam1. Finally, Gam1-E4F1-HDAC1 were detected as components of the same protein complex (Colombo, 2003).

Many studies show the importance of HbX protein (Hepatitis B virus protein x) in the process of oncogenesis as patients positive for hepatitis B virus develop severe hepatocarcinomas. Hbx was found to interact with E4F1 *in vitro* and to repress its transcriptional activity (Rui, 2006). However, the physiopathological consequences of this interaction have not been studied yet.

2.4. Structure and functions of E4F1

2.4.1. Protein structure

E4F1 is a ubiquitously expressed protein, present, with high conservation of its sequence, in all vertebrates, including chicken, African clawed frog, fugu and zebrafish. Indeed, the homology of the E4F1 sequence between human and mouse protein is high (86%). On the other hand, no E4F1 sequence was identified in invertebrates whose genome was entirely

sequenced (*Caenorhabditis elegans, Drosophila melanogaster* etc.). This incomplete analysis suggests that E4F1 appeared late during the evolution process.

E4F1 gene is localised in the 13.3 region of human chromosome 16 (16p13.3) and in the A3.3 region of mouse chromosome 17 (17A3.3). In both human and mouse, E4F1 gene consists of 14 exons distributed over 11 kilobases (kb).

E4F1 is a zinc-finger protein, which belongs to the GLI-Kruppel family (Fognani, 1993). The protein structure consists of six classical C2H2 zing-finger domains (Fig. 16): two at the N-terminal end of the protein responsible for DNA binding, and four other domains at the C-terminal end of the protein, which together with the bHLH (basic helix-loop-helix) domain, are responsible for protein-protein interactions (Fajas *et al.*, 2000). E4F1 also possesses three atypical zing-finger domains C2HC (Rooney *et al.*, 1998).

Recently Le Cam, Linares *et al.* (2006) identified another functional domain of E4F1. Situated at the N-terminal end of the protein, this domain mediates a very atypical E3-ligase activity. For the entire E4F1 structure, see Fig. 16.

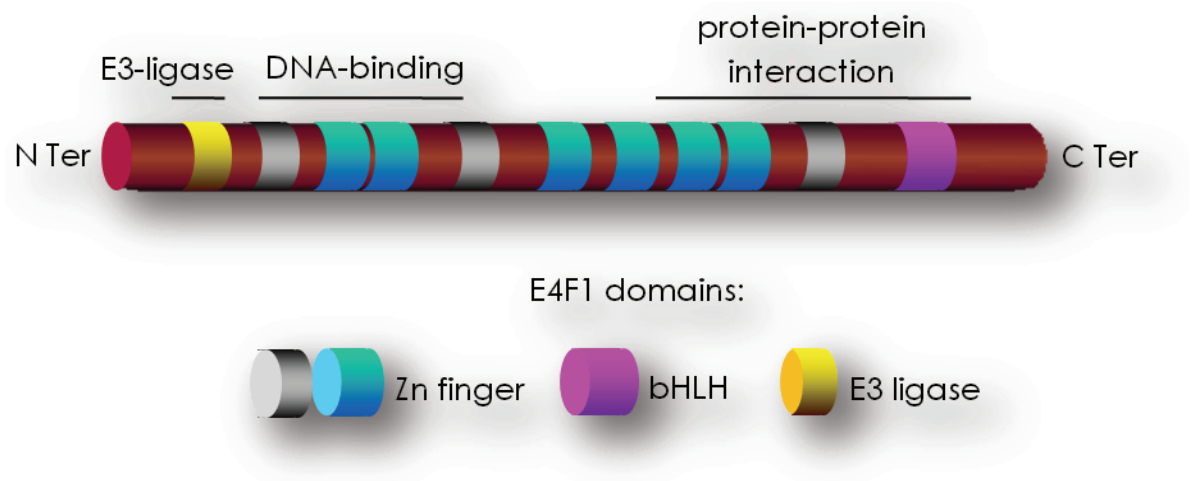

Fig. 16 | **Structure of the E4F1 protein: E4F1 functional domains.**

2.4.2. Functions

2.4.2.1. Transcription regulation

It is now well established that E4F1 can directly bind DNA. So far, only a few genes were shown to be E4F1 targets, such as *cyclin A2* gene (Fajas *et al.*, 2001), *aromatase* (Yang *et al.*, 1998) and *Igf2* (Julien, Paul, Le Cam and Sardet, personal communication). However, recent genome-wide ChIP-seq experiments have extended the number of potential direct E4F1 target genes. Surprisingly, none of the previously identified E4F1 targets were confirmed by ChIP-seq (Kirsh and Sardet, personal communication, 2010).

Over-expression of E4F1 in U2OS cells leads to cell-cycle arrest in the G1 phase (Fajas *et al*., 2001). In these cells E4F1 was found associated to the promoter of *cyclin A2* gene; more specifically E4F1 was binding to the cyclic AMP-responsive element site (CRE) of the promoter and thereby repressing expression of the *cyclin A2* gene. In addition, enforced expression of cyclin A2 released E4F-arrested cells from the G1 block.

Aromatase is a ubiquitously expressed enzyme involved in the conversion of androgens into estrogens. Aromatase is encoded by the *CYP19* gene and its expression is controlled by four different promoters (PI, PII, PIII, PIV), whose activities vary depending on the cell type (Mahendroo *et al.*, 1991; Means *et al.*, 1991; Mahendroo *et al.*, 1993). The strongest promoter, PII, is active in ovaries and prostate and is constitutively repressed in all other tissues *via* the repressor element S1 (Wang and Chen, 1992; Zhou and Chen, 1998). Screening of a cDNA bank of human mammary cells led to the identification of several factors capable of fixing the S1 sequence; among them were nuclear receptors (ERRα-1, RAR) and E4F1 (Yang *et al.*, 1998). This result was confirmed in Dr. Sardet's laboratory by chromatin immunoprecipitation (CHiP) and gel retardation techniques. Nevertheless, the exact role of E4F1 in the control of *aromatase* gene expression remains to be further elucidated.

IGF2 is an important growth factor implicated in the development of many organs. *Igf2* was identified as a positively regulated gene by E4F1 in a transcriptome analysis of E4F1-overexpressing U2OS cells. This study showed

that E4F1 regulates *Igf2* independently of p53 and that *Igf2* is a direct target of E4F1 even though no consensus sequences for binding E4F1 were found on the promoters of *Igf2* gene (Julien, Paul, Le Cam and Sardet, in preparation).

Being a transcriptional factor, E4F1 could be involved in the regulation of expression of senescence-associated genes as it directly interacts with p53 (Sandy et al., 2000; Le Cam, Linares et al., 2006), Rb (Fajas et al., 2000), ARF (Rizos et al., 2003) and Bmi-1 (Chagraoui et al., 2006).

2.4.2.2. E4F1 is an atypical ubiquitin E3 ligase

E4F1 was shown to be an atypical ubiquitin E3 ligase for p53 (Le Cam, Linares *et al.*, 2006). This study revealed that E4F1 is a key post-translational regulator of p53, which modulates its effector functions involved in alternative cell fates, growth arrest or apoptosis.

Strikingly, E4F1 E3 ligase domain has no sequence homology with previously described ubiquitin E3 ligases (domains RING, HECT or B-box) (Pickart and Eddins, 2004). Nevertheless, this domain shows a weak but significant homology with the IR domain of the SUMO-E3 ligase RanBP2 (Pichler *et al.*, 2004; Tatham *et al.*, 2005) (Fig. 17).

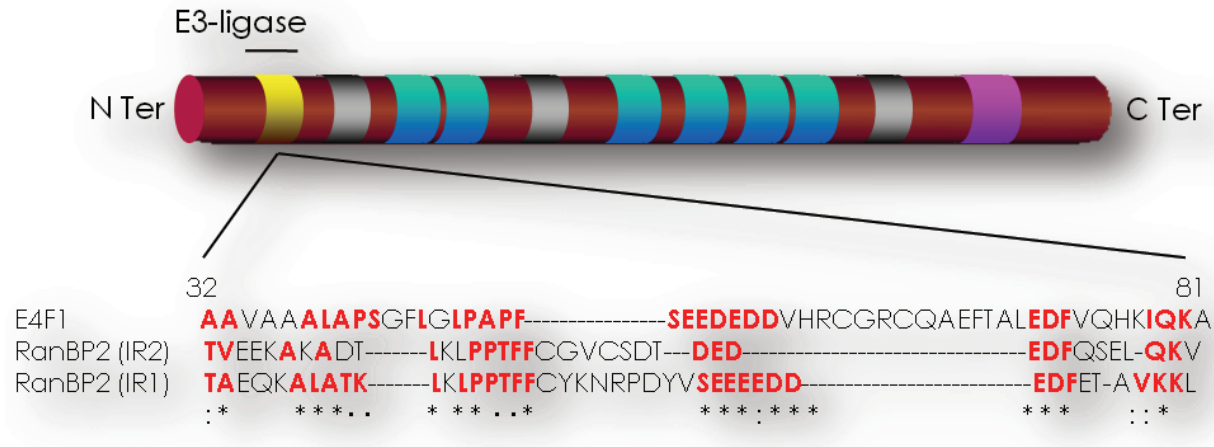

Fig. 17 | **Sequence alignments of the IR1 and IR2 domains from RanBP2 with the N-terminal region of E4F1 (aa 32–81).** Stars indicate residues shown to be essential for RNABP2 sumo E3 ligase activity (after Le Cam, Linares *et al.*, 2006).

E4F1 E3 ligase domain stimulates ubiquitylation of p53 protein both *in vitro* and *ex vivo*, even when the domain is separated from the rest of the protein (Linares, Le Cam and Sardet, unpublished data). Interestingly, ubiquitylation of p53 catalysed by E4F1 does not lead to p53 degradation *via* the proteasome. It is worth mentioning that this is in part explained by the different set of lysines that are targeted by E4F1 (compared to Mdm2). Then, E4F-ubiquitylated forms of p53 localise on chromatin suggesting that this ubiquitylation is implicated in the control of p53 transcriptional activities. This new mechanism of regulation was proposed to specify p53 transcriptional activities towards a cell cycle arrest programme, at the expense of a pro-aptototic response (Le Cam, Linares *et al.*, 2006) (Fig. 18).

More recently, preliminary results obtained by Laëtitia Linares suggested that E4F1 is also capable of regulating p53 SUMOylation *in cellulo*. Ubiquitylation and SUMOylation of p53 both involve the same E3 ligase domain of E4F1, but take place on different lysine residues (lysine 320 and 386, respectively). Mechanism enabling the regulation of these two post-translational modifications remains unknown.

E3-ligase activity of E4F1 seems to be independent of its transcriptional activities as truncated (devoid of E3-ligase domain) E4F1 protein is always capable of binding DNA and regulating the expression of its target genes, such as *Igf2* (Julien, Paul, Le Cam and Sardet, unpublished data). However, the interplay between E4F1 E3 ligase and transcriptional activities is likely more complex since some components of E4F1 multiprotein complexes involved in chromatin remodelling are also targets of E4F1-mediated ubiquitylation (Linares, personal communication).

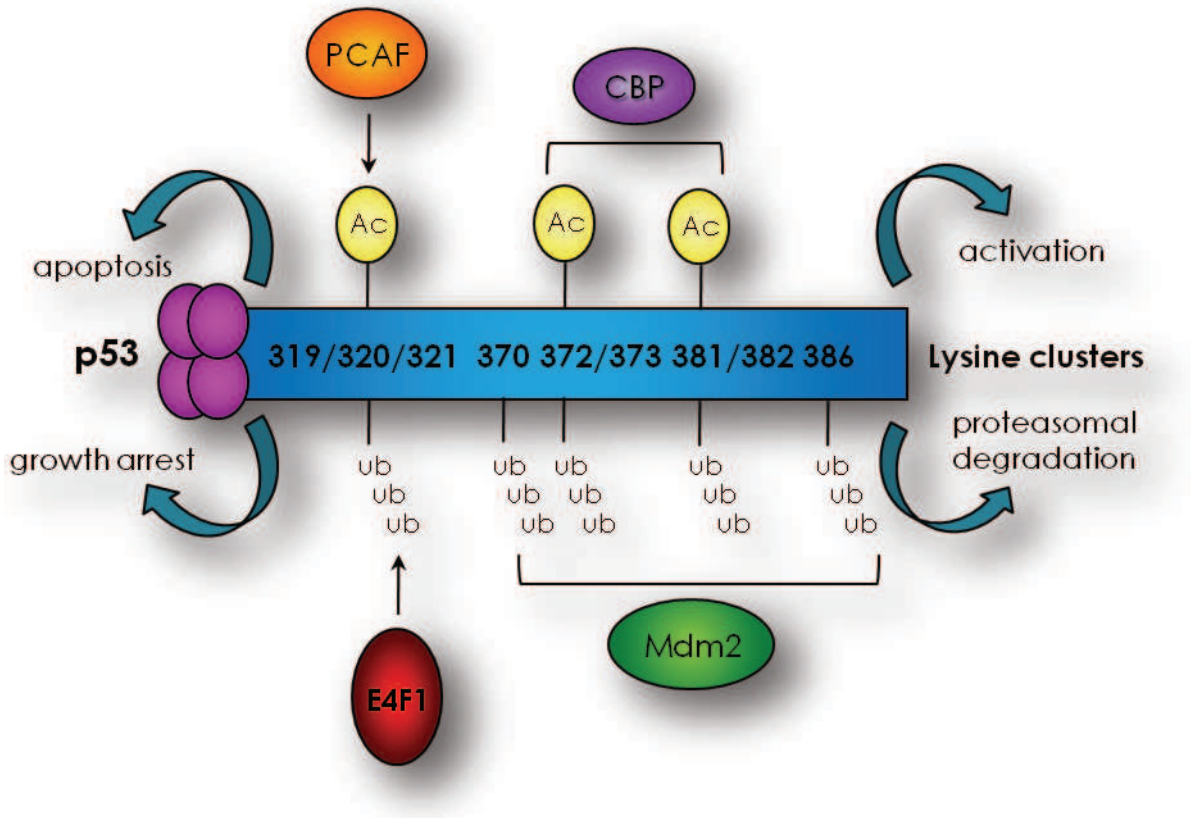

Fig. 18 | **Schematic representation of posttranslational modifications occurring at p53 lysine clusters K319–K321 and K370/372/373/381/382/386.** Both clusters are subjected to mutually exclusive post-translational modifications performed by acetyl-transferases (PCAF and CBP/p300, respectively) and Ub E3 ligases (E4F1 and MDM2, respectively). These modifications have different output signals for p53 effector functions. Competition between PCAF-mediated acetylation and E4F1-mediated ubiquitylation at K320 modulates p53 ability to induce either growth arrest or apoptosis (Le Cam, Linares *et al.,* 2006; modified).

2.5. E4F1 implication in various biological processes

The E4F1 multifunctional protein is implicated in many biological processes, such as embryonic development and mitotic progression (Le Cam *et al.*, 2004), proliferation and programmed cell death (Le Cam, Linares *et al.*, 2006), skin homeostasis (Lacroix, Caramel *et al.*, submitted to PNAS), tumorigenesis (Hatchi *et al.*, in preparation), metabolism and senescence.

Studies of E4F1 functions performed by Le Cam's and Sardet's laboratories are based on *E4F1* gene inactivation in the genetically modified mouse models, constitutive and conditional *E4F1* knockouts (Fig. 19).

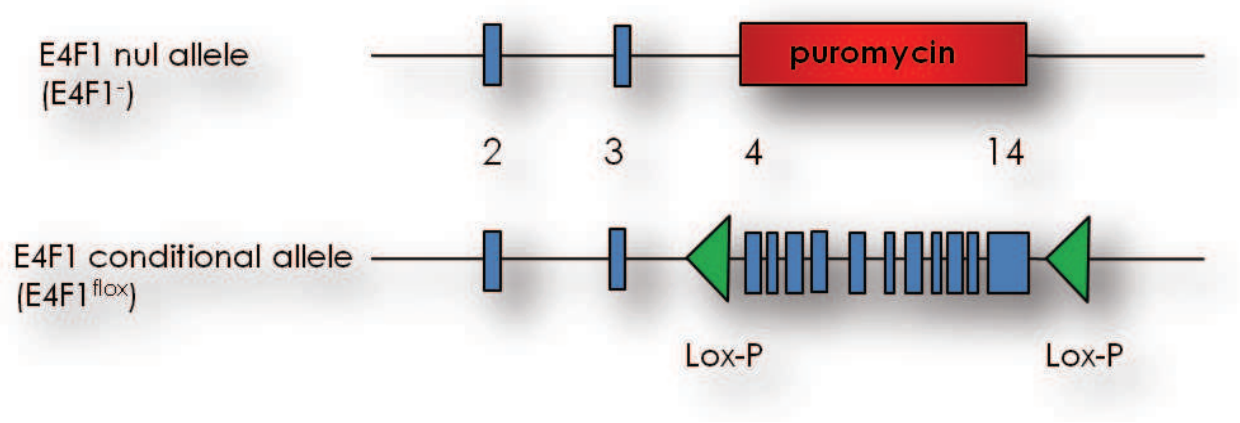

Fig. 19 | **Genetically modified mouse models available in the laboratory to study E4F1 functions (Le Cam *et al.*, 2006).**

2.5.1. E4F1 role in embryonic development

Study by Le Cam *et al.* (2004) showed that *E4F1* is an essential gene during early embryogenesis, as embryos lacking E4F die at the peri-implantation stage (~E5.5), while *in vitro*-cultured *E4F*$^{-/-}$ blastocysts exhibit defects in mitotic progression, chromosomal mis-segregation, and increased apoptosis (Fig. 20). E4F1$^{-/-}$ blastocyst survival until the peri-implantation stage might be explained by the facts that in the early E4F1$^{-/-}$ embryos, stocks of mother E4F1 mRNA are still present and sufficient for early embryo survival. However, those stocks finally become exhausted, which is concomitant with the activation of the zygote genome, and therefore the embryos eventually degenerate.

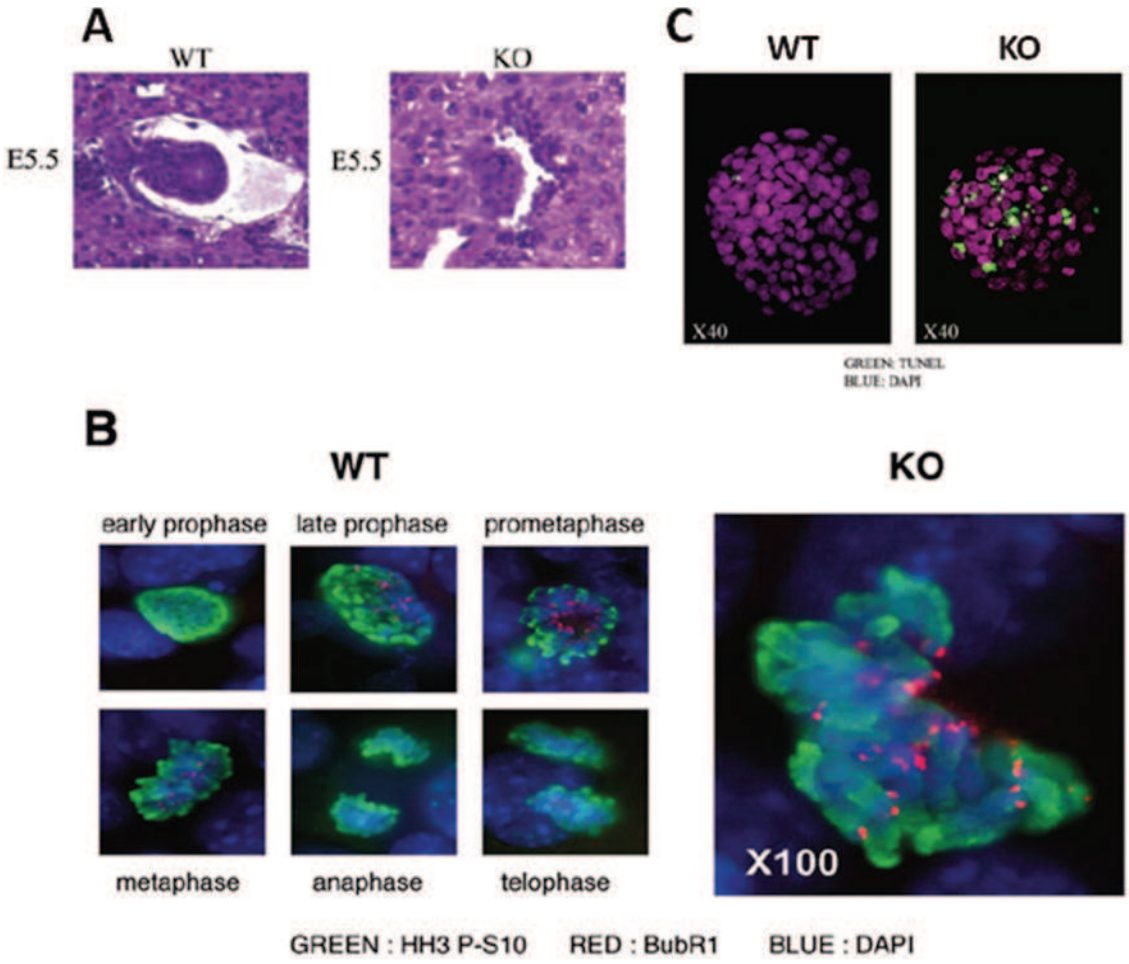

Fig. 20 | **E4F1 is an essential gene during embryogenesis. A** - E4F disruption results in early embryonic lethality; appearance of E5.5 embryos developing *in utero*, as revealed by haematoxylin and eosin staining of histological sections; a typical picture of a WT and of a not yet fully resorbed E4F$^{-/-}$ blastocyst; **B** - Embryonic cells that lack E4F are arrested in prometaphase with an active spindle checkpoint, as revealed by Bub1R immunostaining; **C** - Increased apoptosis in E4F-deficient embryos. Merged image obtained from representative WT and E4F$^{-/-}$ blastocysts cultured for 24 h and processed for DAPI and TUNEL staining (from Le Cam *et al.*, 2006).

2.5.2. E4F1 roles in adult tissues

Because constitutive *E4F1* knockout turned out to be lethal at an early stage of embryogenesis, it was impossible to study E4F1 functions *in vitro* and *in vivo*. To circumvent this inconvenience a conditional *E4F1* KO model, based on the CRE/Lox-P system (see Fig. 19), was developed.

E4F1$^{-/flox}$ mice harbouring *E4F1* conditional KO allele were inter-crossed with several Cre-expressing mouse strains including *Cre-ERT2*$^{KI/KI}$ mice (*RERT*), which express tamoxifen-inducible Cre recombinase-ER^{T2} fusion protein under the control of the ubiquitously active *RNA* pol *II* promoter (equals *E4F1*

inactivation in *all* tissues) (Guerra *et al.*, 2003) or with tissue specific (*K5-Cre*) *Cre/Lox-P* system (E4F1 inactivation *in epidermis*).

2.5.2.1. E4F1 functions in haematopoietic stem cells

E4F1 was recently identified as a novel Bmi-1-interacting partner involved in hematopoietic stem cell (HSC) self-renewal potential. Chagraoui *et al.* (2006) provided evidence that *Bmi-1* and *E4F1* physically and genetically interact in the haematopoietic compartment to regulate HSC self-renewal. Most importantly, it was demonstrated that a reduction of *E4F1* levels through shRNA was sufficient to rescue the clonogenic and repopulating ability of *Bmi-1*$^{-/-}$ haematopoietic stem cells. Using cell lines and MEF, it was also validated that INK4A/ARF and p53 are not essential for functional interaction between Bmi1 and E4F1. More importantly, *Bmi1−/−* foetal liver cells engineered to express low E4F1 levels partially escape senescence but without affecting $p16^{INK4A}$ or ARF protein and mRNA levels.

2.5.2.2. E4F1 functions in epidermal stem cells

Analysis of *E4F1* conditional inactivation in newborn and adult skin revealed an important function for E4F1 in epidermal stem cell maintenance (Lacroix, Caramel *et al.* in press). *E4F1* inactivation in the entire skin or in the basal compartment of the epidermis induced skin homeostasis defects, such as transient hyperplasia in the interfollicular epithelium and alteration of keratinocyte differentiation, followed by loss of cellularity in the epidermis and severe skin ulcerations. Importantly, it was found that *E4F1* depletion alters Epidermal Stem Cells (ESC) clonogenic activity *ex vivo* and ends in exhaustion of the ESC pool *in vivo*. Clonogenic potential of *E4F1 KO* ESC was rescued by Bmi-1 overexpression or by *INK4a/ARF* or *p53* inactivation, indicating that the phenotype of *E4F1* KO ESC depends on the interactions of E4F1 with the Bmi-1-ARF-p53 pathway. Skin phenotype of *E4F1* knockout mice is also delayed in animals with *INK4a/ARF* and *E4F1* compound gene deficiencies. These data strongly suggests the existence of a novel regulatory axis essential for ESC-

dependent skin homeostasis implicating E4F1 and the Bmi-1-ARF-p53 pathway.

* * *

The above examples of E4F1 roles in different adult stem cell compartments lead to two different conclusions. Although both show an important link between E4F1 and Bmi-1, the first example shows that the proliferation defect caused by *Bmi-1* inactivation is rescued by E4F1 knockdown. The second shows that the *E4F1* inactivation phenotype is partially rescued by Bmi-1 over-expression and depends on the ARF-p53 pathway (the phenotype rescued in HSCs does not depend on the INK4a/ARF locus).

It would be of profound importance to see whether the senescent phenotype induced by E4F1 over-expression could be by-passed by Bmi-1 over-expression and whether the *Bmi-1-/-* MEFs, which prematurely enter senescence after just a few passages, escape senescence when *E4F1* is inactivated.

2.5.3. E4F1 Involvement in the control of the cell cycle

Several studies strongly suggest that E4F1 plays an important role in controlling the balance between proliferation and cell survival. E4F1 over-expression in both mouse and human cells provokes cell cycle arrest mainly in the G1 phase (Sandy *et al.*, 2000). This anti-proliferative activity of E4F1 depends on E4F1 interactions with proteins p53 (Sandy *et al.*, 2000), Rb (Fajas *et al.*, 2000) and ARF (Rizos *et al.*, 2003), and leads to transcriptional repression of genes essential for the G1/S transition, such as *cyclin A2* gene (Fajas *et al.*, 2001). E4F1 E3-ligase activity is implicated in establishment of the G1 cell cycle arrest, as E4F1 modulates p53 activity by promoting activation of cell-cycle arrest genes rather than those involved in executing apoptosis (Fig. 21).

It also seems that E4F1 anti-proliferative activity implicates $p21^{CIP1/WAF1}$ through an as yet not well-known mechanism of $p21^{CIP1/WAF1}$ post-transcriptional regulation (Fernandes *et al.*, 1998).

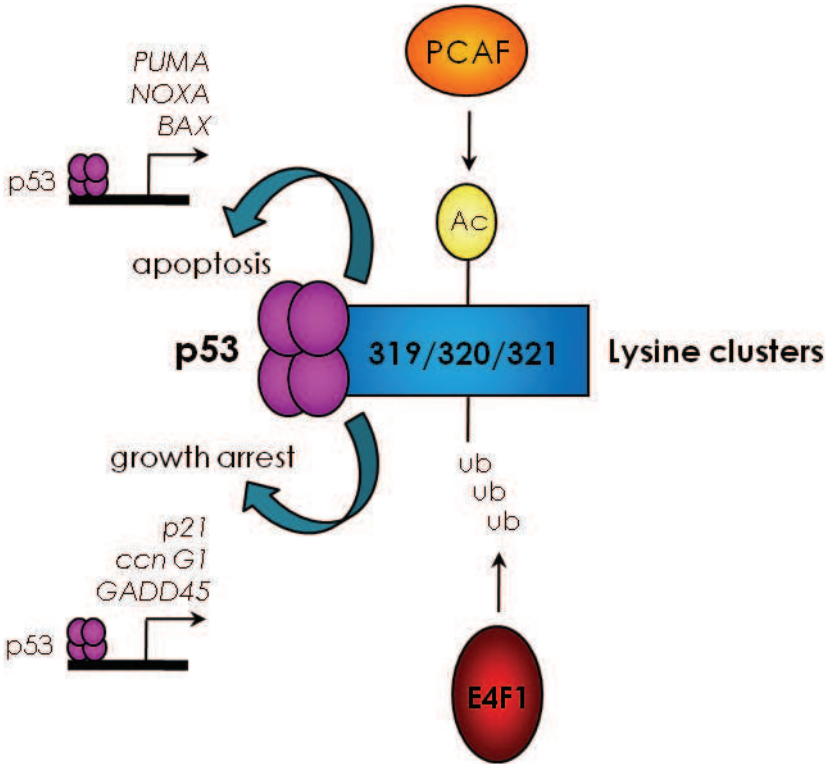

Fig. 21 | **E4F1 acts as a 'router' of p53 response and induces cell cycle arrest rather than apoptosis** (after Le Cam, Linares *et al.*, 2006, modified)

The anti-proliferative effect of E4F1 over-expression might suggest that in the absence of E4F1, cells may present an opposite phenotype and thus proliferate much faster. This notion was confirmed by the work of Chagraoui *et al.* (2006) showing that NIH3T3 immortalised mouse fibroblasts treated with shRNA *against* E4F1 proliferate faster that the cell control group. On the other hand, our laboratory data obtained on mouse embryonic fibroblasts (MEFs) demonstrate that *E4F1* inactivation induces a p53-dependent "mixed" (in G1 and G2) cell-cycle arrest. Moreover, laboratory preliminary data show that E4F1 over-expression in MEFs induce G1 cell-cycle arrest with evidence of premature senescence (Fig. 22).

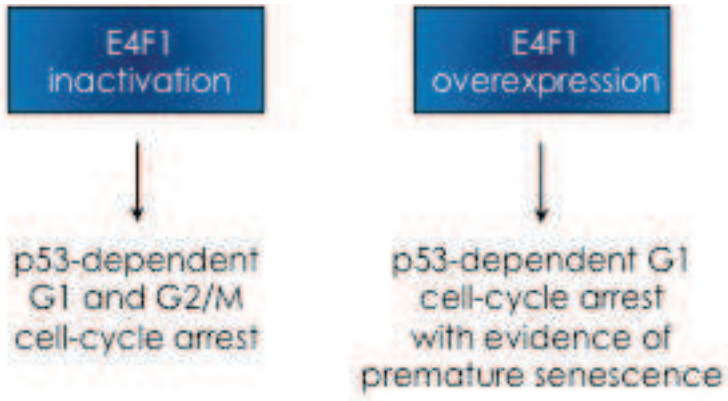

Fig. 22 | **Effects of E4F1 depletion or over-expression in mouse embryonic fibroblasts.**

In addition, E4F1 is important for the mitotic progression during early embryogenesis. Careful examination of mitotic figures in E4F1$^{-/-}$ embryos revealed that an unusually large proportion of them corresponded to the prometaphase stage (phase during which chromosomes are already fully condensed but not yet aligned on the metaphase plate). Consequently, metaphase, anaphase and telophase mitotic figures were observed less frequently in *E4F1*-deficient embryos than in their WT counterparts. The lack of *E4F1* resulted in a chromosomal misalignment, which led to the chronic activation of the spindle checkpoint.

2.6. Cancer-related studies and E4F1

E4F1 physically interacts with tumour-suppressor proteins: p53 (Sandy *et al.*, 2000; Le Cam, Linares *et al.*, 2006), Rb (Fajas *et al.*, 2000), ARF (Rizos *et al.*, 2003) and RASSF1A (Fenton *et al.*, 2004) as well as the oncogenes Bmi-1 (Chagraoui *et al.*, 2006) and HMGA2 (Tessari *et al.*, 2003)(Fig. 23).

Through its anti-proliferative role E4F1 could be considered to have a tumour- suppressor activity. Unfortunately, embryonic lethality of the *E4F1* constitutive knockout did not allow such activity to be assessed and heterozygous (E4F1$^{+/-}$) mice do not develop spontaneous tumours with increased occurrence compared to their wild-type (E4F1$^{+/+}$) littermates. This demonstrates that inactivation of one *E4F1* allele is not sufficient to modify tumour susceptibility.

Nevertheless, the assessment of full *E4F1* depletion was needed to reach a concluaion on E4F1 implication in tumorigenesis. The answer came with the work of Hatchi *et al.* (manuscript in preparation). Using hematopoietic tumor prone mouse model based on *INK4a-ARF* KO and *E4F1* conditional knockout allele, it was demonstrated that inactivation of the *E4F1* gene in *INK4a/ARF* null mice leads to visible tumour regression, results in massive cell death of tumour cells and therefore increases the lifespan of the animal.

Notably, E4F1 interacts with proteins that have a crucial role in the establishment and maintenance of cellular senescence, p53, Rb, ARF and Bmi-1. Preliminary data obtained in the laboratory showed that E4F1 over-expression in mouse embryonic fibroblasts leads to G1 p53- dependent cell-cycle arrest with evidence of premature senescence.

2.6.1. E4F1 and Rb

Rb–E4F1 complexes are formed between the hypophosphorylated (non-active) form of Rb and E4F1 only in cell-cycle arrested cells (Fajas *et al.*, 2000). Rb enhances both E4F1 DNA binding activity and E4F1 mediated promoter repression. By using Rb-deficient cells it was demonstrated that the anti-proliferative effect of E4F1 is, at least in part, dependent on the presence of a functional *Rb* gene. E4F1–Rb interaction involves the C-terminal part of Rb, downstream of the A/B pocket domains. Beside E4F1, this C-terminal region of Rb was reported to bind other proteins important for cell proliferation, including the high mobility group (HMG) box-containing transcription factor UBF (Voit *et al.*, 1997), the c-Abl tyrosine kinase (Whitaker *et al.*, 1998) and the transcription factor c-Jun (Nead *et al.*, 1998).

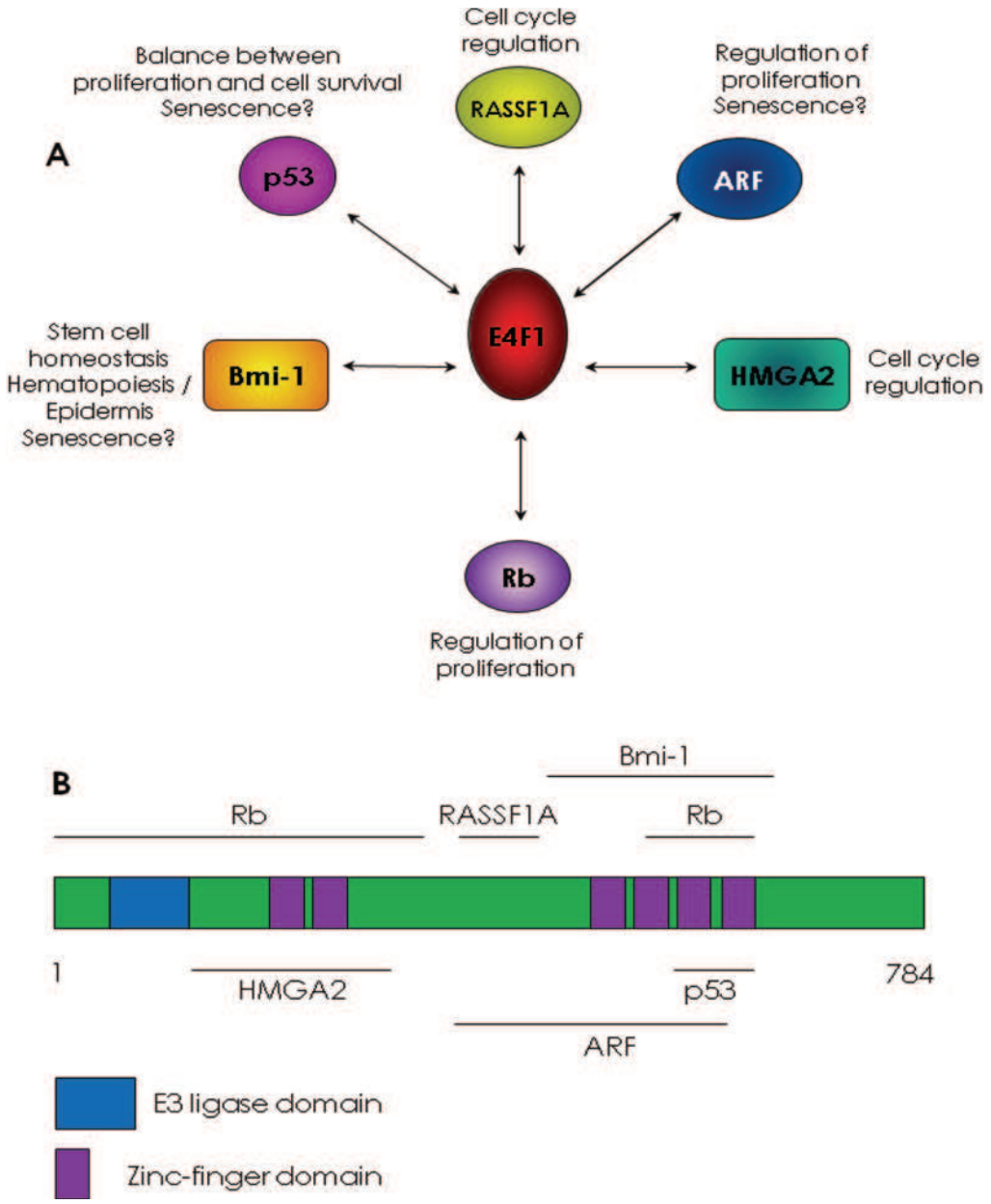

Fig. 23 | **E4F1 interacts with crucial regulators of cell cycle: A** – proteins that physically interact with E4F1; **B** – Regions of E4F1 implicated in protein-protein interactions.

Consistent with the key role played by these factors in growth control, it was shown that the C-terminal part of Rb contributes to Rb-mediated growth suppression. Two E4F1 domains, localized at the N- and C-terminal end of the protein, interact with Rb (Fig. 23B, Fajas *et al.*, 2000). Significance of this interaction during the cell cycle progression of normal and transformed cells remains to be better characterised.

2.6.2. E4F1 and p53

As mentioned earlier in the introduction, abnormalities of the p53 tumour-suppressor gene are among the most frequent molecular events in human and animal neoplasia. A large body of evidence places the transcription factor p53 at a critical nodal point of converging pathways from diverse cellular insults that can elicit coordinated cellular responses that result in adaptation to the insult. p53 is one of the most studied proteins in the whole of contemporary biology; it was identified in 1979 by Lionel Crawford, David P. Lane, Arnold Levine and Lloyd Old (Lane and Crawford, 1979; Linzer and Levine, 1979; De Leo *et al.*, 1979). It had been hypothesised to exist before as the target of the SV40 virus, a strain that induced development of tumours. The murine *Tp53* gene was first cloned by Peter Chumakov (Chumakov *et al.*, 1982) and independently in 1983 by Moshe Oren (Oren and Levine, 1982). The human *Tp53* gene was cloned in 1984 (Matlashewski *et al.*, 1984). p53 was initially presumed to be an oncogene due to the use of mutated cDNA following purification of tumour cell mRNA. Its character as a tumour-suppressor gene was finally revealed in 1990 by the laboratory of Bert Vogelstein (Baker *et al.*, 1990).

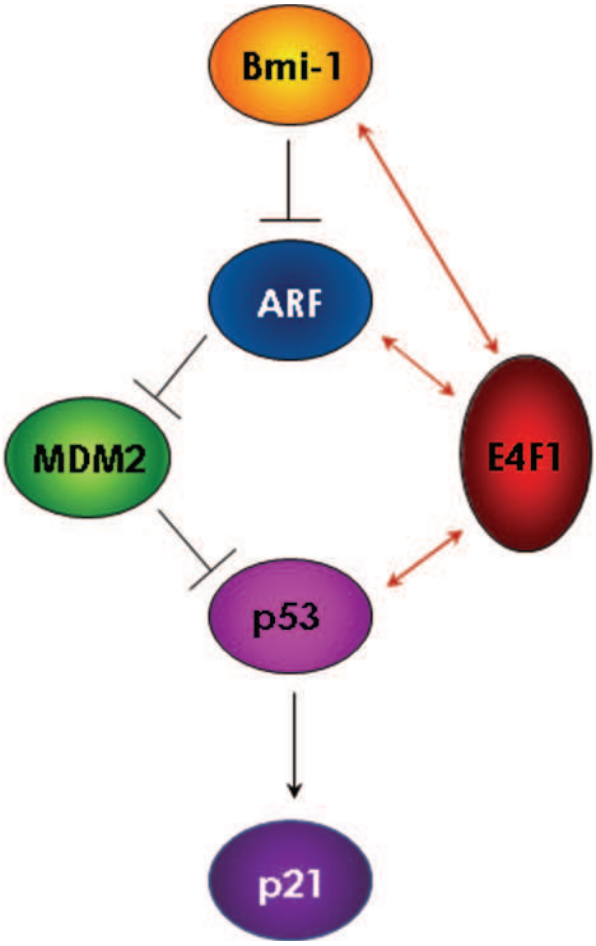

Fig. 24 | **Simplified representation of p53 pathway: E4F1 and p53 pathway.**

In normal, unstressed cells, p53 activity is maintained at low levels through a combination of p53 proteasomal degradation and direct transcriptional squelching, principally mediated by the MDM2 E3-ubiquitin ligase (Fig. 24). By contrast, oncogenic signalling and stress or DNA damage signals (induced by UV, IR, or chemical agents such as hydrogen peroxide) elicit a marked increase in p53 activity, mostly by hindering its interactions with MDM2, which triggers p53 accumulation and unleashes its transcriptional activity. MDM2 activity is regulated by ARF, which induces the p53 pathway by inhibiting MDM2 E3-ligase activity. This leads to stabilisation and activation of p53. Bmi-1, with its repressory function towards *INK4a-ARF* locus, is situated on the top of the p53 signalling pathway. Bmi-1 over-expression represses ARF expression and therefore blocks p53 stabilisation. On the other hand, Bmi-1 knockdown results in de-repression of *INK4a-ARF* locus, ARF expression and finally p53 activation.

p53 functions are regulated by many post-translational modifications, therefore it is not surprising p53 is a central player in many cellular processes (Fig. 25).

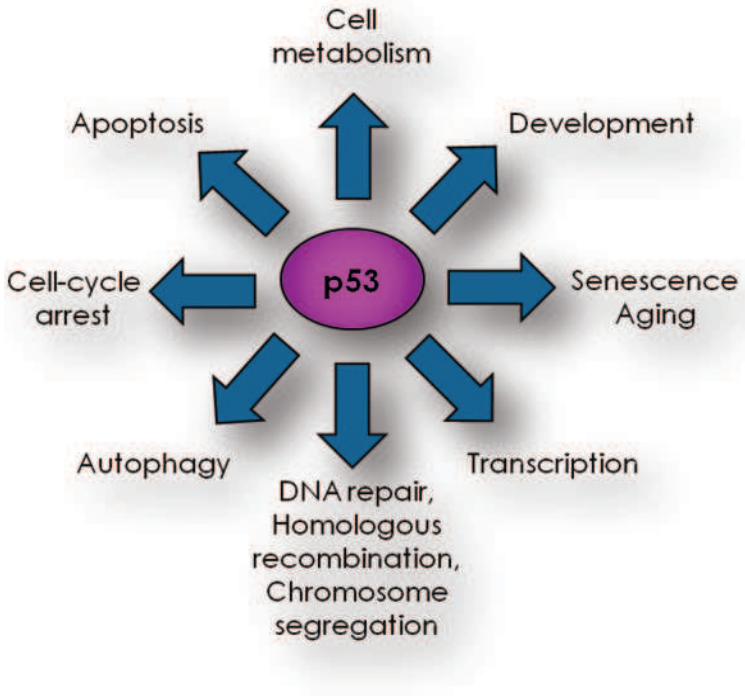

Fig. 25 | **p53 plays a central role in many cellular processes.**

p53 binds E4F1 *in vivo* and *in vitro* and their interaction involves the carboxy-terminal half of E4F1 (Fig 23B) and sequences located at the end of the sequence-specific DNA-binding domain of p53 (Sandy *et al.*, 2000). Ectopic expression of E4F1 results in a block of cell proliferation in several human and murine cell lines and this effect requires the association with wild-type p53 (the growth suppression induced by E4F1 over-expression is severely reduced in a cell line that contains mutant p53).
Interaction between E4F1 and p53 is not only confined to simple protein-protein interaction, it also involves p53 post-translational modifications catalysed by E4F1. This implicates, mentioned earlier in the text, E3-ligase domain of E4F1.

Preliminary data from my laboratory showed involvement of p53 protein in the establishment of E4F1-induced senescence. These data will be presented further, in the chapter concerning the results of my work.

2.6.3. E4F1 and ARF

Locus *INK4a-ARF* is located in the 9p21 region in humans and is often inactivated in tumours with similar frequency to p53. This locus has a unique organisation and encodes for two proteins: ARF (also known as $p19/p14^{ARF}$) and $p16^{INK4a}$ (Fig. 26).

ARF forms a complex with E4F1: E4F1 contacts both ARF and p53 to form a ternary complex *in vivo* and enhances ARF-induced G2 cell cycle arrest in a p53-dependent manner (Rizos *et al.*, 2003). The interaction of ARF and E4F1 forms an important link between the ARF and p53 tumour-suppressor proteins, both of which exhibit enhanced cell cycle inhibitory activity in the presence of this transcriptional repressor.

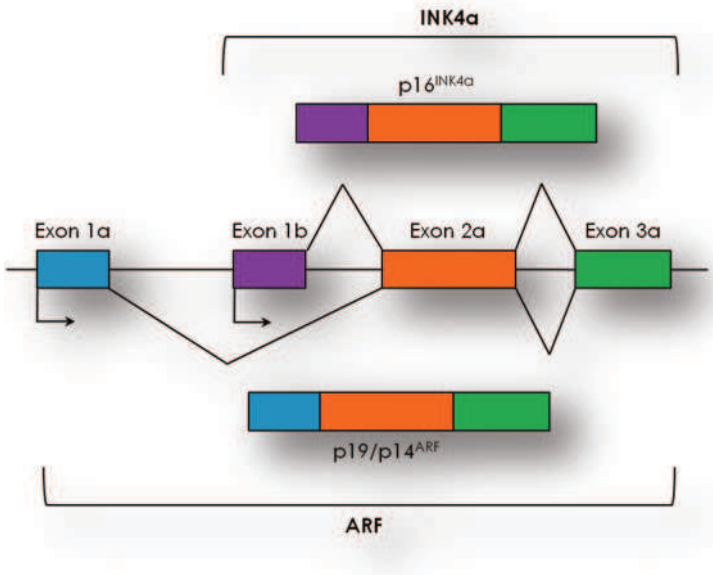

Fig. 26 | **Organisation of the *INK4a-ARF* locus.**

2.6.4. E4F1 and Bmi-1

As mentioned earlier in the introduction (paragraph 2.3.2.1.) Chagraoui *et al.* (2006) have identified Bmi-1 as a new binding partner of E4F1 and provided comprehensive evidence that E4F1 functionally interacts with Bmi-1 to regulate cell proliferation. Several lines of evidence supported this

conclusion: firstly, Bmi-1 was shown to interact physically and genetically both in cell lines (NIH 3T3) and in primary cells (MEF and haematopoietic cells) to control cell proliferation. Secondly, the physical interaction between these two proteins is important for this genetic interaction since the removal of the E4F1-interacting domain of Bmi1 eliminates its ability to rescue the proliferative defect induced by E4F1. Moreover, *INK4A/ARF* and *p53* are independently not required for the genetic interaction between *E4F1* and *Bmi-1* in selected cell lines and in MEFs. Finally and most importantly, the loss of E4F1 function in *Bmi1*$^{-/-}$ haematopoietic cells leads to the rescue of their clonogenic and re-populating ability without affecting levels of ARF or p16^{Ink4a}, thus indicating that the effects of *E4F1* knockdown are not mediated through down-regulation of these two proteins.

2.6.5. E4F1 interaction with other proteins

HMGA2, an architectural transcription factor, which is directly involved in cell proliferation, senescence and neoplastic transformation, associates with E4F1, resulting in the induction of the *cyclin A2* gene (Tessari *et al.*, 2003). Therefore, HMGA2-mediated cyclin A2 induction is due to interference with E4F1 repressing activity. HMGA2 activates the *cyclin A2* gene through binding to the CRE site of *cyclin A2* promoter, which is also recognized by E4F1 (Fajas *et al.*, 2001). This raises the question of how the access of E4F1 to this site and subsequent transcriptional regulation are controlled. HMGA proteins can modulate the access of transcription factors to DNA and are regarded as highly connected nodes of protein-DNA and protein-protein interactions. Indeed, Tessari *et al.* (2003) showed in their report that HMGA2-dependent activation of the *cyclin A2* gene through the CRE site also occurs in the absence of ectopic expression of E4F1. This could be explained by assuming a competition with the endogenous E4F1, but it could also be due to an effect of HMGA2 on other transcription factors binding to CRE. The question remains unanswered.

RASSF1A (RAS association domain family 1 isoform A) is a tumour suppressor whose inactivation (mainly due to its promoter methylation) is

implicated in the development of many human cancers. RASSF1A inhibits the *cyclin A2* promoter through increasing E4F1-mediated repression; it affects the binding of E4F1 to the *cyclin A2* promoter both *in vitro* and *in vivo* (Choudhury *et al.*, 2005).

Research Questions and Experimental Model

3. Research questions

To study senescence, and especially involvement of a particular protein in this cellular process, it is necessary to possess an adequate genetic model. Such model should allow investigating senescence in the presence of a specific protein, in its absence and during its forced overexpression. Therefore, in order to obtain a conclusive data it is extremely important to compare physiological to non-physiological conditions

Preliminary data obtained in our laboratory showed that overexpression of E4F1 in mouse embryonic fibroblasts led to G1-phase cell-cycle arrest with an evidence of premature senescence. These data and the fact that E4F1 interacts with proteins inextricably related to senescence, such as p53, ARF, Bmi-1, strongly suggest a substantial role of E4F1 in signalling pathways involved in cellular senescence.

The biological question that I attempted to answer was to address the significance of E4F1-mediated activities in cellular senescence, to investigate in depth the E4F1-induced senescent phenotype and to try to characterise mechanisms involved in its establishment.

4. Experimental Model

In order to answer the biological question of my PhD thesis a good selection of experimental models was needed. Majority of my laboratory work is based on *in vitro* studies and implicates the *E4F1* conditional knockout mouse model. This particular choice of experimental model enabled me to pursue the studies of E4F1 *in vitro* functions as this model permits to control *E4F1* expression in time and space (*E4F1* constitutive knock-out is lethal at perimplantational state of embryogenesis, Le Cam *et al.*, 2004). Schemes illustrating the experimental model and scientific approach are presented on Fig. 27 and 28.

As a "direct" model I used primary mouse embryonic fibroblasts (MEFs) originating from mouse embryos at the E13.5 stage of gestation. Mouse crosses were established in a particular manner so as to obtain only $E4F1^{+/flox}$ and $E4F1^{-/flox}$ genotypes (Fig. 27). Isolation of mouse embryonic fibroblasts was performed accordingly to a classical laboratory protocol.

The study of E4F1 involvement in senescence is based on two major approaches, which are: E4F1 overexpression and E4F1 depletion in MEFs (Fig. 28). First approach consists of a simple stable overexpression of E4F1 with or without additional pro-senescent stimulus (H-RAS^{V12}) (Fig. 28 A); second comprises *E4F1* depletion combined with targeting the p53 pathway on three different levels and with three different pro-senescent stimuli (Fig. 28 B): (1) expression of constitutively active RAS mutant – H-Ras^{V12}, (2) silencing Bmi-1 expression and therefore de-repressing *INK4a-ARF* locus, and finally (3) a direct induction of DNA damage. Moreover, some experiments were performed in 3 different genetic backgrounds, that is: wild-type background, $p53^{-/-}$ and $INK4a\text{-}ARF^{-/-}$ backgrounds.

As a complementary model I used WI-38 primary human fibroblasts in order to recapitulate E4F1 overexpression experiments performed in mouse embryonic fibroblasts.

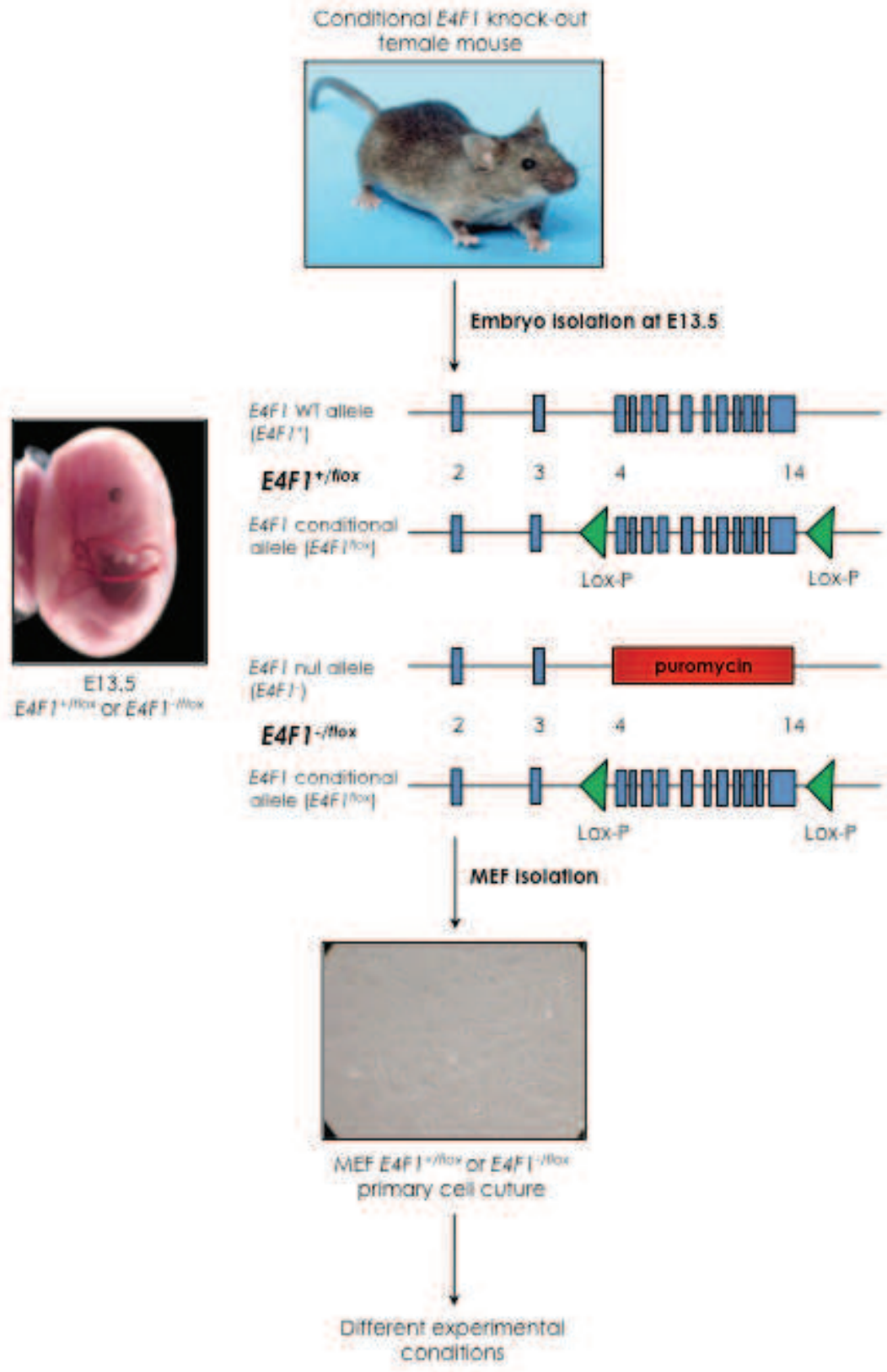

Fig. 27 | **Mouse experimental model used during my PhD thesis** (mouse photo taken from: http://brainmaps.org/index.php?p=info-mus-musculus; embryo photo taken from: http://www.nikonsmallworld.com/gallery/year/2008/79).

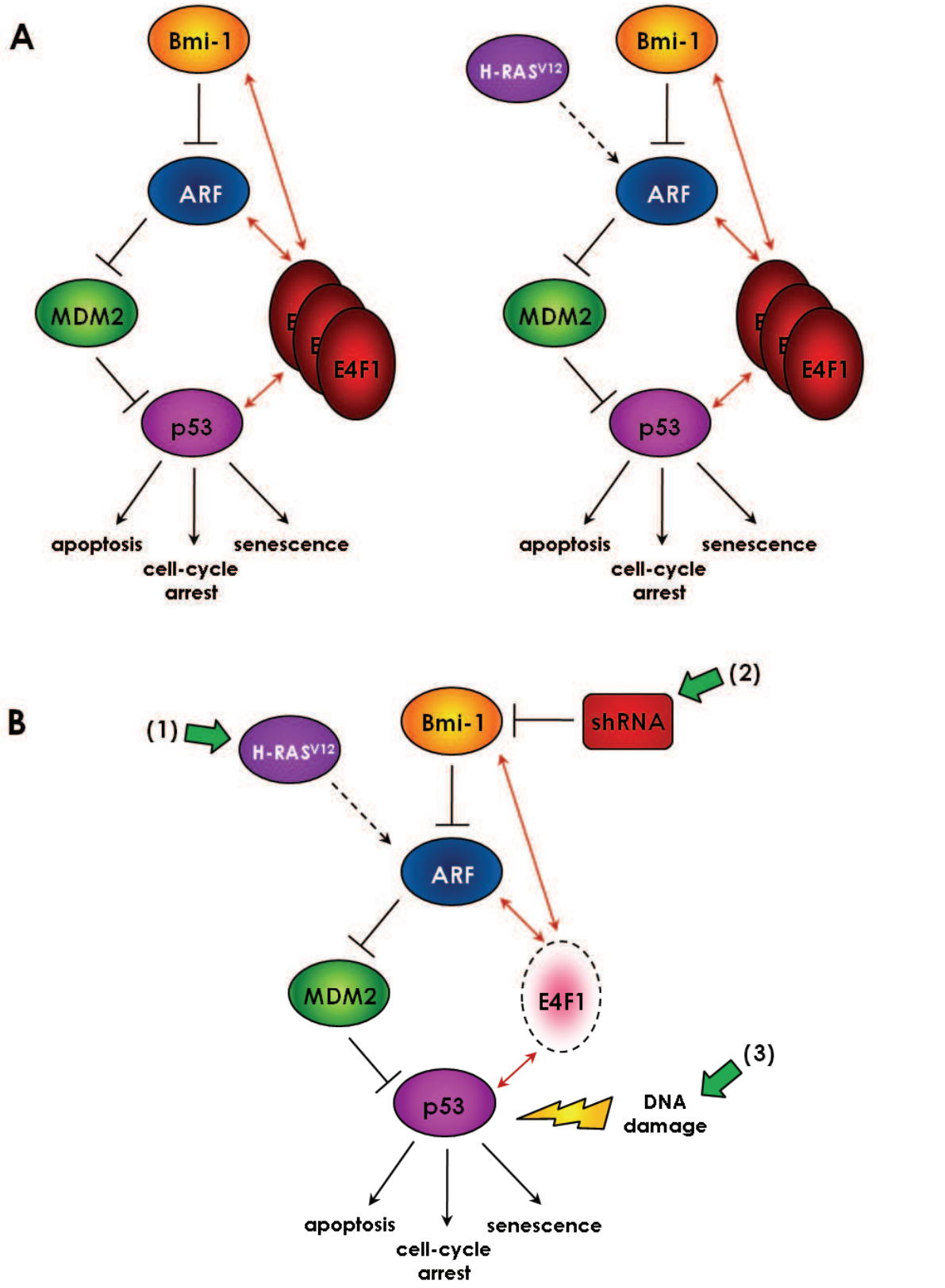

Fig. 28 | **Two approaches to study in vitro E4F1-mediated senescence**. **A** – E4F1 overexpression alone or E4F1 and H-RAS^{V12} simultaneous overexpression; **B** – *E4F1* depletion and either H-RAS^{V12} overexpression (1) or Bmi-1 knockdown (shRNA) (2), or finally treatment with DNA damaging drugs (doxorubicin, etoposide) (3). Those three different experimental conditions are indicated with green arrow.

Materials and Methods

Generation of *E4F1^{flox} RERT* mice

E4F1^{flox} mutant mice were generated as described before (Lacroix, Caramel *et al.,* in press). *E4F1^{flox}* mice were crossed with *Cre-ER^{T2}* knock-in mice (*RERT*) (Guerra *et al.,* 2003) that ubiquitously express tamoxifen-inducible fusion protein CRE-ER^{T2} under the control of the locus encoding the large subunit of RNA polymerase II. These mice were then intercrossed to obtain appropriate genotypes and maintained on a mixed 129Sv/J/DBA/C57BL/6 background.

E4F1^{flox}; RERT mice were genotyped by PCR on tail genomic DNA using the following primers: i) *E4F1* wild type (*E4F1^{+}*) and conditional knock-out flox alleles (*E4F1^{flox}*): 5'-CCTTGAGCACGGAGGAGAGC-3' and 5'-GCCCTAGCCTGCTC-TGCCATC-3', ii) *E4F1* constitutive knock-out allele (*E4F1^{-}*): 5'-CACTGCCTTGGAGGACTTTG-3' and 5'-CCTCTGTTCCACA-TACACTTCATTC-3', iii) wild type and knock-in *RERT* alleles: 5'-GTCAGTACACATACAGACTT-3', 5'-TGAGCGAACAGGGCGAA-3' and 5'-TCCATGGAGCACCCAGTGAA-3' respectively.

E4F1^{flox}; RERT mice were also crossed with *INK4a/ARF$^{-/-}$* (Serrano *et al*., 1996) or *p53$^{-/-}$* mice (Jacks et al., 1994) to generate *E4F1^{flox}; RERT$^{KI/KI}$; INK4a-ARF$^{-/-}$* or *E4F1^{flox}; RERT$^{KI/KI}$; p53 $^{-/-}$* animals.

All animal experiments were approved by and conform to the standards of a regional ethic committee.

Isolation of Primary Mouse Embryonic Fibroblasts (MEFs), Cell Lines and Culture Conditions

E4F1$^{flox/flox}$; RERT$^{KI/KI}$ females were mated with *E4F1$^{+/-}$*; RERT$^{KI/KI}$ males and 12–16 hours later females were examined for the presence of a vaginal ejaculatory plug. The day of plug detection was determined as E0.5 of pregnancy. *E4F1$^{+/flox}$; RERT$^{KI/KI}$* and *E4F1$^{-/flox}$; RERT$^{KI/KI}$* MEFs were harvested from E13.5 embryos according to standard procedures (Manipulating the Mouse Embryo: A Laboratory Manual, Third Edition). Cells trypsinised and replated

from dishes receiving the embryonic cell suspensions were designated as passage 0 (p.0). MEFs were seeded on to cell culture dishes and cultured at 37°C and 5% CO_2 in DMEM + GlutaMAX™ – I (GIBCO), 10% FBS (GIBCO) and antibiotic-antimycotic mix (GIBCO). Cells were split every two days and cells no older than p.4 were used for further experiments.

For a number of experiments, appropriate crosses were established in order to obtain *E4F1* ^{WT}; *INK4a-ARF* $^{-/-}$ and *E4F1* ^{WT}; *INK4a-ARF* $^{+/+}$; *E4F1* ^{WT}; *p53* $^{-/-}$ and *E4F1* ^{WT}; *p53*$^{+/+}$.

WI38 human primary fibroblasts were obtained from ATCC and cultured in the same conditions and medium as MEFs.

Growth curves for some experiments were performed as follows: 2 x 10^6 cells were plated in 10 cm Petri dishes and counted every two to three days. At least three time points were counted.

Production of Retroviral Particles and Transduction of Primary Cells

293T cells (1.8 x 10^6) were plated in 10cm dish, incubated for 24h and then transfected using jetPEI™ reagent with 3μg of a retroviral plasmid (for a minimum of 5h at 37°C). The following retroviral plasmids were used: HR-MMP Cre GFP, empty vector pMSCV hygro, pMSCV hygro H-RAS ^{V12}; pRS blasticidin shGFP, pRS blasticidin shBmi1; empty vector pSCVEF-1 puromycin, pSCVEF-1 puromycin E4F1 p120. Cell transfection was performed twice at intervals of 24h and supernatant was collected and filtered (0.45μm Minisart® filter, Sartorius Stedim Biotech) 48h after the second transfection. MEFs were transduced either with freshly collected or thawed viral supernatants for at least 8h at 37°C in the presence of polybrene (8μg/ml, Sigma). Appropriate antibiotic selection was administered to infected cells 48h after retroviral infection. Selection was maintained for three days (puromycin, blasticidin) or six days (hygromycin).

Proliferation and Cell Death Assay

Proliferation was assessed on cells pulsed for 4h with bromodeoxyuridine (BrdU, 75 μM, Sigma). Cells were detached with trypsin, fixed in cold 70% ethanol, and treated as follows (DPBS (GIBCO) washes between each step): 2M HCl - 0.5% Triton X-100 for 30 min at room temperature; wash with borate buffer (pH = 8.5; Sigma); incubation with non-conjugated mouse monoclonal anti-BrdU antibody (Becton Dickinson, #556028, C36634K; diluted 1:30 in PBS – 0,5% Tween 20 – 1% BSA) for 2-3h at room temperature; incubation with anti-mouse FITC-coupled secondary antibody (diluted 1:300 in PBS – 1% BSA) for 1h at room temperature, and, finally, 5μg/ml propidium iodide and 200μg/ml RNAse A in PBS overnight at 4°C. Samples were analysed on a FACS Calibur (Becton Dickinson) and data were processed with Flowjo software (Treestar).

Cell death assay was performed with Annexin V - FITC/7-AAD kit (Becton Dickinson, PN IM3614) according to the manufacturer protocol and samples were submitted to FACS analysis as described above.

Western Blotting Analysis

Cells were washed twice with DPBS and lysed in a lysis buffer (50mM TRIS-HCl [pH 7.4], 1% Triton–X, 100mM NaCl, 5mM EDTA, 5mM NaF, 1mM orthovanadate, 1μM DTT, 1mM PMSF and protease inhibitors. After 30 minutes on ice, lysates were cleared by centrifugation at 12000 rpms Samples corresponding to 40μg of protein were separated on 8, 10 or 12% SDS-PAGE gels and transferred to PROTRAN® membranes (PerkinElmer). Western blot analysis was carried out according to standard procedures using ECL detection (PerkinElmer).

The following primary antibodies were used (1:1000 unless otherwise indicated): rabbit anti-E4F1 (1:500, Fajas et al., 2000), mouse anti-p53 (1C12, Cell Signaling), mouse anti-Bmi1 (clone F6, Millipore), rabbit anti-p16 (M-156, sc-1207, Santa Cruz), rabbit anti-p19 ARF (ab80-100, Abcam), goat anti-p21 (C19, sc-397-G, Santa Cruz), mouse anti-phospho ERK1/2 (9106, Cell

Signaling), rabbit anti-ERK1/2 (9102, Cell Signaling), rabbit anti-RAS (3965, Cell Signaling), mouse anti-α tubulin (Sigma), rabbit anti-β actin (Sigma).

Horseradish peroxidase-conjugated goat anti-mouse (115-035-003, Jackson Laboratories) or goat anti-rabbit (111-035-003, Jackson Laboratories) were used as secondary antibodies.

RNA isolation and Quantative RT-PCR

Total RNA was isolated by TRIzol® (Invitrogen) according to the manufacturer's RNA purification protocol. cDNA was synthesized from 1μg of total RNA using random primers (Invitrogen) and MMLV Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Q-PCR reactions were carried out in a total volume of 10μL using LightCycler® 480 SYBR Green I Master Mix (Roche) in a LightCycler® 480 Real-Time PCR System from Roche Diagnostics. Primers used are given in Table X. QPCR analysis of GAPDH mRNA expression was performed as positive control and for normalization.

Table 10 | **Sequences of primers used for gene expression analysis.**

Primer	*Sequence 5' – 3'*
$p16^{INK4a}$ fwd	GTGTGCATGACGTGCGGG
$p16^{INK4a}$ rev	GCAGTTCGAATCTGCACCGTAG
$p19^{ARF}$ fwd	TCGCAGGTTCTTGGTCACTGT
$p19^{ARF}$ rev	TGCTACGTGA ACGTTGCCCA
$p21^{CIP1/WAF1}$ fwd	TCTATCACTCCAAGCGCAGA
$p21^{CIP1/WAF1}$ rev	CACACAGAGTGAGGGCTAAGGC
E4F1 fwd	CTGGGCATTCTTGGTTTTGT
E4F1 rev	CCAAAGCCTACCTGCTCAAG
Bmi1 fwd	CCAGCAAGTATTGTCCTATTTGTGA
Bmi1 rev	TTGAAGAGTTTTATCTGACCCTTATGTT
DEC1 fwd	GGCGGGGAATAAAACGGAGCGA
DEC1 rev	CCTCACGGGCACAAGTCTGGAA
Dcr2 fwd	AGCTAACCCAGCCCATAATCGTC
Dcr2 rev	AGTTCCCTTCTGACAGGTACTGGC

GAPDH fwd	GCTCACTGGCATGGCCTTCCGTGT
GAPDH rev	TGGAAGAGTGGGAGTTGCTGTTGA

Senescence-Associated β-Galactosidase Assay

SA-β-gal activity was detected as previously described (Debacq-Chainiaux et al., 2009). Cells were plated on glass cover slips at a density of 15 x 10^4 per well and per cover slip in a 6-well plate. 24h later the cells were washed twice with DPBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde in DPBS for five minutes. Then the cells were thoroughly washed with DPBS and submitted to staining in X-GAL solution: 1mg/ml X-GAL (Sigma), 5mM $K_3Fe(CN)_6$ (Sigma), 5mM $K_4Fe(CN)_6$ (Sigma), 2mM $MgCl_2$ in PBS at pH 6.0, overnight at 37°C.

Immunofluorescence

Cells were grown on glass cover slips (15 x 10^4 cells per well in a 6-well plate), fixed with 2% paraformaldehyde for 15 minutes and permeabilised by 0.5% Triton-X for 20 minutes at room temperature. After 1h of blocking in DPBS – 3% BSA cells were incubated overnight with primary antibodies: goat anti-HMGA2 (S-15, sc-23684, Santa Cruz). Next day the cells were washed and incubated for 1 h with secondary antibodies: Alexa Fluor 594 donkey anti-goat (1:1000, A11058, Invitrogen). Finally the cells were stained with DAPI for 15 minutes, mounted with Prolong® Gold antifade reagent (Invitrogen) before viewing.

Other Chemical Reagents

Final concentrations of other chemical reagents used in this study were 30nM doxorubicin (Sigma), 900nM etoposide (Sigma), 1μM 4-hydroxytamoxifen (Sigma).

Results

5. Mouse Embryonic Fibroblasts

5.1. Overexpression of E4F1 induces cell-cycle arrest in wild-type mouse embryonic fibroblasts.

To start with, I decided to confirm the preliminary data obtained in the laboratory. Using a retroviral infection technique I overexpressed *E4F1* in wild-type (WT), *p53* knock-out (*p53* KO) and *INK4a-ARF* knock-out (*INK4a-ARF* KO) primary mouse embryonic fibroblasts (MEFs). For those experiments only cell populations from littermates were used. A human *E4F1* cDNA was introduced into these cells using recombinant replication-deficient retroviruses as described in Material and Methods. The retroviral vectors co-expressed a puromycin gene that allowed selection for pure populations of transduced cells within 4 days (see Fig. 29 for experimental details).

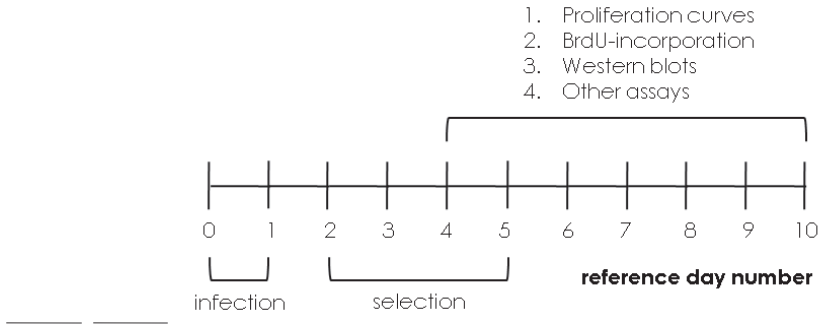

Fig. 29 | **Experimental design and reference time frame.** Cells were infected with *E4F1* retrovirus at day 0. 48hr after infection cells were subjected to puromycin selection during 3 to 4 days. Different analyses were performed starting from the day 4 until day 10.

Overexpression of E4F1 produced morphological changes in WT MEFs: by day 5, a significant number of *E4F1*-transduced WT MEFs acquired a flat and enlarged morphology and apparently were growth arrested (Fig. 30 A, B and C). No changes in morphology were detected in cell populations transduced with empty viral vector or cells transduced with *E4F1* or empty vector and deficient in *p53* or *INK4a/ARF*.

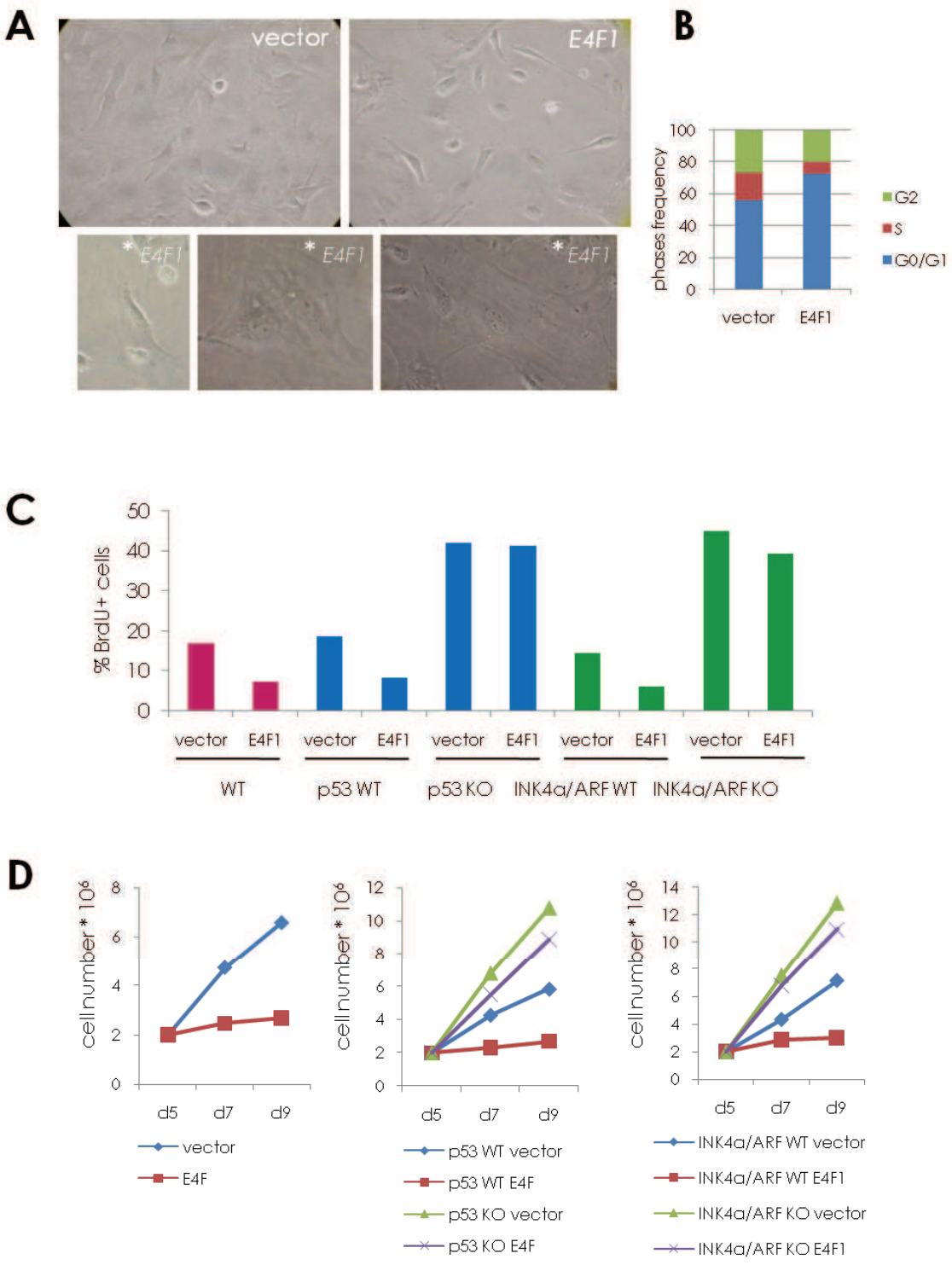

Fig. 30 | **E4F1 overexpression induces a *p53*- and *INK4a/ARF*-dependent cell cycle arrest with evidence of premature senescence.** **A** – growth-arrest of WT MEFs infected with *E4F1* retrovirus, live-cell imagining shows a significant difference in cell density at day 5; *E4F1*-infected cells present a flat and enlarged morphology (*); **B** – E4F1 overexpression leads to cell-cycle arrest in G1 phase; **C** – BrdU analysis shows that E4F1-induced growth arrest is both *p53*- and *INK4a/ARF*-dependent; **D** – Growth curves of MEFs obtained from wild-type, *p53* and *INK4a-ARF* KO mice transduced with an empty retroviral vector (vector) or its derivatives expressing *E4F1* (E4F1). MEFs, mouse embryonic fibroblasts; WT, wild-type; KO, knock-out.

The proliferative properties of cell populations were monitored by growth curves and by BrdU-labelling followed by flow cytometry (Fig. 30 B, C and D). All MEF populations transduced with *E4F1* or with empty vector were plated at low density ($2x10^6$ cells per 10 cm culture dish) to ensure that cells were never arrested by confluency, and cell numbers were counted every 2 days. Wild-type control populations grew steadily and doubled their number within 2 days of culture, control populations lacking *p53* or *INK4a/ARF* grew faster than WT MEFs as they were immortalized. By contrast, WT *E4F1*-transduced cells were evidently growth-arrested. Moreover, E4F1-overexpressing *p53* and *INK4a/ARF* KO MEFs did not cease proliferation and their growth rate was comparable to their control counterparts (Fig. 30 D). BrdU incorporation analysis revealed that *E4F1*-transduced WT populations retained a very small percentage of BrdU-positive cells at day 6 and they were mainly arrested in G1 phase of the cell cycle (Fig. 30 B and C). The proportion of BrdU-positive cells in control WT populations was relatively high (varying between 14 and 19% of BrdU-positive cells). In MEFs deficient in *p53* or *INK4a/ARF*, both in control and E4F1-overexpressing groups, the percentage of BrdU-positive cells was higher (~40% BrdU-positive cells).

Those results led us to conclude that E4F1-ovexpression induces a permanent, *p53*- and *INK4a/ARF*-dependent growth arrest in G1 phase of the cell cycle.

5.2. E4F1- induced growth arrest has characteristics of premature senescence

To further explore the obtained phenotype and decipher the nature of observed cell-cycle arrest I performed SA-β-GAL assay. This test enables to detect at pH 6.0 the activity of senescence-associated β-galactosidase. Cells presenting such activity stain blue and are considered *senescent*. My observations confirmed previous results of the laboratory indicating that E4F1-induced growth arrest possesses characteristics of premature senescence (Fig. 31 A, graph and photographs of SABG staining). MEFs overexpressing *E4F1* but lacking *p53* or *INK4a/ARF* do not enter senescence. This is due to the

disruption of the functional p53 and/or Rb pathways, which are crucial for establishment of senescence in mouse primary fibroblasts.

To complete the characterisation of the nature of the proliferation cease provoked by E4F1 overexpression, I also examined the expression of several cell-cycle regulatory proteins by immunoblotting (Fig. 31 B).

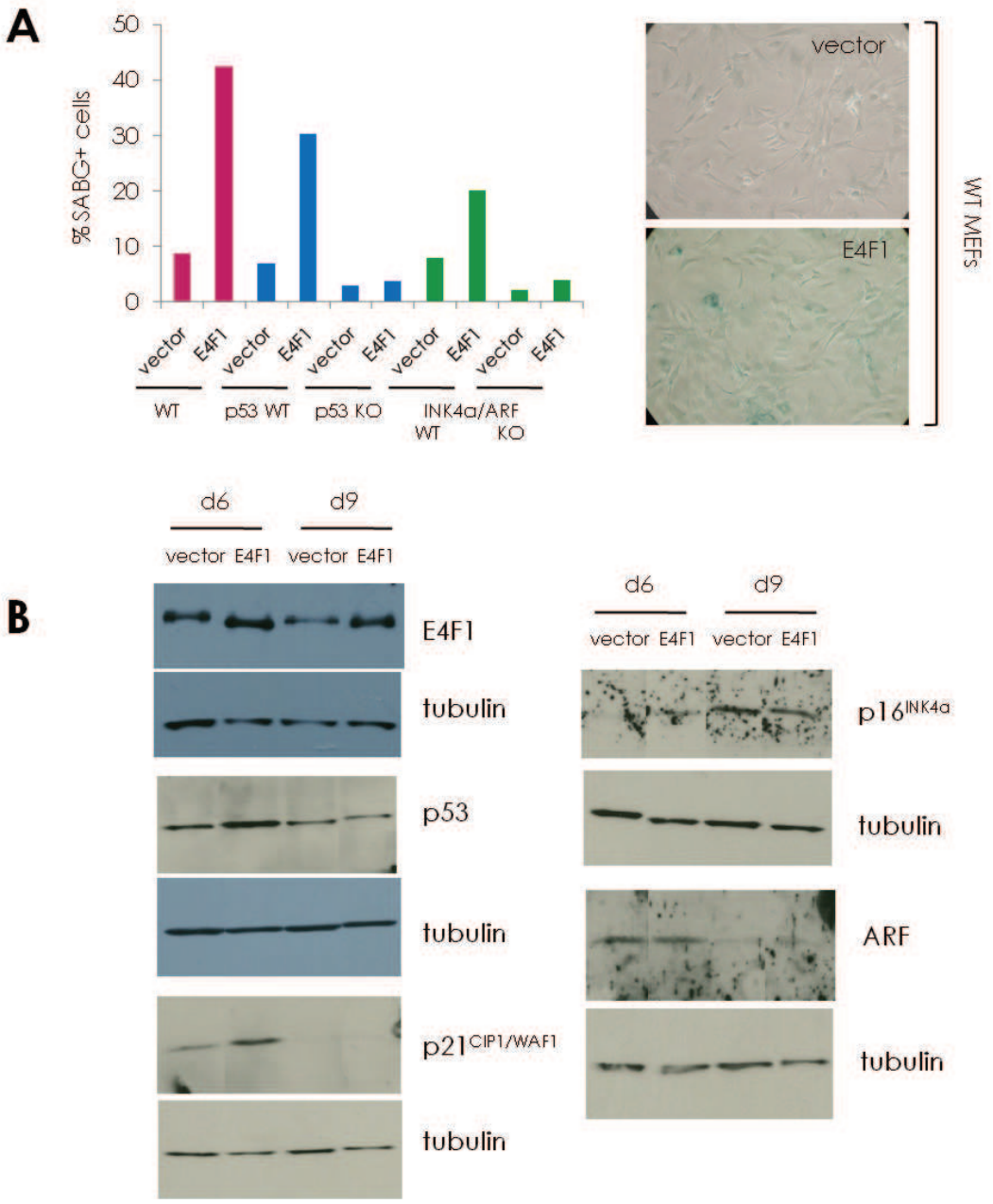

Fig. 31 | **E4F1 overexpression induces growth arrest that possesses characteristics of cellular senescence. A –** percentage of SABG positive cells shows an increase in SA-β-GAL activity in mouse fibroblasts overexpressing *E4F1* and WT for *p53* and *INK4a/ARF;* photographs of the WT MEFs stained for SABG (pH 6.0) activity at day 6 **B** – Effect of *E4F1* on some cell-cycle regulatory proteins in WT MEFs. SABG, SA-β-GAL, MEFs, mouse embryonic fibroblasts; WT, wild-type.

In my experimental conditions, E4F1 overexpression resulted in transient increase in p53 and $p21^{CIP1/WAF1}$ protein levels (Fig. 31 B), which was observed at day 6 but not at day 9. Surprisingly, E4F1 overexpression did not elevate $p16^{INK4a}$ and ARF levels.

Therefore, obtained results suggest that E4F1 overexpression indeed induces senescent phenotype and that establishment of senescence is both *p53*- and *INK4a/ARF*-dependent even though in the latter case E4F1 overexpression does not lead to up-regulation of $p16^{INK4a}$ and ARF – contrary to p53 and its target gene $p21^{CIP1/WAF1}$.

5.3. Simultaneous overexpression of E4F1 and H-RAS^{V12} has a synergic effect and accelerates senescence

In mouse embryonic fibroblasts expression of oncogenic form of RAS – H-RAS^{V12} (mutated by replacement of glycine 12 with valine, which results in a constitutively active RAS protein) – induces a permanent growth arrest in G1 phase of the cell cycle. Cell-cycle arrest induced by RAS is accompanied by accumulation of p53 and $p16^{INK4a}$, and is phenotypically indistinguishable from cellular senescence (Serrano *et al.,* 1997).

I tested a hypothesis that co-expression of E4F1 and H-RAS^{V12} might have a synergic effect and accelerate senescence. Experiment design is presented on Fig. 32.

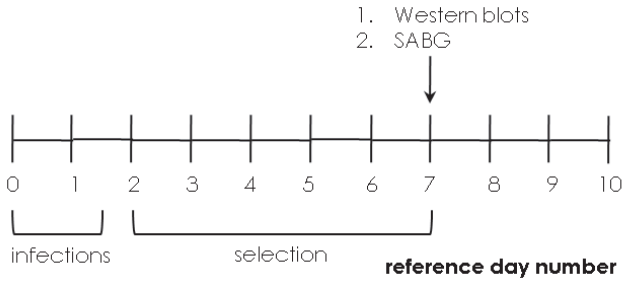

Fig. 32 | **Experimental design and reference time frame for E4F1 and oncogenic RAS co-expression.** Cells were infected in following order: day 0 – *E4F1*, day 1 – *H-RAS*^{V12}; selection was administered 48h after every infection (puromycin for *E4F1*, hygromycin for oncogenic *RAS*). Immunoblots and SABG staining were performed at day 7. SABG, SA-β-GAL.

First, I analysed the expression of some cell-cycle regulatory proteins by immunoblotting. Total cell lysates were prepared at day 7. Expression of oncogenic RAS provoked an increase in p53, $p21^{CIP1/WAF1}$, $p16^{INK4a}$ and ARF levels (Fig. 33 A). E4F1 overexpression also led to p53 stabilisation and induction of $p21^{CIP1/WAF1}$. However, no $p16^{INK4a}$ and no ARF induction were observed. Co-expression of E4F1 and $H\text{-}RAS^{V12}$ also resulted in p53 and $p21^{CIP1/WAF1}$ induction that was comparable to levels obtained when E4F1 or oncogenic RAS were overexpressed alone (Fig. 33 A).

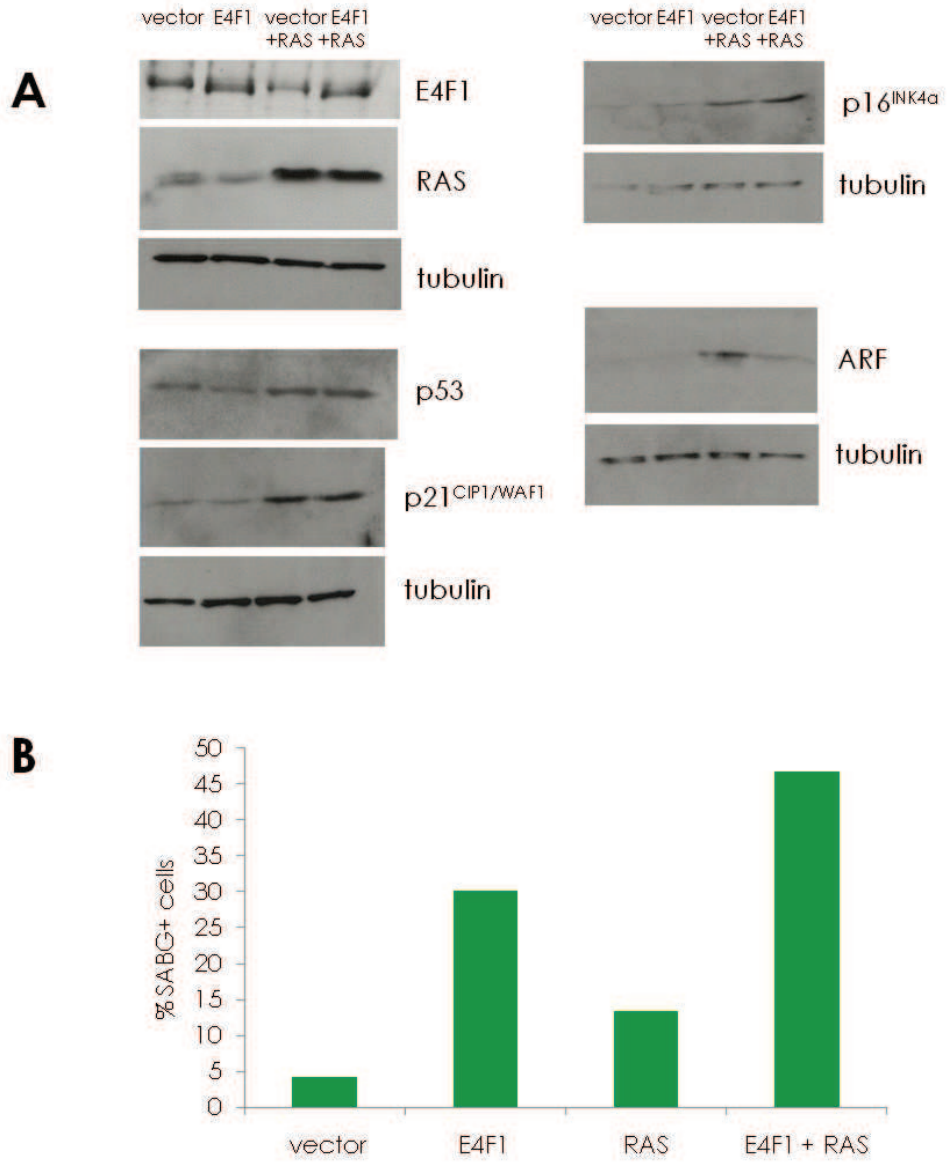

Fig. 33 | **E4F1 and $H\text{-}RAS^{V12}$ cooperate to induce cellular senescence. A** - effect of $H\text{-}RAS^{V12}$ on several cell-cycle regulatory proteins; **B** – SABG staining showing that co-expression of E4F1 and oncogenic RAS potentialises establishment of cellular senescence. SABG, SA-β-GAL

By contrast, simultaneous overexpression of E4F1 and RAS caused stronger expression of $p16^{INK4a}$ protein and, strikingly, a significant decrease in ARF levels. This could give an evidence of independent *INK4a-ARF* locus regulation these preliminary results have to be confirmed.

To explore further the potential co-operation between E4F1 and H-RAS^{V12} in induction of senescence I examined an additional senescence-associated marker: SABG activity. In my experimental conditions oncogenic RAS efficiently induced senescence: at day 7 I observed 4% of SABG-positive staining in the control cells versus 13,3% of SABG-positive cells upon transduction with *RAS* (Fig. 33 B). As showed on figure 33 B, E4F1 also promotes senescence (30% of SABG-positive cells). Simultaneously expressed E4F1 and RAS co-operate and the pro-senescent signal become enforced which results in higher (46,6%) percentage of SABG-positive cells comparing to cells expressing either E4F1 or RAS alone (Fig. 33 B). Hence, this result shows a synergy of action of those two pro-senescent proteins.

5.4. Expression of H-RAS^{V12} leads to cell cycle arrest in both *E4F1* WT and KO MEFs

Another way of deciphering E4F1 role in cellular senescence is the *E4F1*-gene inactivation approach. I "switched off" *E4F1* using an inducible CRE-lox system. For details on the gene-inactivation procedure see Material and Methods, Research Questions and Experimental Model, and Fig. 34. As continuous expression of the Cre recombinase in cultured mammalian cells is toxic and causes decreased growth of cells, a self-excising CRE retroviral vector was used in all experiments. To ensure a good depletion of *E4F1* only RERT MEFs were used, since administration of 4-OH-tamoxifen additionally induced *E4F1* excision.

The first approach involved studies of *RAS*-induced senescence in wild-type (WT) and *E4F1* knockout (*E4F1* KO) MEFs.

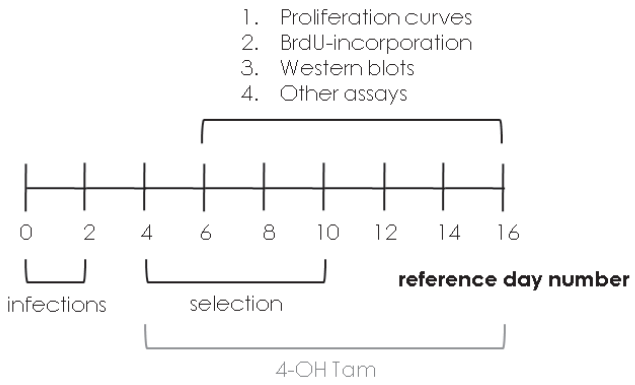

Fig. 34 | **Experimental design and reference time frame for H-RAS^{V12} induced senescence.** At first, cells were infected with *CRE* retrovirus (day 0) then with *H-RAS^{V12}* (day 1). 48hr after infection with oncogenic *RAS* cells were subjected to hygromycin selection. The selection was maintained at least 5 days. In order to ensure a good rate of *E4F1* depletion 4-OH-tamoxifen was added together with selection antibiotic and maintained during all duration of the experiment. Different analyses were performed starting from the day 6 until day 16.

Expression of H-RAS^{V12} produced morphological changes in mouse embryonic fibroblasts (Fig. 35 A). Initially, both WT and KO *E4F1 H-RAS^{V12}*-infected cells displayed a refractile cytoplasm with thin and long projections. By day 7 a significant proportion of *H-RAS^{V12}*-transduced WT MEFs became flat and enlarged and was growth arrested. *E4F1* KO MEFs, which were transduced either with empty vector or *H-RAS^{V12}*, displayed a slightly different morphology than their wild-type counterparts: cells were less enlarged but also presented a growth-arrest phenotype. No changes in morphology were detected in wild-type cell populations transduced with empty viral vector.
Proliferation curves revealed that *E4F1* KO, *E4F1* WT and KO expressing *H-RAS^{V12}* MEFs ceased proliferation (Fig. 35 B). BrdU incorporation combined with DNA-content analysis showed that *E4F1* KO, *H-RAS^{V12}*-transduced *E4F1* WT and KO populations retained a small percentage of BrdU-positive cells at day 6 but virtually none at day 12. At early time point (day 6) *E4F1* KO and all *H-RAS^{V12}*-transduced cell populations accumulated preferentially in G1, later, at day 12, the proportion of cells in G2 phase visibly increased (Fig. 35 C). The proportion of BrdU-positive cells in *E4F1* WT control population was high, and declined successively with passages (compare day 6 and day 12).

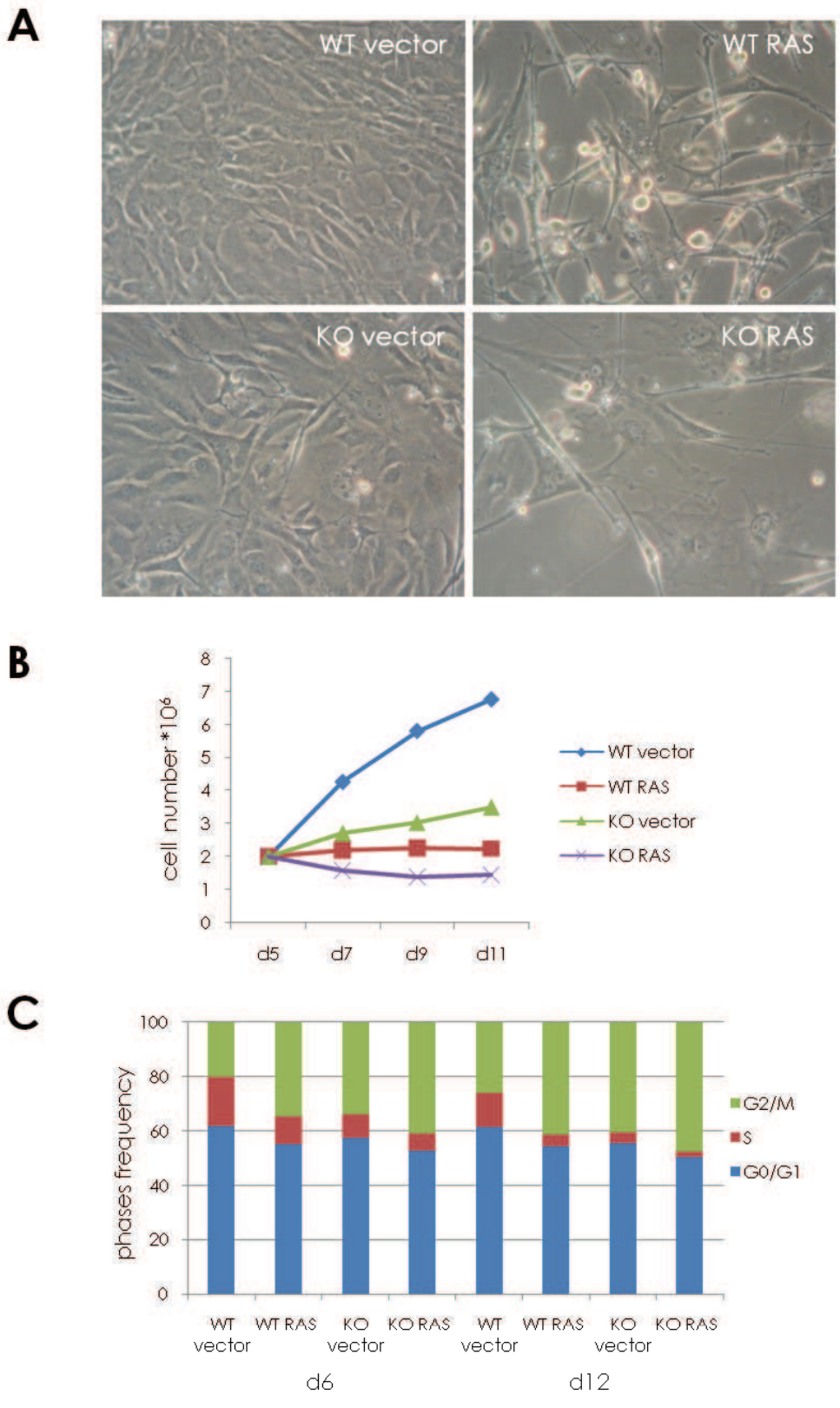

Fig. 35 | **Effect of H-RAS^{V12} on *E4F1* WT and KO mouse embryonic fibroblasts. A –** Cell morphology at day 4. Photographs are at the same magnification**; B –** Representative growth curves corresponding to *E4F1* WT and KO cell cultures transduced with empty vector or with H-*RAS*^{V12}-expressing retroviruses. Each curve was performed at least twice. **C** – Cell-cycle distribution of H-*RAS*^{V12}-expressing and control populations of *E4F1* WT and KO MEFs as measured by BrdU incorporation and DNA-content flow cytometry analysis. WT, wild-type; KO, knock-out; MEFs, mouse embryonic fibroblasts; d, day.

Obtained data show that H-RAS^{V12} provokes cell-cycle arrest in both *E4F1* WT and KO MEFs.

5.5. Constitutive expression of H-RAS^{V12} induces apoptosis in *E4F1* KO MEFs

In order to compare the nature of H-RAS^{V12}-induced cell-cycle arrest in *E4F1* WT and KO MEFs first I analysed the expression of proteins implicated in cell-cycle regulation. Total cell lysates were prepared at day 6 and 12. Control immunoblots of E4F1 depletion (Fig. 36 A) and RAS overexpression (Fig. 36 B) were performed in all experiments. I also verified whether E4F1 influenced MAP kinase signalling pathway and no significant differences in ERK1/2 activation/levels were observed (Fig. 36 B). Analysis of p16^{INK4a} and ARF expression revealed decreased levels of p16^{INK4a} and ARF in *E4F1* KO MEFs infected either with empty vector or H-RAS^{V12}. Such decline was observed both at day 6 and 12 (Fig. 36 C). To see if this effect was transcriptional I performed RT-qPCR analyses of *p16^{INK4a}* and *ARF* mRNA expression (Fig. 37 A). It turned out that levels of both – *p16^{INK4a}* and *ARF* mRNA – were reduced in cells deficient in *E4F1* and infected either with empty or *H-RAS^{V12}* vector comparing to their wild-type counterparts. Analysis of levels of other genes implicated in senescence – *p21$^{CIP1/WAF1}$* and *Dcr2* – showed increase in mRNA levels when cells were transduced with oncogenic *RAS*. No significant difference was observed between *E4F1* WT and KO cells.

To further analyse the obtained cell phenotypes I examined the expression of senescence-associated beta galactosidase (SABG). I found that *E4F1* KO MEFs presented less staining (4,85% of SABG-positive cells) when compared to *E4F1* WT MEFs (7,35%) (Fig. 38 A and B). *E4F1* KO cells transduced with *H-RAS^{V12}* also showed decreased percentage (9,5%) of SABG staining in comparison with *E4F1* WT; *RAS*-transduced cells (16,65%).

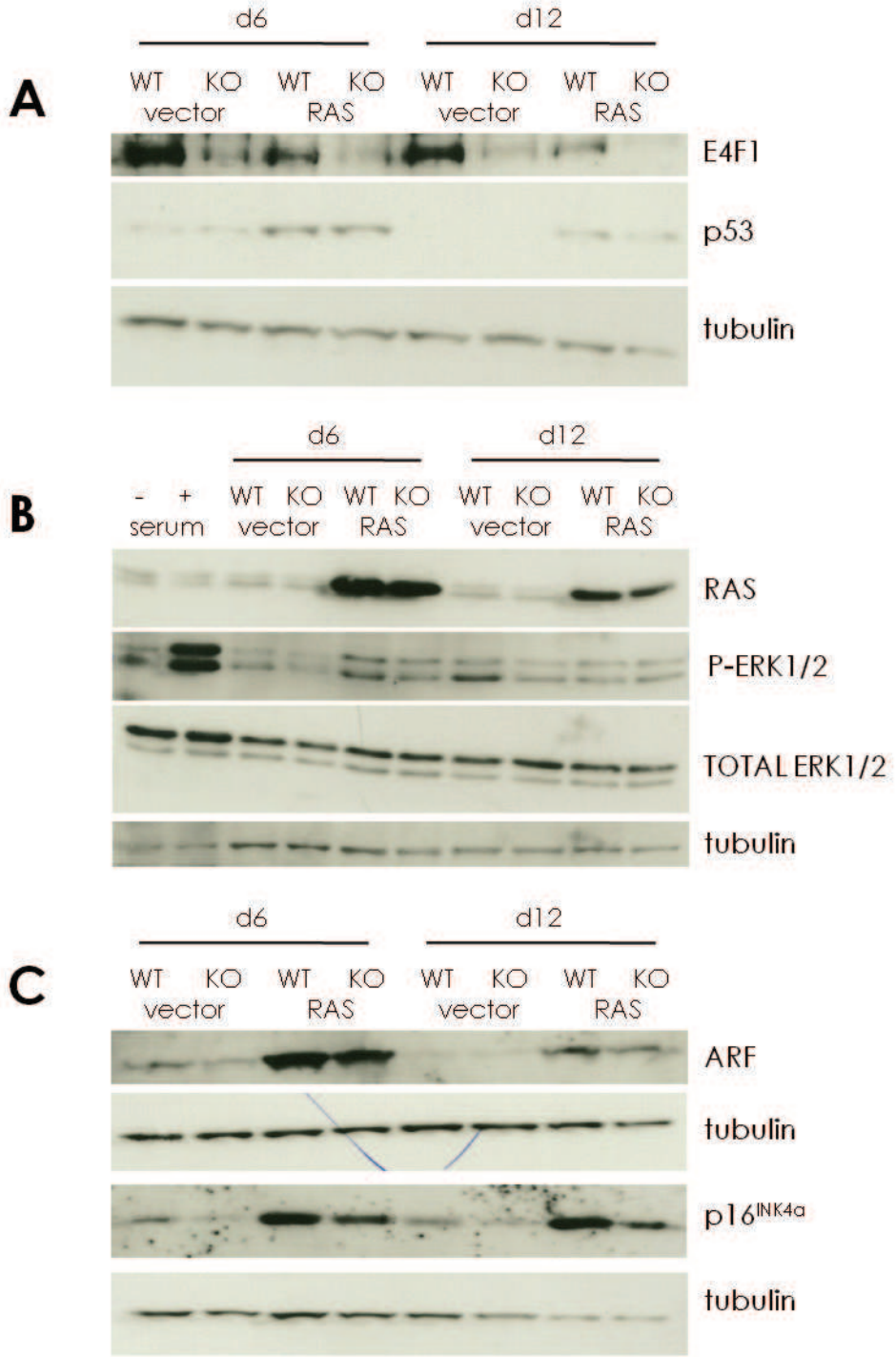

Fig. 36 | **Effect of H-RAS^{V12} on Cell-Cycle Regulatory Proteins. A** – control western blot of E4F1 depletion and p53 induction while overexpressing *H-RAS^{V12}*, no differences in p53 levels were observed between WT and KO *E4F1*; **B** – Control RAS western blot showing good protein overexpression; P-ERK1/2 and TOTAL ERK1/2 blots showing no E4F1-mediated effect on MAPK signalling pathway; **C** – p16^{INK4a} and ARF western blots demonstrating p16^{INK4a} and ARF decrease in KO *E4F1* MEFs – both control and *H-RAS^{V12}*-transduced. WT, wild-type; KO, knock-out; MEFs, mouse embryonic fibroblasts, P, phospho; MAPK, MAP kinase.

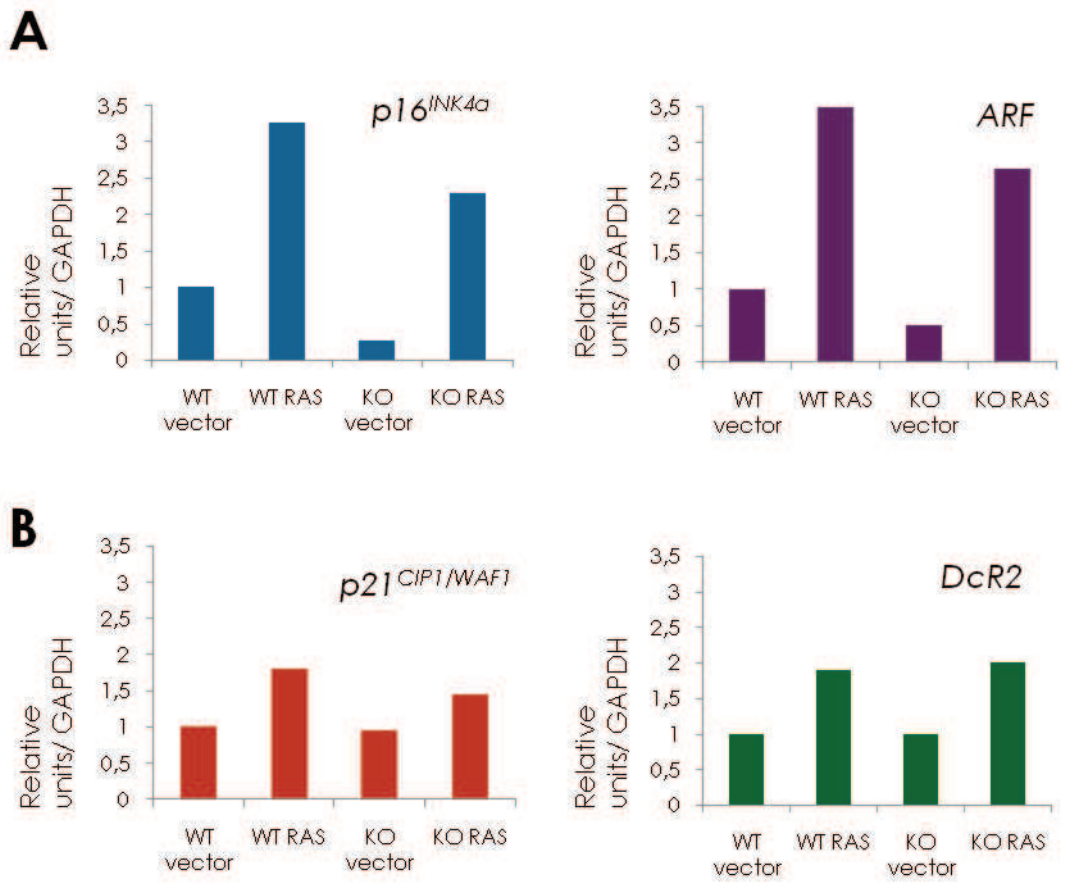

Fig. 37 | **RT-qPCR analysis of several senescence-related genes at day 6.** A- RT-qPCR analysis of the mRNA levels of *p16*^{INK4a} and *ARF* in the indicated cells; B – gene-expression analysis of p21$^{CIP1/WAF1}$ and DcR2.

During live-cell observations I also remarked a visible "loss" of *E4F1* KO MEFs infected with *H-RAS*^{V12} (see Fig. 35 B for the growth curve). Additionally, around day 10 I noticed a considerable number of floating cells in the culture medium. Those observations suggested cell death and, therefore, FACS analysis was performed upon staining with annexin-V. The result shows (Fig. 38 B) that *E4F1* KO MEFs infected with oncogenic *RAS* seem to change their "fate" and instead of senescence enter apoptosis. RAS-induced cell death is p53-independent since *p53* knockout MEFs devoid of *E4F1* and infected with oncogenic *RAS* still enter apoptosis (Geneviève Rodier personal observations). RT-qPCR analysis of *NOXA* mRNA expression, which is a pro-apoptotic gene, revealed a slight induction of this gene expression in *RAS*-transduced *E4F1* KO MEFs (Fig. 38 C). Taken together, these results show a change of the cellular response to oncogenic RAS in MEFs devoid of *E4F1*: instead of senescence those cells enter apoptosis.

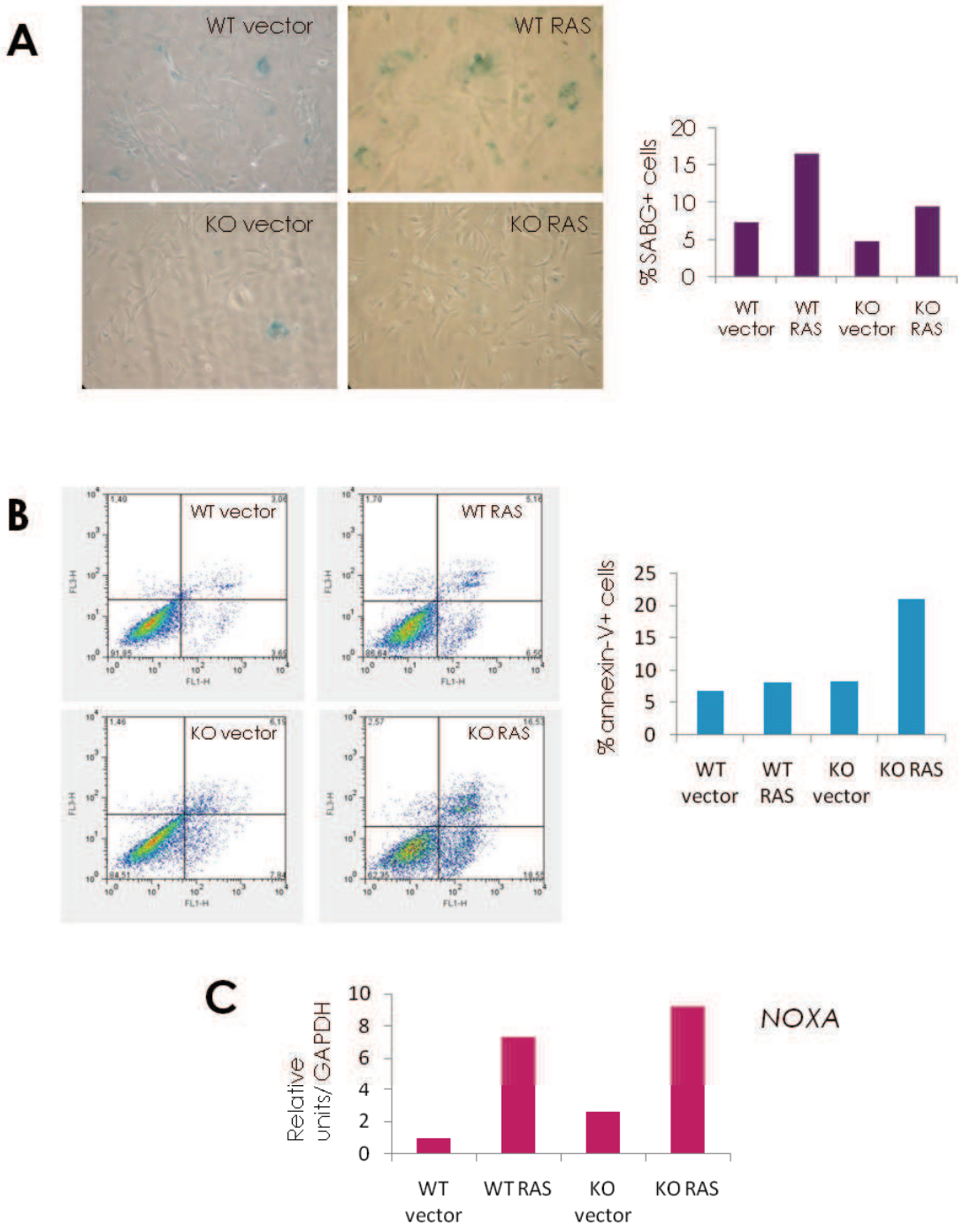

Fig. 38 | **Constitutive H-RAS^{V12} overexpression in *E4F1* knockout MEFs impairs establishment of senescence and leads to apoptosis. A –** Percentage of SABG positive cells shows a decrease in SA-β-GAL activity in *E4F1*-deficient MEFs transduced with control or H-RAS^{V12} vectors, photographs of the MEFs stained for β-gal (pH 6.0) activity at day 12. **B** – Annexin-V staining showing an apoptotic response in E4F1 KO H-RAS^{V12}-transduced MEFs, FACS profiles of four experimental conditions and corresponding graph; analysis was performed between day 10 and day 16; **C** – RT-qPCR analysis of *NOXA* mRNA expression at day 12. KO, knockout; WT, wild-type, MEFs, mouse embryonic fibroblasts; FACS, fluorescence-activated cell sorting.

5.6. *E4F1* KO MEFs seem to be « more resistant » to other stimuli promoting cellular senescence

To strengthen the hypothesis that *E4F1* deficiency slows down senescence I induced senescence in MEFs targeting the p53 pathway on different levels – either more upstream (sh*Bmi-1*) or more downstream (genotoxic anti-cancer drugs) than *H-RAS*^{V12} (see Research Questions and Experimental Model).

For experimental details see Fig. 39. Similarly to H-RAS^{V12} experiments I used the *E4F1* depletion approach. The sole difference was the pro-senescent signal, which was either shRNA directed against *Bmi-1* or DNA-damaging drugs: doxorubicin or etoposide.

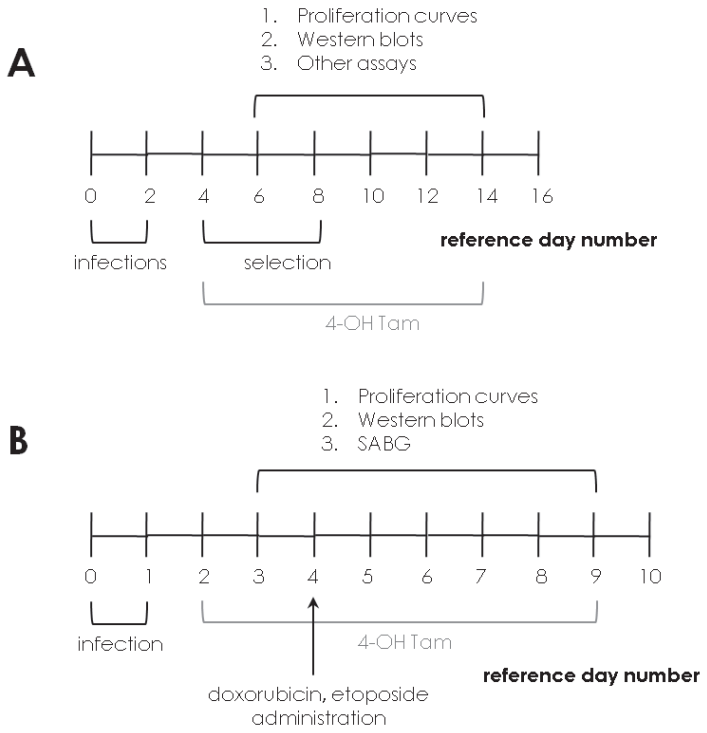

Fig. 39 | **Experimental design and reference time frame for sh*Bmi*-1 and DNA damage-induced senescence. A** – cells were infected with *CRE* retrovirus (day 0) then with shRNA targeting *Bmi-1*mRNA and control vector (day 1). 48hr after sh*Bmi-1* infection cells were subjected to blasticidin selection. The selection was maintained 4 days. In order to ensure a good rate of *E4F1* depletion 4-OH-tamoxifen was added together with selection antibiotic and maintained during all duration of the experiment. Different analyses were performed starting from the day 6 until day 14; **B** – cells were infected with CRE (day 0) and at day 4 treated with either doxorubicin or etoposide. 4-OH-tamoxifen was administered 48h after CRE infection to ensure high rate of E4F1 depletion. Analyses were performed starting from day 3 and until day 9.

I examined cell proliferation by evaluating appropriate growth curves (Fig. 40 A and B). *Bmi-1* silencing leads to cell cycle arrest (Fig. 40 A) – its establishment takes more time in comparison with the proliferation cease induced by oncogenic RAS. Such difference can be explained by only a 50% reduction in Bmi-1 protein (Fig. 41 B) and mRNA levels (Fig. 42 A). Administration of doxorubicin or etoposide efficiently ceases proliferation of both WT and *E4F1* KO MEFs (Fig. 42 B).

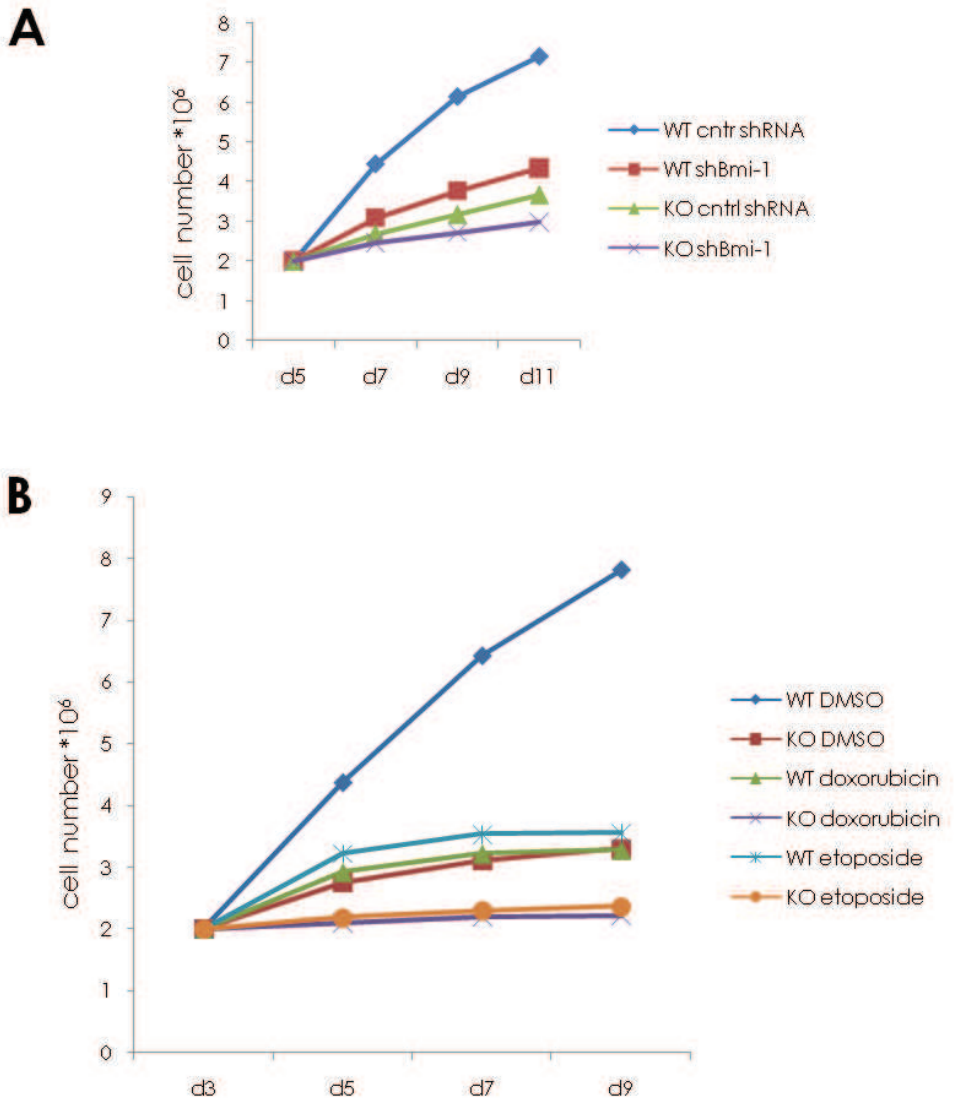

Fig. 40 | **Effect of sh-*Bmi1* and chemotherapy drugs on WT and KO *E4F1* mouse embryonic fibroblasts.** Growth curves of following experimental populations of MEFs for **A** – shBmi-1 and **B** – DNA-damaging drugs experiments. WT, wild-type; KO, knockout; MEFs, mouse embryonic fibroblasts.

In order to investigate the nature of sh*Bmi-1*-induced cell-cycle arrest in WT and KO *E4F1* MEFs I examined the expression of proteins implicated in cell-cycle regulation. Total cell lysates were prepared at day 6 and 12. Control immunoblots confirmed E4F1 and Bmi-1 depletion (Fig. 41 A and B). p53 and $p21^{CIP1/WAF1}$ induction was transient – observed at day 6 but not at day 12 (Fig. 41 A). $p16^{INK4a}$ and ARF immunoblots demonstrated reduced levels of $p16^{INK4a}$ and ARF in KO *E4F1* MEFs infected either with empty vector or sh*Bmi-1* (Fig. 41 C). Such decline was observed both at day 6 and 12 and confirmed previous findings obtained during *H-RAS*^{V12} experiments.

RT-qPCR analysis revealed that levels of *p16*^{INK4a} and *ARF* mRNA were reduced in cells deficient in *E4F1 and* infected either with control shRNA or *shBmi-1* (Fig. 42 B). Those results were again in agreement with previous findings obtained with *H-RAS*^{V12}.

To complete the analysis of the phenotype, SABG staining was performed. MEFs lacking *E4F1* and infected with control shRNA or sh*Bmi-1* were less positive for the staining (Fig. 43 A). Similar results were obtained in *E4F1*-deficient cells treated with DNA-damaging drugs and in non-treated *E4F1* KO cells (Fig. 43 B).

These data confirm earlier observations that *E4F1* inactivation impairs the execution of cellular senescence program.

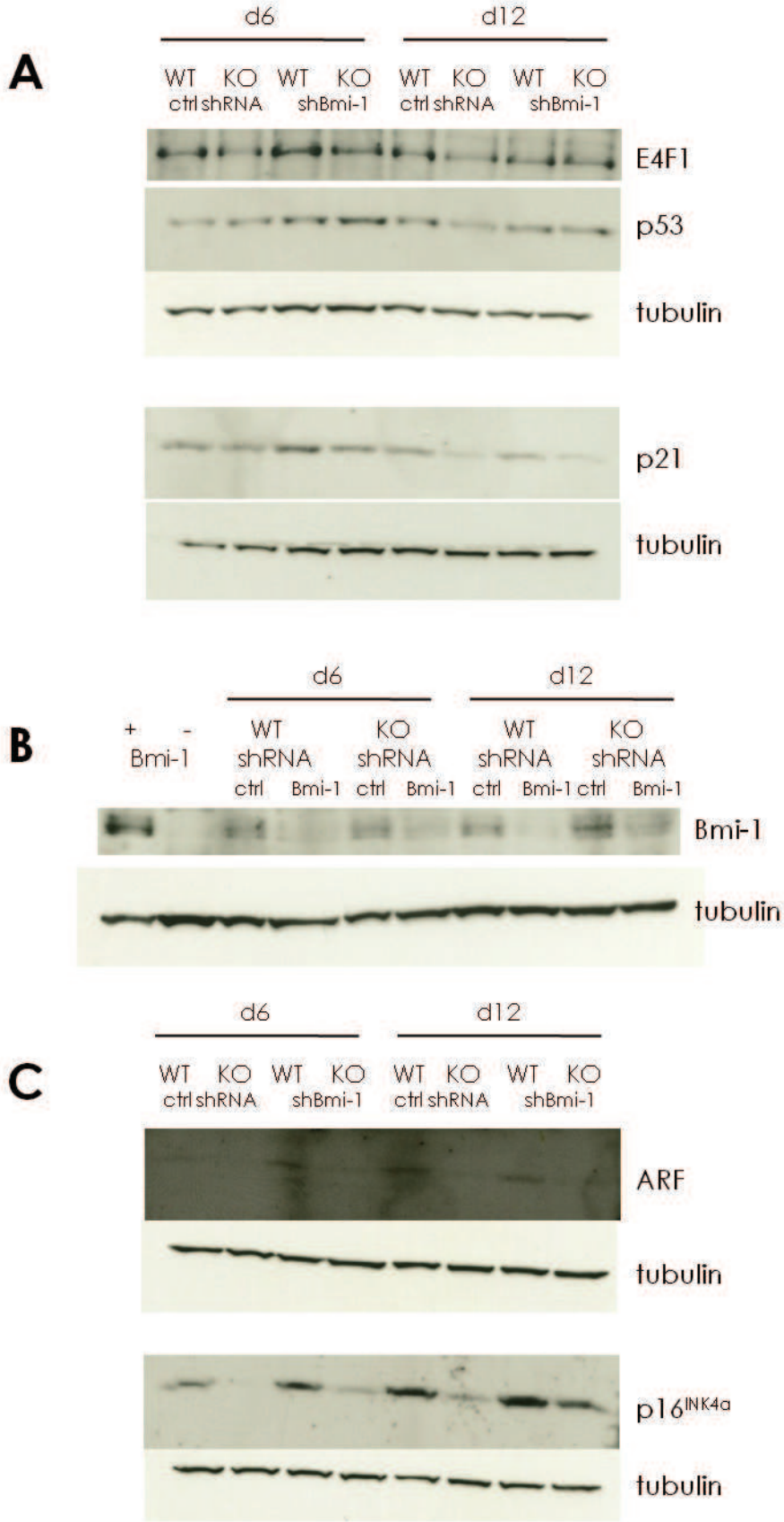

Fig. 41 | **Effect of sh*Bmi-1* on Cell-Cycle Regulatory Proteins. A** – control western blot of *E4F1* depletion, p53 and p21$^{CIP1/WAF1}$ induction at day 6 but not day 12; **B** – Control Bmi-1 western blot showing good protein knockdown; **C** – p16^{INK4a} and ARF western blots demonstrating p16^{INK4a} and ARF decrease in KO *E4F1* MEFs – both control and sh*Bmi-1*-transduced; WT, wild-type; KO, knockout; MEFs, mouse embryonic fibroblasts,

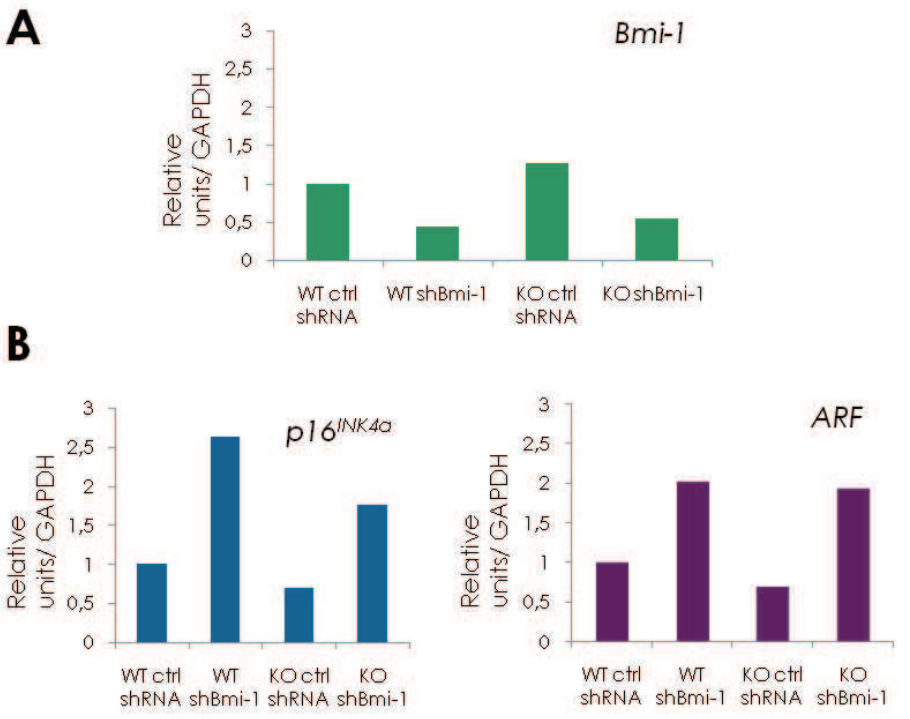

Fig. 42 | **QRT-qPCR analysis. A –** expression levels of *Bmi-1* mRNA; **B –** *p16INK4a and ARF* mRNA expression at day 6.

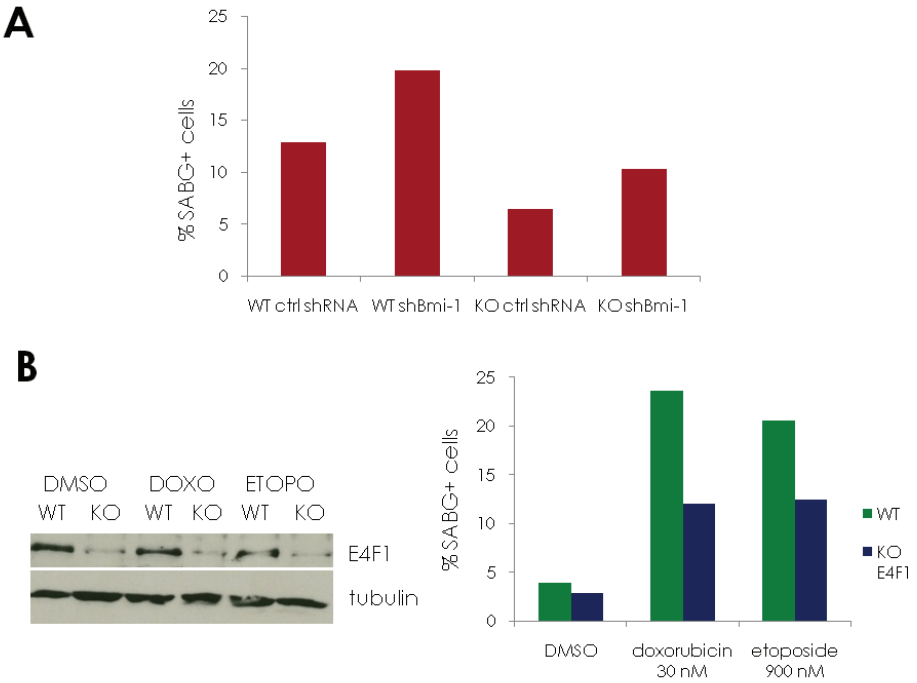

Fig. 43 | **Lack of *E4F1* delays the onset of senescence. A –** Percentage of SABG positive cells shows a decrease in SA-β-GAL activity in *E4F1*-deficient MEFs transduced with control shRNA or sh*Bmi-1*; **B** – E4F1 depletion control blot for cancer drug experiments; visibly lower percentage of SABG-positive cells in *E4F1*-deficient cells treated with doxorubicin, etoposide or with vehicle.

6. Human primary fibroblasts

6.1. E4F1 overexpression leads to cell-cycle arrest with evidence of premature senescence

To further explore E4F1 implication in senescence, I extended the analysis to WI-38 human primary fibroblasts. Experiments were performed according to the scheme presented on Fig. 44.

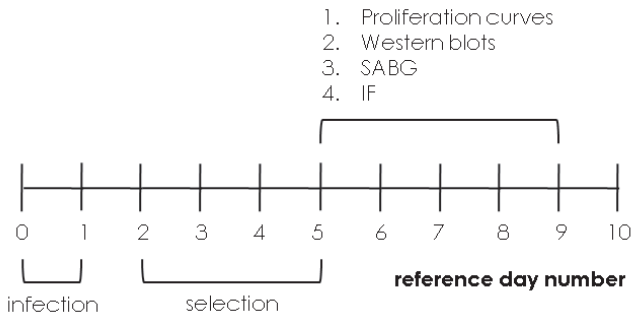

Fig. 44 | **Experimental design and reference time frame for *E4F1*-overexpression experiments in WI-38 cells.** Human primary fibroblasts were infected with *E4F1* retrovirus at day "0". 48h after infection cells were subjected to puromycin selection during 3 to 4 days. Different analyses were performed starting from the day 5 until day 9.

WI-38 cells expressing high levels of *E4F1* underwent a fast cell-cycle arrest and displayed features of senescence, including flat morphology and elevated SABG activity (Fig. 45 A and B). No changes in morphology were detected in cell populations transduced with empty viral vector.

Cells overexpressing *E4F1* presented a high percentage of SABG staining (34,8%) contrary to cells transduced with the empty vector (18,4%) (Fig. 45 B). Therefore, *E4F1* is able to induce a senescent phenotype also in human primary fibroblasts.

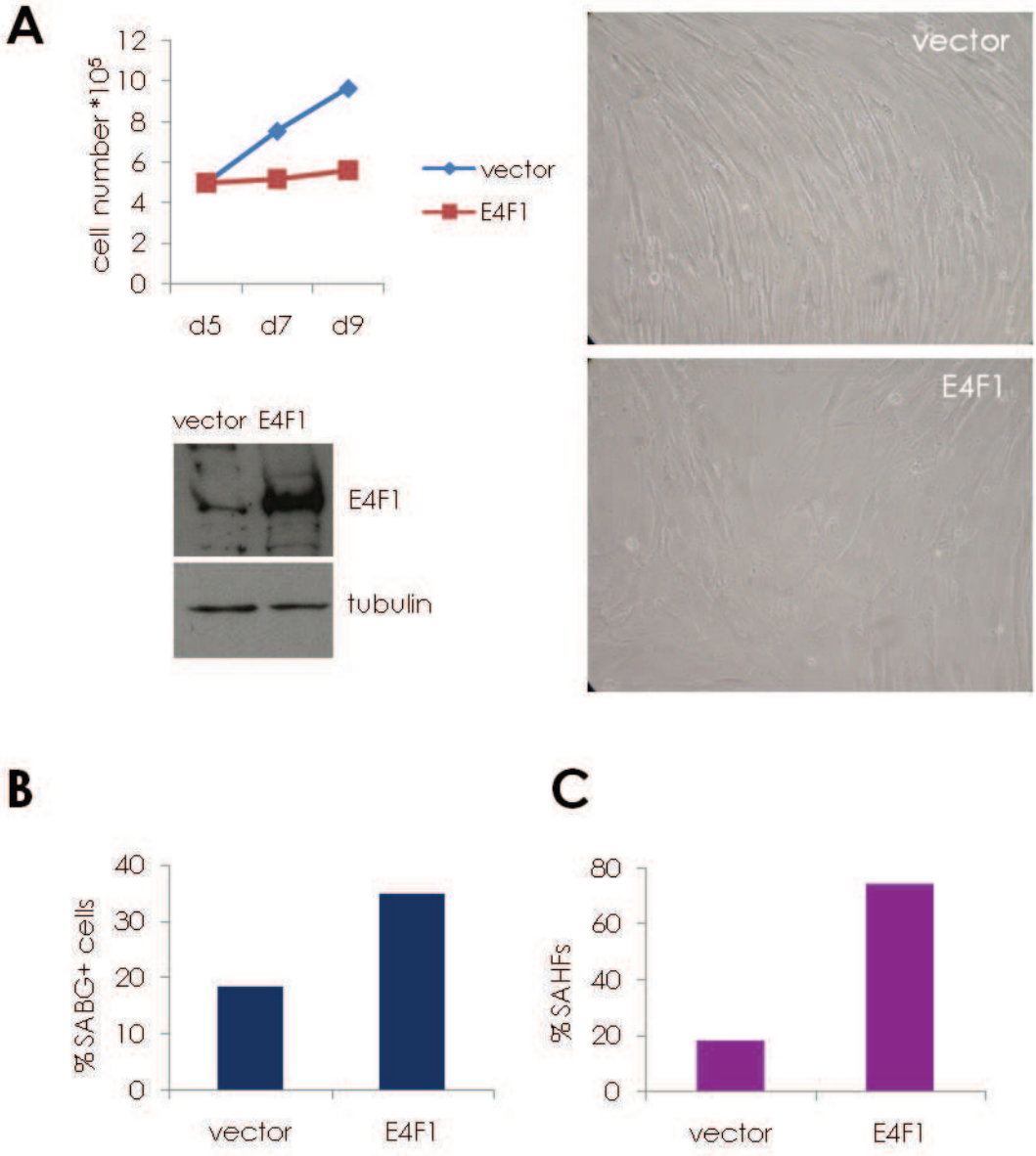

Fig. 45 | **Effect of E4F1 on WI-38 primary human fibroblasts. A -** Growth curves of WI-38 cells transduced with an empty retroviral vector (vector) or its derivatives expressing *E4F1* (E4F1); control western blot of *E4F1* overexpression and visibly flat morphology of *E4F1*-infected WI-38 fibroblasts; **B** – Percentage of SABG-positive cells shows an increase in SA-β-GAL activity in *E4F1*-transduced WI-38 cells; **C** – Significant increase in occurrence of SAHFs in cells overexpressing E4F1. SABG, SA-β-GAL

6.2. Overexpression of E4F1 induces SAHFs formation in WI-38 human primary fibroblasts

It was previously described that senescent WI-38 cells are characterised by the presence of specific heterochromatin structures called senescence-associated heterochromatin foci (SAHFs). I checked whether overexpression of *E4F1* promoted SAHFs formation. Using the immunofluorescence technique I observed that DAPI staining revealed I observed that DAPI staining revealed numerous cells exhibiting condensed heterochromatin foci corresponding to SAHFs in cells overexpressing E4F1 and not in cells infected with control vector (Fig. 46). I also examined the subcellular localization of HMGA2 protein. In cells infected with empty viral vector HMGA2 was expressed diffusely throughout the nucleus (Fig. 46). In contrast, in *E4F1*-infected cells, HMGA2 was predominantly localized to DAPI-dense SAHFs. Therefore, ectopic expression of E4F1 in human primary fibroblasts is capable of inducing SAHFs.

The promyelocytic leukaemia protein, PML, is a tumour suppressor that distinctively localises to punctate nuclear structures that are interspersed between chromatin (Melnick *et al.,* 1999). These structures, are now simply called PML-nuclear bodies (PML-NBs). Recent evidence implies that, although they appear to be uniform, PML-NBs are structurally and functionally heterogeneous and are dynamic structures. It was reported that an increase in the number and size of PML-NBs occurs, *inter alia*, in response to the induction of premature senescence by overexpression of Ras^{V12} (Jiang *et al.,* 1997 ; Ferbeyre *et al.,* 2000 ; Pearson *et al.*, 2000). I verified the number and size of PML-NBs in WI-38 *E4F1*-infected cells and cells expressing high levels of *E4F1* had bigger PML-NBs (Fig. 46) when compared to the control cells.

Taken together, these findings support a model by which E4F1 induces senescence in WI-38 cells with SAHFs formation and PML induction.

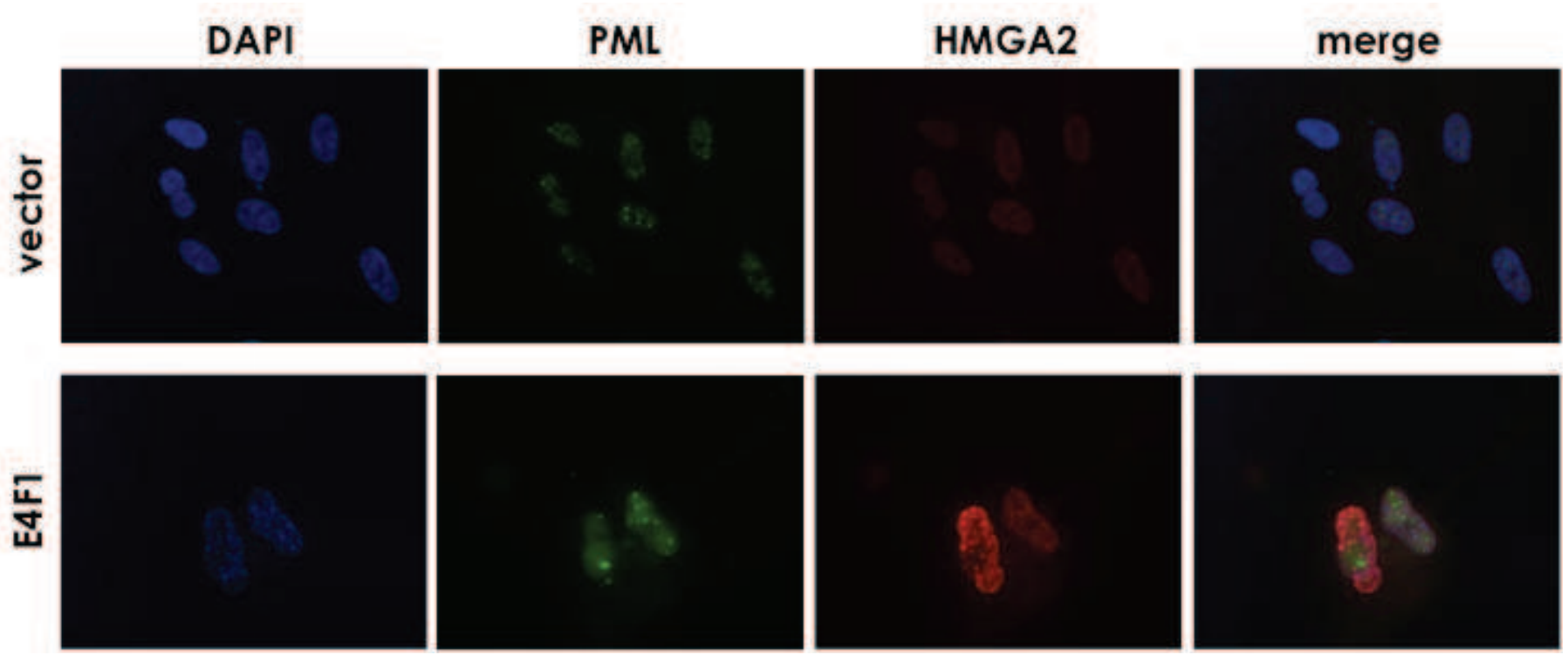

Fig. 46 | **High levels of E4F1 cause SAHFs formation and PML induction.** Representative images of indirect immunofluorescence of HMGA2 and PML, nuclei were counterstained with DAPI.

Discussion and Perspectives

My observations expand our understanding of *E4F1* functions, especially its role in cellular senescence. Using primary mouse embryonic fibroblasts originating from genetically engineered *E4F1* conditional KO mice, I evaluated E4F1 functions by two opposing strategies: protein overexpression and depletion.

E4F1 overexpression in mouse embryonic fibroblasts promotes a cell-cycle arrest in G1 phase and MEFs overexpressing E4F1 exhibit morphological (flat morphology) and molecular (SABG staining) evidences of premature senescence. Characterised by p53 stabilisation and induction of its target gene $p21^{CIP1/WAF1}$, E4F1-induced senescence is bypassed in *p53* null and *INK4a-ARF* null MEFs showing that p53 signalling pathway is crucial for establishment of the E4F1-mediated senescence. Surprisingly, overexpression of E4F1 does not induce high expression of $p16^{INK4a}$ and ARF proteins. It might be possible that presence of those proteins at basal levels is sufficient for the onset of E4F1-induced senescence and the mechanism involves mainly p53 and $p21^{CIP1/WAF1}$ proteins. One could also imagine that E4F1-induced cellular response depends not on "the quantity" of $p16^{INK4a}$ and ARF proteins but rather on "the quality" that is post-translational modifications of those proteins that could fine-tune their functions as regulators of senescence.

Further experiments demonstrated that simultaneous overexpression of E4F1 and oncogenic RAS results in a visible acceleration of senescence showing a synergy between those two pro-senescent signals. It would be of great importance to investigate whether overexpression of E4F1 combined with other pro-senescent signals, such as the Bmi1 knockdown or DNA damage, also accelerates the onset of senescence. Additionally, it will be interesting to see what phenotypic effect one could observe while simultaneously overexpressing E4F1 and Bmi1. It was previously showed that E4F1 and Bmi1 interact together and the premature senescence observed in *Bmi* null hematopoietic stem cells (HSC) was rescued upon shRNA-mediated depletion of *E4F1* (Chagraoui *et al.,* 2006). In contrast, MEFs overexpressing Bmi1 become immortal and exhibit decreased expression of $p16^{INK4a}$ and ARF

(Jacobs *et al.,* 1999). Therefore, it would be of a great interest to evaluate whether Bmi1 overexpression alters the pro-senescent activity of E4F1 as this particular experiment was partially performed in Chagraoui *et al.* – ectopic expression of Bmi1 bypass cell proliferation defects induced by ectopic expression of E4F1 in NIH 3T3.

E4F1 overexpression experiments performed in WI-38 primary human fibroblasts recapitulated those performed in MEFs. Forced expression of E4F1 leads to cell cycle arrest with features of premature senescence : flat cell morphology and high percentage of SABG positive cells. Additionally, E4F1 overexpression leads to establishment of SAHFs in WI-38 cells. This result raises the question about E4F1 localisation within the SAHFs. Does it colocalise with such proteins as HMGA2 and therefore participate in formation of those heterochromatin structures? It remains to be determined how E4F1 induces SAHFs and whether this implicates its transcriptional or E3 ligase activity.

E4F1 depletion in MEFs also leads to a growth arrest, but in this case cells arrest in G1 and G2/M phases. Paradoxically, although we found that this arrest is also p53 dependent, *E4F1* inactivation in MEFs impairs establishment of senescence and attenuates the induction of pro-senescent signals after ÷ ectopic expresion of oncogenic RAS or shRNA-mediated depletion of Bmi1. Hence, $p16^{INK4a}$ and ARF are not upregulated in the absence of E4F1 and percentage of SABG positive cells is visibly lower comparing to E4F1 WT cells. RT-q-PCR analysis suggested that the effect on $p16^{INK4a}$ and ARF is transcriptional so one can presume that E4F1 regulates transcription of the *INK4a/ARF* locus. The exact mechanism is unknown and remains to be elucidated. Recently, Agherbi *et al.* (2009) described a new mechanism of *INK4a/ARF* locus regulation during senescence. It was showed that in young proliferating mouse embryonic fibroblasts the Polycomb Repressive Complex 2 (PRC2) member EZH2 together with Polycomb Represseive Complex 1 (PRC1) members BMI1 and M33 are strongly expressed and localise at the *INK4a/ARF* regulatory domain (RD) identified as a DNA late replication origin. Such localisation of both PRC1 and PRC2 complex members allows repression

of the *INK4a/ARF* locus. When MEFs enter senescence the binding to RD of both PRC1 and PRC2 complexes is lost leading to a decreased level of histone H3K27 trimethylation (H3K27me3). This loss is accompanied with an increased expression of the histone H3K27me3 demethylase Jmjd3 and with the recruitment of the MLL1 protein, and correlates with the expression of $p16^{INK4a}$ *and* ARF. Interestingly, preliminary data from our laboratory and our collaborators (Dr. Sardet, IGMM in Montpellier and Pr. Sicinski at the DFCI in Boston) suggested that E4F1 is part of a multiprotein complex containing MLL-binding protein (Wdr5). The hypothesis that E4F1 directly participates in *INK4a/ARF* regulation through Wdr5/MLL is tempting but will require additional studies.

To sum up, both overexpression and E4F1 inactivation impinge on p53 pathway but induce different output signals. That might suggest that E4F1 could act as a 'router' for p53.

Although E4F1 may contribute to the regulation of senescence-associated genes since it directly interacts with p53 (Sandy et al., 2000; Le Cam, Linares et al., 2006), Rb (Fajas et al., 2000), ARF (Rizos et al., 2003) and Bmi-1 (Chagraoui et al., 2006), its anti-proliferative activity might also reflect its ability to induce p53 post-translational modifications. It was previously showed that ectopic expression of E4F1 in U2OS cells leads to a p53-dependent growth arrest. The mechanism of this growth arrest relies on E4F1-catalysed p53-ubiquitylation and subsequent p53 stabilisation. (Le Cam, Linares et al., 2006). Therefore, it is possible that E4F1-mediated post-translational modifications could participate in its pro-senescence activities. Indeed, some post-translational modifications of essential regulators have been associated with cellular senescence.

Firstly, It was shown that p53 is modified by SUMO-1 at a single site K386, in the C-terminus end of protein (Gostissa *et al.*, 1999; Rodriguez *et al.*, 1999). Gostissa *et al.* employed a luciferase reporter construct containing the natural p53 responsive promotor of $p21^{CIP1/WAF1}$, and observed an up to three-fold increase in luciferase activity upon overexpression of either SUMO-1 or

Ubc9 (Ubiquitin-like protein SUMO-1 conjugating enzyme). This increase was observed only for wt p53 but not for a point mutant that can not be modified with SUMO-1. Similar experiments by Rodriguez *et al.* with the reporter construct PG13-luciferase, which contains multiple copies of a synthetic p53 binding site, led to comparable results. However, a more recent study by Kwek et *al.,* which employed reporters containing either the p53 responsive promoter of the adenosine deaminase gene or the $p21^{CIP1/WAF1}$ promoter, concluded that SUMOylation has no effect on p53 transcriptional activity (Kwek *et al.,* 2001).

Similarly, Schmidt and Müller (2002) also failed to find an increase of p53 dependent transcription upon co-expression of SUMO-1. To add to the confusion, conflicting results were also reported regarding the effect of the E3 ligase PIAS1 on p53 transactivation. First, Schmidt and Müller found that PIAS proteins strongly repress transcriptional activity of p53 in reporter assays when SUMO-1 is co-expressed. This depends on the catalytic cystein of PIAS1, but does not depend on p53's known SUMOylation site (Schmidt and Müller, 2002). One possible explanation of these results is that PIAS1-dependent SUMOylation of an unknown protein inhibits p53 activation. In contrast, Megidish et *al.* found that ectopic expression of PIAS1 stimulates transactivation of a PG13-luciferase construct by p53 two-fold, and also stimulates expression of endogenous $p21^{CIP1/WAF1}$ (Megidish *et al.,* 2002). Surprisingly, these effects are also observed with a PIAS1 construct lacking the RING domain necessary for E3 ligase activity, which implies that the observed effects are independent of p53 SUMOylation. In addition to comparing wt and SUMOylation deficient p53 for transactivation, one study analysed potential differences in localisation and growth inhibition. Mutating the p53 SUMO acceptor lysine to arginine had no obvious effect in colony formation assays or on p53 localisation (Kwek et *al.,* 2001) even though the acceptor site for modification, lysine 386, is in close proximity to one of three nuclear localization sequences of p53 (residues 379-384) (Hayon and Haupt, 2002).

Finally, abolishing SUMOylation only marginally reduced p53's potential to induce apoptosis in Saos-2 cells (Hershko and Ciechanover, 1998).

More recently, p53 and Rb were found to be modified by SUMO-2/3 (Li et al., 2006) Site-directed mutagenesis studies showed that lysine 386 of p53 is the modification site for SUMO-2/3. Li *et al.* also demonstrated that overexpression of SUMO-2/3 in cultured cells results in premature senescence : cells acquire characteristic flat morphology supported by early growth arrest and they are characterised by upregulation of the $p21^{CIP1/WAF1}$ protein. In contrast, cells overexpressing non-conjugatable forms of SUMO-2/3 showed neither an apparent senescent phenotype nor elevated $p21^{CIP1/WAF1}$. Moreover, knocking down p53 or Rb using small interfering RNA significantly alleviated the premature senescence phenotypes in SUMO-2/3 overexpressing cells. Finally, overexpression of SUMO-1 appears not to directly affect the process of cellular senescence (Li *et al.,* 2004).

Protein inhibitors of activated STAT (PIAS) have been shown to function as E3 SUMO ligases and data from various SUMO conjugates make them one of the largest families of SUMO-specific ligases (Schmidt and Müller, 2003). During replicative senescence the levels of endogenous PIASy, a member of this protein family, are significantly increased compared to presenescent cells (Bischof *et al.,* 2006). This effect is considered specific to PIASy, as no other member of the PIAS protein family appears to have a similar activity. In addition, overexpression of PIASy can induce cellular senescence or apoptosis (Bischof *et al.,* 2006). Induction of senescence is conferred by the E3 ligase activity of PIASy ; PIASy associates with p53 and Rb and stimulates p53 SUMOylation and subsequent activation of p53 target promoter regions, such as the activation of the $p21^{CIP1/WAF1}$ promoter. Expression of $p21^{CIP1/WAF1}$ keeps Rb in its active, repressive state. PIASy interacts with Rb, creating a repressive heterochromatin state that buffers expression of E2F-response genes involved in cell-cycle progression.

Mutation of the PIASy E3 ligase active site abolishes the pro-senescent effect of PIASy - an extended lifespan is observed in cells that overexpress mutant

forms of PIASy with an inactive E3 ligase site. In further support of this process, deletion of PIASy in mouse embryo fibroblasts results in a significant delay of the onset of senescence. Even after induction of p53 expression by a pro–senescence signal, for example through oncogenic RAS, p53 target genes such as $p21^{CIP1/WAF1}$ and MDM2 are not upregulated in the absence of PIASy. It it formally possible that E4F1 acts as a E3 SUMO ligase for p53 and have similar protein functions as PIASy. Both of them induce senescence in MEFs when overexpressed and their inactivation delays the onset of senescence. PIASy as well as E4F1 were described to have a E3-ligase activity towards p53 – E4F1 being an atypical ubiquitin E3 ligase and PIASy being a SUMO E3 ligase. It would be therefore of extreme interest to check if E4F1 E3 ligase domain could be also responsible for p53 SUMOylation.

Preliminary data from our laboratory indicate that in E4F1 KO MEFs p53 SUMOylation by SUMO-2/3 is decreased comparing to WT E4F1 MEFs. Additionally, induction of senescence with oncogenic RAS leads to visible p53 SUMOylation by SUMO-2/3 in WT *E4F1* MEFs but not in KO *E4F1* MEFs. Overexpression experiments performed with E3-ligase E4F1 mutant suggest that the observed senescent phenotype might partially depend on the E3-ligase activity of E4F1 protein. Nevertheless, further studies will be required to confirm those premiminary data. It would be also of great importance to investigate p53 post-translational modifications in E4F1 overexpression model and E3-ligase mutant of E4F1. In order to provide a direct proof that p53 SUMOylation plays a direct role in E4F1-induced senescence, we planned to complement *E4F1* flox; *p53* null MEFs with WT, K320R and K386R p53 mutants.

Finally, as a future project, it would be of profound importance to address whether E4F1-mediated effects on cellular senescence impacts on organismal aging. To address this hypothesis, our laboratory is currently generating an appropriate mouse model. E4F1 ROSA knock-in animals that will allow inducible (based on the Cre/Lox-P system) overexpression of WT E4F1 or E3 domain- or DNA-binding domain mutants, will be essential to answer this important biological question and determine the relative

importance of E4F1-associated transcriptional and E3 ligase activities. We suspect that these mice will develop phenotypic alterations observed in previous mouse models displaying perturbed p53 activity such as reduced lifespan, lordokyphosis, weight loss, osteoporosis, altered stress tolerance etc... Such (and other) mouse models might be instrumental to provide data supporting the controversial role of premature senescence during organismal aging.

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RESUME en français

Le facteur de transcription E4F1 fût initialement identifié comme une cible cellulaire de l'oncoprotéine virale E1A au cours de l'infection par l'adénovirus sérotype V. E4F1 est une protéine multifonctionelle essentielle au cours du dévelopment embryonnaire précoce et joue des rôles importants dans l'équilibre entre proliferation/survie de differents types cellulaires, notamment des cellules souches. Au niveau moléculaire, E4F1 possède des activités transcriptionelles intrinsèques mais possède également une activité ubiquitine E3 ligase atypique dirigée contre d'autres facteurs de transcription tel que le suppresseur de tumeurs p53. Récemment, il a été demontré qu'E4F1 régule les voies oncogéniques impliquant p53 and Rb qui jouent un rôle essentiel au cours de la sénescence cellulaire. La sénescence, qui est définie par un arrêt irréversible du cycle cellulaire, est consideré comme un mécanisme suppresseur de tumeurs essentiel au cours des phases précoces du dévelopment tumoral. L'objectif de ma thèse a été d'évaluer le rôle d'E4F1 au cours de la sénescence cellulaire. Au travers d'études menées sur des fibroblastes humains primaires et des fibroblastes embryonnaires murins dérivés de souris genetiquement modifiées pour le gène E4F1, j'ai examiné comment la perturbation des activités d'E4F1 module l'initiation ou le maintien de la sénescence prématurée induit par l'oncogène RAS, la depletion du membre de la famille polycomb Bmi1, ou par les dommages à l'ADN. Mes résultats suggèrent que la déplétion d'E4F1 protège partiellement contre l'induction de la sénescence alors que l'expression éctopique d'E4F1 accélère la sénescence par son implication dans la voie INK4A/ARF-p53. L'ensemble de mes resultats supportent la notion qu'E4F1 est un régulateur important de la sénescence cellulaire.

TITRE en anglais

Roles of the multifunctional protein E4F1 in cellular senescence

RESUME en anglais

E4F1 was originally identified as a cellular target of the viral oncoprotein E1A during adenoviral infection. E4F1 is a multifunctional protein that is essential during early embryogenesis and plays important roles in the proliferation/survival balance of different cell types including stem cells. At the molecular level, E4F1 exhibits intrinsic transcriptional activities but also an ubiquitin E3 ligase function that targets other transcription factors, including the p53 tumor suppressor. Recent studies indicate that E4F1 impinge on several pathways, including the Rb and p53 pathways, that are known to influence cellular senescence, an irreversible state of cell cycle arrest that is considered to be an essential tumor suppressor mechanism during early steps of tumorigenesis. The objective of my thesis was to evaluate the roles of E4F1 during cellular senescence. Using human primary fibroblasts and mouse embryonic fibroblasts derived from genetically engineered mouse models, I investigated how perturbations of E4F1 activities modulated the initiation or the maintenance of premature senescence induced by oncogenic Ras, depletion of the polycomb member Bmi1 or DNA damage. My results suggest that E4F1 depletion partly protects from the induction of cellular senescence whereas ectopic expression of E4F1 accelerates premature senescence through its implication in the Ink4a/ARF-p53 pathway. Altogether, my results support the notion that E4F1 is an important regulator of cellular senescence

DISCIPLINE

Biologie cellulaire et moléculaire

MOTS-CLES

E4F1, sénescence, fibroblastes primaires, p53, INK4a/ARF, souris génétiquement inactivées pour E4F1 (KO)

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