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Mechanism of Maintenance of Cellular Senescence

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Abstract of the Dissertation

Mechanism of Maintenance of Cellular Senescence

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Cellular senescence is a stable form of cell cycle arrest that limits the proliferation of cells subjected to a variety of stresses. Much like apoptosis, senescence acts as a natural barrier to tumor development. Although the molecular mechanisms underlying the process remain poorly characterized, the key players– p53, p16, Rb – are tumor suppressors commonly mutated in most cancers. Our studies led to the identification of a distinct form of facultative heterochromatin (**S**enescence-**a**ssociated **h**eterochromatic **f**oci) that accumulates in some senescent fibroblasts. At the onset of senescence the retinoblastoma (Rb) tumor suppressor and heterochromatic proteins are recruited to E2F-responsive promoters concomitant to SAHF formation, resulting in the stable repression of E2F target genes. SAHF formation and the silencing of E2F-target genes are unique to the senescent cell cycle arrest and require an intact $p16^{Ink4a}$/Rb pathway. At the time we hypothesized that formation of these heterochromatic structures underlies the stability of the senescent arrest.

Through variety of genetic manipulations to interfere with the $p16^{Ink4a}$/Rb pathway we showed that cells that acquire morphological features of senescence but fail to form SAHFs can resume growth upon inactivation of p53. In contrast, SAHF-positive fibroblasts are unable to cycle upon p53 inactivation. We propose a model in which $p16^{ink4a}$ upregulation during senescence engages the Rb pathway to produce a permanent arrest by altering the chromatin state of growth regulatory genes. The failure to form SAHF, and therefore stably repress E2F-target genes, in cells sustaining ink4a or Rb mutations compromises the stability of the arrest. Our findings suggest the existence of two fundamentally distinct states of senescence, each of varying stability that correlates with SAHF formation.

We then focused our attention on the mechanisms maintaining SAHF-positive senescence. We showed that the adenoviral oncoprotein E1A, known to prevent the establishment of senescence, can force SAHF-positive fibroblasts into S phase. SAHF dissolution precedes the onset of DNA synthesis. The cells fail to divide and die despite the disassembly of heterochromatin and the re-expression of E2F target genes; thus uncovering and additional layer of complexity in the maintenance of the senescent arrest. The same result is obtained when a shRNA targeting Rb is used, indicating that Rb is the cellular target of E1A relevant to the maintenance of SAHF and the arrest.

Our work identifies Rb as being required for both the formation as well as the maintenance of heterochromatin in senescent cells; thus providing the first insight into the mechanism of maintenance of cellular senescence.

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Chapter One:

Introduction

1.1 Biology of senescence

1.1.1 Replicative Senescence

In 1961 Experimental Cell Research published an article that would forever challenge the notion that cells explanted in culture are immortal. This article was the work of Hayflick and Moorehead, showing that a population of normal human fibroblasts can double in culture a finite number of times, after which the cells stop dividing and enter what the authors then termed phase III phenomenon (Hayflick and Moorhead, 1961). This phase III phenomenon is now known as replicative senescence. Once cells reach their replicative capacity, they exit the cell cycle and remain viable and metabolically active but are unable to replicate; even when subjected to physiologic mitogens. Senescent cells have a characteristic enlarged, flatten morphology, with vacuoles and an enlarged nucleus (Figure 1.1).

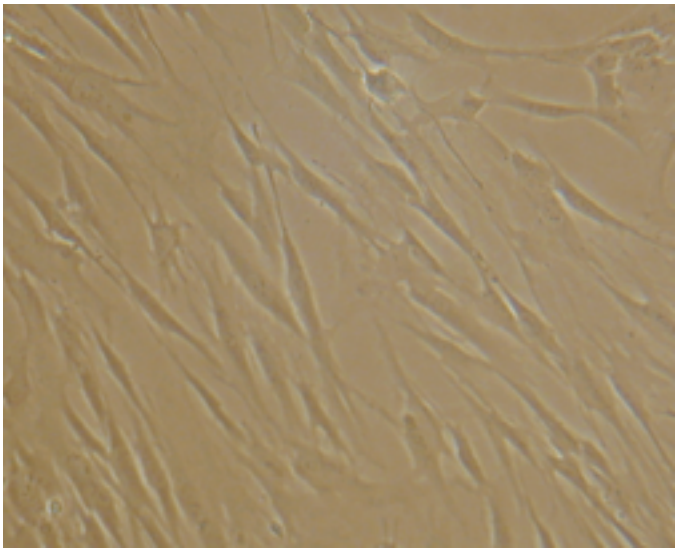
The best-known marker for senescence is the presence of β-galactosidase activity at a pH of 6.0 (Dimri et al., 1995). β-galactosidase is a lysosomal enzyme that normally functions at pH 4.0 to cleave β-linked galactosyl residues from gangliosides, glycoproteins, glycosaminoglycans and a variety of artificial substrates (Morreau et al., 1989). When the activity of the enzyme is measured at a non-physiological pH of 6.0 (5.5 for mouse cells) there is a strong correlation between measurement of activity and senescence. The mechanism underlying this senescence-associated β-galactosidase

Figure 1.1

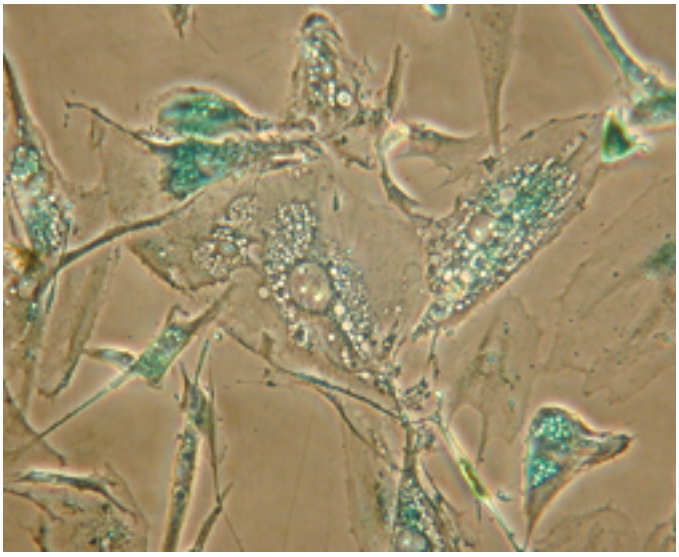
Morphology of growing and quiescent IMR90 human diploid fibroblasts.

Phase contrast image of IMR90 cells at passage 15 and Ras senescent cells at post-selection day 7. Senescent cells display the characteristic enlarged, flatten morphology, with vacuoles and an enlarged nucleus as well as positive staining for senescence-associated β-galactosidase activity.

GROWING



SENESCENT



activity is unknown, and it is likely it represents an upregulation of lysosomes in senescent cells (Kurz et al., 2000). Indeed, early reports have shown that senescent cells have an increase in both the number and the size of lysosomes (Robbins et al., 1970). Nevertheless, measurement of SAβ-gal activity appears to be a good marker of senescence, particularly because it is not related to the growth arrest per se; therefore allowing for the distinction between senescent and quiescent or terminally differentiated cells both in vitro and *in vivo*.

More recently, work from our lab resulted in the addition of senescence associated heterochromatic foci (SAHF) as markers of senescence (Narita et al., 2003). SAHF represent a novel type of facultative heterochromatin that is directed by the p16/Rb pathway and is believed to contribute to the stable silencing of E2F-target genes in senescent cells (see detailed discussion below).

Expression of p16 has been used as a marker of senescence (Serrano et al., 1997). Investigation of the signaling pathways that lead to OIS has revealed several important players that, similar to p16INK4a, are involved in the initiation of senescence, such as the promyelocytic leukemia protein (PML) (Ferbeyre et al., 2000), or the transcription factors ID1 (Zheng et al., 2004) and ETS1 and ETS2 (Foulds et al., 2004). However, the value of these molecules as markers of senescence is limited because downstream alterations can cancel their effects on senescence. Work by Collado et al. recently identified p15INK4b (also known as CDKN2B), Dec1 (BHLHB2) and DcR2 (TNFRSF10D) as de novo markers of OIS (Collado et al., 2005). Although they were able to see these markers along with SA βgal in pre-malignant lesions it remains to be explored whether these are universal markers of senescence.

1.1.2 Premature senescence

In 1997 our laboratory showed that expression of oncogenic Ras in primary human or rodent cells results in a permanent G1 arrest that is accompanied by accumulation of p53 and p16, and is phenotypically indistinguishable from cellular senescence (Serrano et al., 1997). It wasn't long before reports emerged indicating that the senescence phenotype could be induced in early passage cells by a variety of cellular stresses, including DNA damage, oncogenic stress, oxidative stress, aberrant mitogenic stimuli and suboptimal culture conditions (Robles and Adami, 1998; Roninson et al., 2001; Serrano et al., 1997). This type of senescence has been referred to as "premature senescence", "stress-induced premature senescence" (SIPS) or "stasis", to distinguish it from replicative senescence, and to indicate the program is executed before the set time dictated by telomere length. More recently, the term "oncogene induced senescence" or OIS has also been used referring specifically to senescence induced by expression of activated oncogenes.

The term cellular senescence was later coined to refer to both replicative and premature senescence. This terminology represents the view that the endpoint of replicative senescence and premature senescence is the same, with telomere attrition and uncapping being just another way of activating the senescent response (see below).

1.1.3 Senescence *in vivo*

As more people studied senescence in a variety of *in vitro* models, and mechanistic insights were attained, it became clear that cellular senescence had the potential to suppress cancer. The first report to suggest senescent cells might exist *in vivo* showed that senescence-associated β-galactosidase activity could be detected in dermal fibroblasts and epidermal keratinocytes of aging individuals (Dimri et al., 1995). An increased frequency of SA-β-Gal-positive cells was also reported in chronic diseases (Paradis et al., 2001; Wiemann et al., 2002). Scattered in the literature were reports that suggested senescence could be triggered *in vivo* by short telomeres (Satyanarayana et al., 2004), DNA damage (Schmitt et al., 2002), and oncogenic stress (Lazzerini Denchi et al., 2005).

Although the work from Clemens Schmitt in our lab using a mouse model of lymphoma showed that part of the anticancer action of a chemotherapeutic agent occurs by induction of a senescence program (Schmitt et al., 2002), it had not yet been determined whether cancer progression could be halted by senescence in tumors triggered by oncogenic stimuli. It took a single issue of Nature to solve that problem. On August 4, 2005 four different studies were published in Nature, showing for the first time that endogenous oncogenic signals that are thought to initiate cancer do provoke stress-induced senescence *in vivo*.

A brief communication by Collado et al. utilized a microarray screen to identify genes whose expression correlates with senescence induced by ERK and then employed these newly identified markers alongside known markers of senescence (SA β-gal, p16,

SAHF) to show the presence of senescent cells in premalignant lung, pancreas, and skin lesions (Collado et al., 2005). They also observed that advanced tumors isolated from these animals lacked expression of senescence markers; which suggests that, as predicted, it is necessary to bypass senescence to allow tumor progression.

Braig and colleagues studied senescence as an anticancer mechanism in a mouse model of lymphoma in which tumors are initiated by the expression of an oncogenic form of N-Ras (Braig et al., 2005). These animals develop a non-lymphoid neoplasia with a long latency. In this system proliferation of primary lymphocytes is directly stalled by a Suv39h1-dependent, H3K9me-related senescent growth arrest, thereby canceling lymphomagenesis at an initial step. Accordingly, the Eμ-N-Ras transgenic mice harboring targeted heterozygous deletions at the Suv39h1 locus succumb to invasive T-cell lymphomas. The same result is achieved in Eμ-N-Ras transgenic mice harboring targeted heterozygous deletions at the p53 locus. Suv39h1-deficient lymphoma cells grow rapidly but, unlike p53-deficient cells, remain highly susceptible to adriamycin-induced apoptosis. In contrast, only control, but not Suv39h1-deficient or p53-deficient, lymphomas senesce after drug therapy when apoptosis is blocked. These results identify H3K9me-mediated senescence as a novel Suv39h1-dependent tumor suppressor mechanism whose inactivation permits the formation of aggressive but apoptosis-competent lymphomas in response to oncogenic Ras.

Michaloglou et al. (Michaloglou et al., 2005) worked with cultures of human melanocytes and nevi. They found that sustained BRAFV600E expression in human melanocytes induces cell cycle arrest, which is accompanied by the induction of both p16INK4a and SA β-gal activity. The authors hypothesized that human nevi are formed

of senescent, irreversibly arrested melanocytes; which would explain the lack of growth of this benign tumors. The same hypothesis had been previously formulated by Dorothy Bennett (Bennett, 2003). The data from Michaloglou et al. supports this hypothesis, as congenital naevi are positive for SA β-gal, and possess mosaic induction of p16INK4a. Thus, both in vitro and *in vivo*, BRAFV600E-expressing melanocytes display classical hallmarks of senescence, suggesting that oncogene-induced senescence represents a genuine protective physiological process in humans.

Chen et al. identified senescent cells in early-stage prostate abnormalities of mice engineered to sustain prostate-specific deletion of the PTEN tumor-suppressor gene (Chen et al., 2005). Under their experimental conditions loss of PTEN in prostate epithelial cells only results in a high-grade prostatic intraepithelial neoplasia. While loss of p53 does not initiate prostate tumors, co-deletion of PTEN and p53 promotes full-blown prostate cancer. In mice with concomitant loss of p53 and PTEN, senescence markers such as SA β-gal are absent, which indicates that p53-mediated senescence normally restricts the proliferation of PTEN-mutant cells *in vivo*. Notably, the tumor suppressor function of p53 in this context is not to induce apoptosis but to induce senescence. The authors show that SA β-gal staining can be observed specifically in specimens from early-stage human prostate cancers removed from patients but not in wild-type tissue or advanced prostate tumors. Their results suggest that progression from early, PTEN-deficient prostate lesions to advanced prostate cancer *in vivo* requires loss of p53 function to alleviate a senescent cell cycle arrest.

One interesting observation to come out from these studies is that low levels of expression of oncogenic RAS and BRAF (i.e. similar to endogenous levels) are sufficient

to trigger senescence (Braig et al., 2005; Collado et al., 2005). High concentrations of members of the RAS pathway are known to induce senescence, but it is not clear whether such high concentrations are physiological. Additionally, expression of oncogenic RAS at endogenous levels in mouse fibroblasts has been reported to not induce senescence (Guerra et al., 2003; Tuveson et al., 2004). These reports suggest that, at least in some settings, deregulation of oncogenes without any accompanying overexpression is enough to trigger senescence *in vivo*. Perhaps any level of expression of the oncogene that can result in an aberrant proliferative signal is enough to trigger senescence. The cellular context might therefore dictate the required level of expression for OIS to happen.

These studies show that oncogene-induced senescence acts as a tumor suppressor mechanism in early tumors initiated by an oncogenic activity. Since the reports focus on different human tumor types and mouse-model systems collectively they strengthen the idea that, similar to apoptosis, induction of cellular senescence is a strategy employed by mammalian cells to suppress cancer.

1.1.4 Fate of senescent cells *in vivo*

If we accept that cells senesce *in vivo* both as a result of tissue aging as well as of the execution of an anti-tumor response, then we need to ask what happens to these cells after they senesce. Do they remain there forever? Are they eventually removed? Can they ever resume growth? If melanocytic nevi are truly senescent melanocytes (Michaloglou et al., 2005), they represent an example of senescent cells that apparently can persist *in vivo* for decades. The observation that markers of senescence accumulate in aging tissues

and pre-malignant lesions, with no apparent apoptosis associated with their presence, would also argue that some senescent cells can persist *in vivo*. In a recent study done by our lab reactivation of p53 in a mouse model of liver carcinoma resulted in tumor regression involving the induction of a cellular senescence program that in turn triggered an innate immune response that targeted the tumor cells (Xue et al., 2007). Through the use of a Cre-loxP-based strategy Ventura et al. showed that restoring endogenous p53 expression leads to regression of lymphomas and sarcomas in mice without affecting normal tissues (Ventura et al., 2007). Apoptosis was the main consequence of p53 restoration in lymphomas, while sarcomas exhibited a suppression of cell growth with features of cellular senescence. According to these studies how p53 carries out its anticancer function seems to differ according to the tumor type and its context.

Senescent cells seem to be capable of recruiting the immune system, yet some senescent cells seem to be impervious to this clearance mechanism. It is not clear how such a recruitment would take place but it has bees suggested that it is related to some of the factors senescent cells secrete. The factors that are secreted by senescent cells seem to vary depending on the cell type (Shelton et al., 1999). Among the different mammalian cell types, fibroblasts have been the most thoroughly studied with regard to the senescent secretory phenotype. The reason for this being that fibroblasts synthesize and maintain the stromal support for virtually all renewable epithelial tissues. Senescent fibroblasts secrete high levels of several matrix metalloproteinases, epithelial growth factors, and inflammatory cytokines [reviewed in (Krtolica and Campisi, 2002)].

Work from the lab of Judith Campisi demonstrated that immortal but non-tumorigenic mammary epithelial cells produce undifferentiated tumors when injected into

mice together with senescent, but not pre-senescent, fibroblasts (Krtolica et al., 2001). This observation led them to propose a theory of antagonistic pleiotropy for senescence. According to this theory, senescence may prevent early life cancers by irreversibly arresting the proliferation of potential cancer cells; but later in life, as senescent cells accumulate, they may disrupt normal tissue structure and/or function. Senescent cells may therefore contribute to late life cancers by creating a tissue microenvironment that is permissive for malignant progression of cells that harbor oncogenic mutations. Other reports have provided additional evidence to support this theory (Coppe et al., 2006; Dilley et al., 2003; Roninson, 2002). The main assumptions of this theory are that senescent cell can persist *in vivo* and that they exhibit the same secretory phenotype as senescent cells in culture. Even when senescent cells have been detected *in vivo*, there have been no studies on their secretory phenotype or persistence. Without such data the theory will have to remain just a theory.

1.2 Mechanisms of senescence

1.2.1 Telomere-dependent senescence

More than 40 years after Hayflick introduced the notion of an internal clock or "replicometer" for normal cells we have made significant progress towards understanding the mechanisms underlying cellular senescence. Early studies by Hermann Muller and Barbara McClintock showed that the ends of chromosomes are capped by a structure called the telomere, which prevents chromosome fusions (McClintock, 1941; Muller,

1938). In the 1970's, as the mechanism of DNA replication became better understood, it became evident that DNA polymerase could not replicate the 3' end of linear duplex DNA (Watson, 1972). The end-replication problem was also recognized by the Russian theoretical biologist Alexey Olovnikov, who went further to propose that due to the nature of lagging-strand synthesis telomeres would shorten with each round of replication and that this mechanism could be the cause of replicative senescence (Olovnikov, 1971; Olovnikov, 1973).

Olovnikov model turned out to be incredibly accurate, and telomere shortening is considered to be the main causal mechanism of replicative senescence. It took almost 20 years though to prove the model, since at the time it was proposed nothing was known about the nature of telomeres. Once telomeres were found to be sequences of a hexameric repeat (Blackburn and Gall, 1978; Moyzis et al., 1988) it became possible to measure telomere length. Only then was it possible to show telomere erosion in cultured fibroblasts (Harley et al., 1990). Despite the increasing number of reports that supported the idea of telomere attrition limiting the proliferation of primary cells both in culture and *in vivo* (de Lange et al., 1990; Hastie et al., 1990; Lindsey et al., 1991) it remained unclear how some cells could remain immortal in culture.

Greider and Blackburn had discovered telomerase, the enzyme that synthesizes and elongates telomeres, back in 1985 (Greider and Blackburn, 1985). Telomerase has two components: and RNA template on which new telomeres are made and a catalytic subunit. Although telomerase activity was found in immortalized cell lines and human tumors (Counter et al., 1992; Kim et al., 1994) the definite breakthrough was possible only after the cloning of the catalytic portion of the enzyme (Nakamura et al., 1997). It

was then that a group of scientists at Geron showed that expression of the human telomerase catalytic subunit (hTERT) in retinal pigment epithelial cells and foreskin fibroblasts allowed the cells to bypass replicative senescence (Bodnar et al., 1998). The cells had elongated telomeres and divided vigorously while retaining a normal karyotype after greatly exceeding their normal life span. This study established a causal relationship between telomere shortening and in vitro cellular senescence. The main question then became how telomere shortening triggers cellular senescence.

Electron microscopy analysis of telomeres has revealed them as not linear structures but rather as duplex loops called t-loops (Griffith et al., 1999). The prevailing hypothesis is that these loops stabilize or cap the telomeres and capping may prevent the telomeres from being recognized as DNA damage. The telomeric repeat-binding factors TRF1 (van Steensel and de Lange, 1997) and TRF2 (Smogorzewska et al., 2000) are crucial for the maintenance of the t-loops. In particular TRF2 can remodel linear telomeric DNA into t-loops (Griffith et al., 1999). Inhibition of TRF2 induces apoptotic cell death (Karlseder et al., 1999) while overexpression of TRF2 increases the rate of telomere shortening in primary cells without accelerating senescence (Karlseder et al., 2002).

The fact that TRF2 can reduce the senescence setpoint, (telomere length at senescence) suggests replicative senescence is induced by a change in the protected status of shortened telomeres rather than by a complete loss of telomeric DNA. This notion is supported by the demonstration that hTERT is expressed in cycling primary presenescent human fibroblasts, where its disruption slows cell proliferation, restricts cell lifespan, and alters the maintenance of the 3' single-stranded telomeric overhang without changing the

rate of overall telomere shortening (Masutomi et al., 2003). These observations demonstrate that in normal human cells telomeres are dynamically regulated structures and that telomere length alone is unlikely to be responsible for triggering entry into replicative senescence. It has been proposed that telomere shortening may destabilize or prevent the capping of telomeres, leading to replicative senescence (Shay, 1999).

1.2.2 Molecular pathways involved in senescence.

More than 20 years ago the large T Antigen of the SV40 DNA tumor virus was shown to allow the bypass of senescence in human fibroblasts (Chang et al., 1986; Mayne et al., 1986). The bypass eventually lead to a crisis due to critically short telomeres, with some rare emergence of immortalized clones (Wright et al., 1989). It is known that T antigen binds p53 (Lane and Crawford, 1979; Linzer and Levine, 1979) and Rb (DeCaprio et al., 1988) and interferes with their function. Human fibroblasts immortalized with an inducible SV40 T-antigen become senescent following the de-induction of T-antigen. While a plasmid expressing an alternative source of intact T-antigen can restore proliferation, T-antigen deletion mutants lacking either the RB or p53 binding domains are unable to do so (Shay et al., 1991). These findings point to Rb and p53 as being the main tumor suppressors controlling cellular senescence.

p53 is by far the most studied tumor suppressor gene. An immense amount of literature points to it being a damage sensor, capable of activating genetic programs that can halt the cell cycle transiently, permanently (senescence), or eliminate the cell altogether (apoptosis). A wide variety of reports highlight p53 as playing a critical role in

cellular senescence. Abrogation of the p53 can bypass senescence or reverse it in mouse cells and certain human cells (Beausejour et al., 2003; Dirac and Bernards, 2003; Shay et al., 1991). Enforced expression of p53 can induce a senescent-like phenotype in certain cell types and a variety of stimuli induce senescence in a p53/p21-dependent manner. Telomere shortening or dysfunction as well as oncogene expression induce a DNA damage response mediated by p53 (Bartkova et al., 2006; d'Adda di Fagagna et al., 2003; Di Micco et al., 2006; Mallette et al., 2007). It is not clear how p53 induces senescence but the cell cycle arrest per se appears to occur through the induction of p21 which in turns inhibits the activity of cyclin-dependent kinases (CDKs) resulting in hypophosphorylation of pocket protein family members (Figure 1.2).

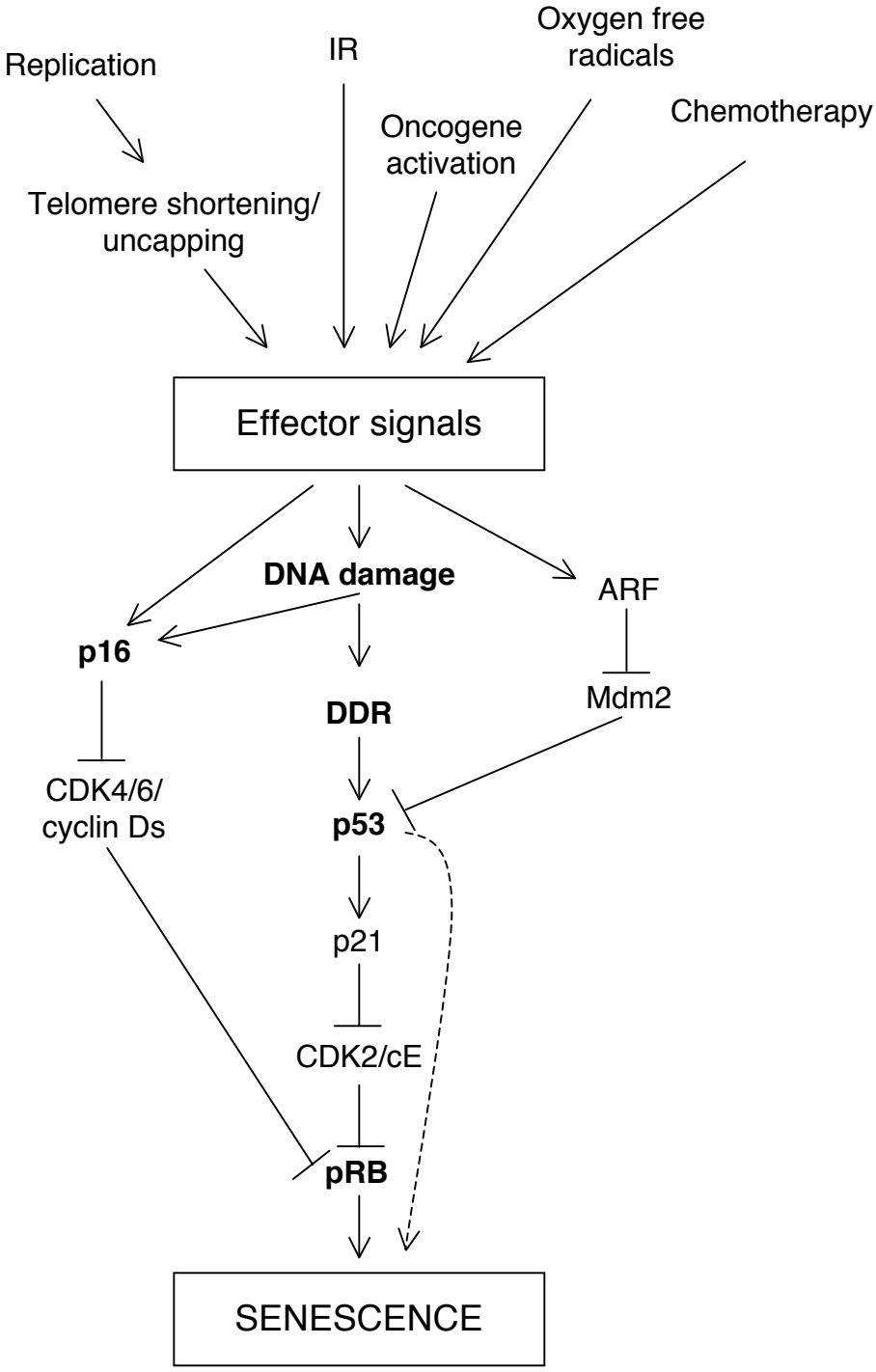
Rb remains hypoposphorylated in senescent cells (Stein et al., 1999), consistent with the downregulation of the activity of CDK4 and CDK6 kinases. It is believed that p16 regulates senescence in an Rb-dependent manner by inhibiting CDK4 and CDK6 activity. In human fibroblasts senescence can be established in the absence of a functional p16/Rb pathway. The cells lack the senescent associated heterochromatic foci ((Narita et al., 2003) see below) and can resume growth following p53 inactivation (Beausejour et al., 2003), consistent with the notion that the Rb and p53 pathways are essential for senescence. Loss of Rb appears to be sufficient for cell cycle re-entry and senescence reversal in MEFs (Sage et al., 2003). Inactivation of a temperature sensitive Rb in senescent Saos-2 cells also results in S-phase reentry (Alexander et al., 2003). These data will be further discussed in Chapter 3.

Figure 1.2

A model for the molecular pathways involved in cellular senescence

A variety of intrinsic (telomeres) and extrinsic signals feed into one program. Induction of p53 and p16 happens through DNA damage-dependent and -independent pathways. They, in turn, transduce the senescence-inducing signals to promote the establishment of senescence. The p16/Rb pathway promotes the formation of SAHF and it is possible that the DNA damage foci contribute to SAHF formation.

IR: Ionizing radiation; DDR: DNA-damage response, pRb: pocket protein family (Rb, p107 and p130).


Replication
IR
Oxygen free radicals
Chemotherapy
Oncogene activation
Telomere shortening/ uncapping
Effector signals
DNA damage
ARF
p16
Mdm2
DDR
CDK4/6/ cyclin Ds
p53
p21
CDK2/cE
pRB
SENESCENCE

Although Rb and p53 represent two important control points in the senescence network, the proteins connecting activating signals to these tumor suppressors and the effector proteins that are downstream of them, are critical for an efficient anti-tumor response. Since senescence is characterized by a permanent cell cycle arrest, significant attention has been paid to p53 and Rb targets that mediate cell cycle arrest. The CDK-inhibitor p21 is a direct p53 target that has been reported as being upregulated in senescence in a variety of systems. Most studies suggest that p21 induction by p53 inhibits CDK2/Cyclin E activity. Activity of CDK4/Cyclin Ds can also be inhibited by p21. Inhibition of activity of CDKs by p21 results in hypophosphorylation of pRB, which is believed to mediate cell cycle arrest during senescence. Whether p21 plays an essential role in the establishment of senescence has not been unambiguously answered. Some studies suggest p21 is dispensable for OIS (Pantoja and Serrano, 1999). p21 levels have been reported to decline in the final stages of senescence, suggesting its upregulation is required for the establishment but not the maintenance of senescence (Alcorta et al., 1996; Stein et al., 1999). There is however no functional basis for this suggestion.

In many instances, p53 and Rb are activated to promote senescence by products of the INK4a/ARF locus [reviewed by (Lowe and Sherr, 2003)]. This locus encodes two tumor suppressors, p16INK4a and p14ARF (p19ARF in mice), expressed from partially overlapping nucleotide sequences read in alternative reading frames. p16INK4a engages the Rb pathway by inhibiting cyclin D-dependent kinases that would otherwise phosphorylate and inactivate Rb. In contrast, p14ARF increases the growth suppressive functions of p53 by interfering with its negative regulator, Mdm2.

ARF is activated by multiple oncogenic stimuli (de Stanchina et al., 1998; Zindy et al., 1998; Zindy et al., 2003) and is required for induction of p53 and senescence in response to oncogenic Ras in mouse primary fibroblasts (Ferbeyre et al., 2002) and keratinocytes (Lin and Lowe, 2001). On the other hand, oncogenic Ras does not induce p53 via ARF in human fibroblasts and, as a consequence, ARF is not required for ras-induced senescence in these cells (Wei et al., 2001). It has been recently reported that MDM2 interacts with Rb and that ARF can disrupt this interaction resulting in Rb accumulation by preventing its proteasome-dependent degradation (Chang et al., 2007). There are some scattered reports in the literature alluding to this functional interplay between Rb and ARF. Its relevance to senescence remains to be explored.

p16INK4a is an inhibitor of cyclin dependent kinases 4 and 6, known to mediate cell cycle arrest in transient assays (Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995; Serrano et al., 1995). Even though p16INK4a is inactivated in a wide variety of tumors (Baylin et al., 1998), its regulation and the stages of tumor progression that are suppressed by the protein remain poorly defined. A significant amount of evidence suggests a role for p16INK4a in senescence of cultured cells. p16INK4a accumulates as fibroblasts, T-lymphocytes, keratinocytes, mammary epithelial cells, uroepithelial cells, and prostate cells senesce. p16 accumulation has only been reported in the context of senescence, suggesting its tumor suppressor abilities result from its function in this process.

Recent work has indicated that downregulation of p16 through a knockdown strategy is not functionally equal to inactivation of Rb (Wei et al., 2003). This result suggests another CDK inhibitor might be relevant in the induction of the senescent

phenotype. It is known that p21 can inhibit CDK2/CyclinE activity, preventing these complex from phosphorylating Rb. Since the senescent-cell-cycle arrest occurs prior to the accumulation of the Cdk4-Cdk6 inhibitor p16, p21 might be sufficient for this event. Accordingly, cyclin D1-associated phosphorylation of pRb at Ser-780 is lacking even in newly senescent fibroblasts that have a low amount of p16. Instead, the cyclin D1-Cdk4 and cyclin D1-Cdk6 complexes in these cells are associated with an increased amount of p21, suggesting that p21 may be responsible for inactivation of both cyclin E- and cyclin D1-associated kinase activity at the early stage of senescence (Stein et al., 1999). p16 and p21 might therefore cooperate to keep Rb hypophosphorylated during senescence. Further work will either strengthen this notion or open up new possibilities.

The role of p15 in senescence is now starting to be explored. The CDKN2b-CDKN2a locus encodes three cell cycle inhibitory proteins: p15INK4b encoded by CDKN2b, p16INK4a encoded by CDKN2a and p14ARF (p19Arf in mice) encoded by an alternative reading frame of CDKN2a (Gil and Peters, 2006). Mice deficient for all three open reading frames (TKO) are more tumor-prone and develop a wider spectrum of tumors than Cdkn2a mutant mice, with a preponderance of skin tumors and soft tissue sarcomas (Krimpenfort et al., 2007). This study by Anton Bern's group showed that the TKO MEFs are substantially more sensitive to oncogenic transformation than p16Ink4a knockout MEFs. p15Ink4b protein levels were elevated in MEFs deficient for p16INK4a under conditions of stress, suggesting that . p15Ink4b can somehow backup p16INK4a function. Since p15 has been identified as an upregulated gene in OIS (Collado et al., 2005), further studies on the role of p15 in senescence are sure to follow.

The promyelocytic leukemia (PML) gene is upregulated during cellular senescence, with PML nuclear bodies increasing both in size and number (Ferbeyre et al., 2000). PML is a RING finger protein that localizes to large nuclear structures called promyelocytic oncogenic domains (PODs), ND10, or PML nuclear bodies. PML along with the autoimmune antigen Sp100 are essential components of nuclear bodies. A variable number of accessory proteins known to regulate processes, such as transcription or repair shuttle in and out of the PML nuclear bodies; suggesting they may act as transient storage sites for regulatory proteins and/or organizing centers where nuclear processes are regulated or executed (for review, see (Borden, 2002; Zhong et al., 2000)).

HP1 proteins have been reported to localize to PML bodies (Everett et al., 1999; Seeler et al., 1998) and work by Peter Adams and co-workers suggest that this localization might be important for SAHF formation during senescence (see below). Ectopic expression of PML induces senescence in a p53-dependent manner (Pearson et al., 2000). Studies by de Stanchina and coworkers showed that ARF and p53 are required for PML upregulation in response to oncogenic Ras and that PML is a direct p53 target gene (de Stanchina et al., 2004). Interestingly, PML can bind MDM2 and sequester it into the nucleolus, therefore protecting p53 from proteasome-mediated degradation (Bernardi et al., 2004). In recent studies done in human fibroblasts PML overexpression induced senescence in a pRb-dependent manner rather than a p53-dependent manner (Bischof et al., 2005; Mallette et al., 2004). Further studies will be needed to dissect the contribution of PML to cellular senescence in human and mouse cells.

Despite our incomplete understanding of the process it seems fair to say that, much like apoptosis, senescence is a genetically controlled process that entails the

activation of molecular pathways that are key to tumor suppression (Figure 1.2). Although significant progress has been made in the last few years, further work will be needed to understand the molecular mechanisms underlying the establishment and maintenance of cellular senescence.

1.2.3 Role of DNA damage in replicative and premature senescence

Since the field of senescence evolved from the notion of a set proliferative lifespan built into cells, intrinsically dictated by telomere length, there was an inherent resistance from people in the field to accept premature senescence as a related process (Ramirez et al., 2001; Rubin, 1997; Rubin, 1998). Despite the fact that the processes were phenotipically indistinguishable and that most findings pertaining the molecular mechanisms involved in them appeared to be the same, the field of senescence remained divided. Those studying replicative senescence focused their attention on the nature of the telomeric signals that triggered senescence and the effect the process had on organismal aging. The researchers studying premature senescence were more concerned with the role the process had in tumor suppression.

As the two fields developed in parallel, a number of observations were made by people studying replicative senescence that were hard to explain. One of them was that telomerase expression was not sufficient to immortalize human keratinocytes or mammary epithelial cells. These cells were reported to require both telomerase expression and inactivation of Rb/p16ink4a to bypass senescence and be immortalized (Kiyono et al., 1998). A similar observation was made in rodent cells, which possess

telomerase activity and have very long telomeres, yet are also able to senesce in culture. In 2003 it was shown that senescent human fibroblasts display molecular markers characteristic of cells bearing DNA double-strand breaks (d'Adda di Fagagna et al., 2003). This work showed that uncapped telomeres were able to directly associate with many DNA damage response proteins and that inactivation of DNA damage checkpoint kinases in senescent cells could restore cell-cycle progression into S phase. The model proposed by the authors was one in which replicative senescence results from the activation of a DNA damage checkpoint by dysfunctional telomeres. This single idea could reduce replicative senescence to one of many signals that can activate cellular senescence. The term cellular senescence now indicates the existence of a variety of intrinsic (telomeres) and extrinsic signals that feed into one program.

A number of recent papers provide evidence that oncogene induced senescence (OIS) involves activation of p53 via the DNA damage response (DDR) (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007). The study by Di Micco et al. showed that senescence, triggered by the expression of an activated oncogene (H-RasV12) in BJ fibroblasts, is accompanied by the activation of a robust DDR. Senescent cells appear to arrest with partly replicated DNA and with DNA replication origins having fired multiple times. In their experimental setting oncogene expression does not trigger a DDR in the absence of DNA replication. Additionally, inactivation of DDR abrogates OIS and promotes cell transformation. They propose that OIS results from the enforcement of a DDR triggered by oncogene-induced DNA hyper-replication.

Barktova et al. also show that oncogene-induced senescence is associated with signs of DNA replication stress, including prematurely terminated DNA replication forks

and DNA double-strand breaks. In their experimental setting inhibiting the DNA double-strand break response kinase ataxia telangiectasia mutated (ATM) also suppressed the induction of senescence. While Bartkova et al. or DiMicco et al. could bypass OIS by interfering the DDR, Mallette et al. only observed a rescue of OIS by inactivation of the DDR in the context of inactivation of the Rb tumor suppressor pathway. Earlier work on OIS in human fibroblasts established that the Rb and the p53 pathways had to be simultaneously inactivated to bypass OIS (Ferbeyre et al., 2000; Serrano et al., 1997). This original work was done in the strain of normal fibroblasts IMR90, the same cell line used in Mallette's study. In contrast, Barktova et al. and Di Micco et al. used BJ foreskin fibroblasts, a cell line in which inactivation of p53 is sufficient to bypass OIS (Voorhoeve et al., 2006). Their results reinforce the idea that p53 is being activated by the DDR, since a prediction stemming from this model is that inactivation of the DDR in BJ cells would bypass OIS. Taken together these studies suggest cellular senescence might be a special case of cell cycle arrest resulting from chronic DNA damage signaling (Figure 1.3).

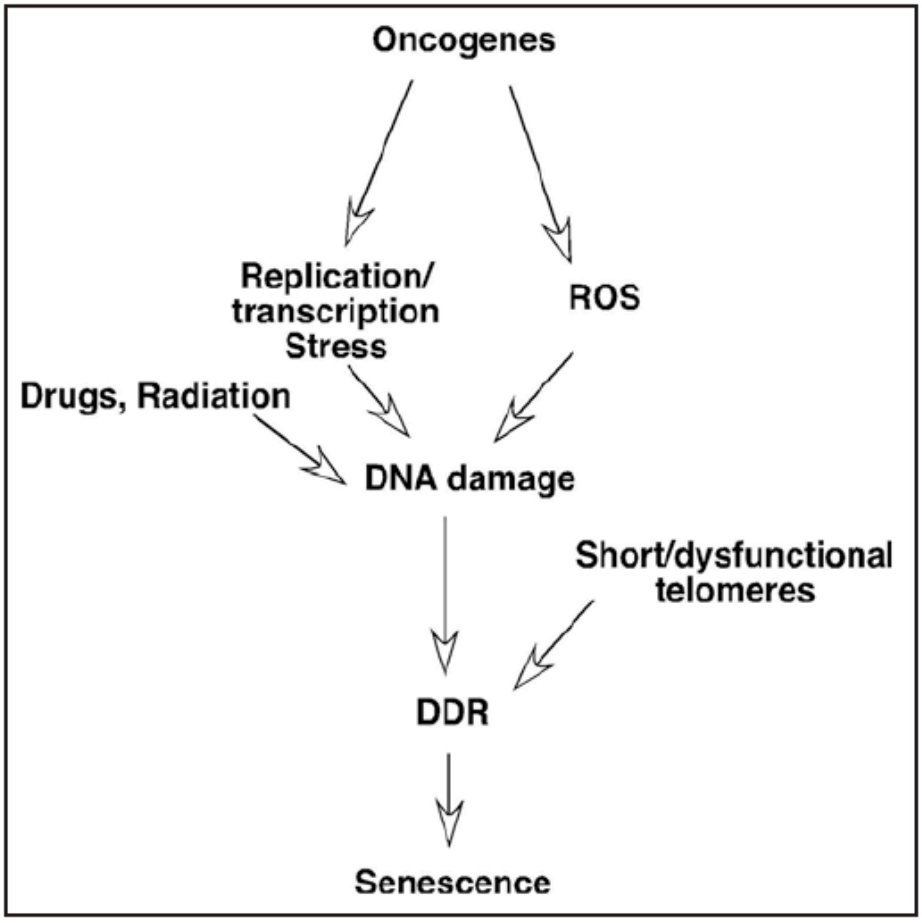
An intriguing observation made in the study by Mallette et al. is that the accumulation of oncogene-induced DNA damage foci (ODDI) is not eliminates in cells that bypass OIS by inactivation of p53 and Rb. This suggests a mechanism that may limit transformation in immortalized cells, since suppression of DNA damage signals would be required for tumor progression.

Figure 1.3

Senescence a general response to DNA damage signals

Oncogenes may induce DNA damage by forcing an aberrant DNA replication process where some replication forks arrest as a result of some yet unknown mechanisms. Oncogenes might also induce the accumulation of ROS, which are known for their reactivity and ability to produce oxidative base damage and DNA breaks. (Mallete, F.A. amd Ferbeyre, G. Cell Cycle 6:15, 1831-1836, 1 August 2007).

DDR: DNA-damage response; ROS: Reactive Oxygen Species



[Cell Cycle 6:15, 1831-1836, 1 August 2007]

1.3 Chromatin biology and senescence

1.3.1 Background on chromatin

The term chromatin is used to describe the complex of DNA and proteins that makes up chromosomes. In eukaryotes the lowest level of chromatin compaction is a 10 nm fiber, in which DNA is wrapped around 4 pairs of histones to form a nucleosome (Kornberg, 1974). Exceptions aside, the nucleosome repeats roughly every 200 base pairs (bp), with about 146 bp of dsDNA wrapped in 1.65 left-handed superhelical turns around four identical pairs of histones (collectively known as the histone octamer) and the remaining 50 bp being the "linker DNA". A chain of nucleosomes can be arranged in a 30 nm fiber that is stabilized by Histone H1. The structure of chromatin is poorly understood beyond this level of compaction, but the common belief is that the 30 nm fiber is arranged into loops along a central protein scaffold to form transcriptionally active euchromatin. Further compaction leads to transcriptionally inactive heterochromatin.

Heterochromatin is classically defined by condensation throughout the cell cycle, replication in late S phase and gene inactivity. There are regions of DNA that exist as heterochromatin in all cells for a given species. This type of heterochromatin is known as constitutive heterochromatin, and is believed to never convert back to euchromatin. It is generally found at centromeres and telomeres, although it does occur throughout chromosomes. Facultative heterochromatin refers to regions of heterochromatin that are not consistent within the different cell types of a species. Formation of facultative heterochromatin is a regulated event that is often associated with morphogenesis or

differentiation. The best known example of facultative heterochromatin is the mammalian inactive X chromosome (Beutler et al., 1962; Ohno et al., 1961).

1.3.2 Histone modifications

To date 8 distinct types of modifications, dispersed among more than 60 different residues have been reported to exist on core histones [reviewed by (Kouzarides, 2007)]. Table 1.1 summarizes the different classes of modifications and the residues they modify. Most of these modifications remain poorly understood. Substantial progress has been made in the understanding of lysine acetylation and methylation. The current view is that lysine acetylation correlates with chromatin accessibility and transcriptional activity while lysine methylation can have different effects depending on which residue is modified. In general methylation of histone H3 lysine 4 (H3K4) and H3 lysine 36 is associated with transcribed chromatin, while methylation of H3 lysine 9 (H3K9), H3 lysine 27 (H3K27), and H4 lysine 20 (H4K20) generally correlate with transcriptional repression. To make things even more complicated there seems to be cross-talk between modifications, since an enzyme may recognize its substrate more or less effectively in the context of other modifications (Fischle et al., 2003).

A wide variety of enzymes that direct these histone modifications have been identified in the last few years (Table 1.2). Enzymes capable of removing the modifications have also been identified, except for arginine demethylases. The specificity

Table 1.1

Overview of the different classes of modifications identified on histones

(Kouzarides, T. Cell 128, 693-705, February 23, 2007)

Table 1. Different Classes of Modifications Identified on Histones

Chromatin Modifications	Residues Modified
Acetylation	**K**-ac
Methylation (lysines)	**K**-me1 **K**-me2 **K**-me3
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me2s
Phosphorylation	**S**-ph **T**-ph
Ubiquitylation	**K**-ub
Sumoylation	**K**-su
ADP ribosylation	**E**-ar
Deimination	**R > Cit**
Proline Isomerization	**P-cis > P-trans**

Table 1.2

Overview of the different histone-modifying enzymes

Human and yeast histone-modifying enzymes of known specificity. Yeast enzymes are distinguished by the prefix Sc (Saccharomyces cerevisiae) or Sp (Saccharomyces pombe).

(Kouzarides, T. Cell 128, 693-705, February 23, 2007)

Table 2. Histone-Modifying Enzymes

Enzymes that Modify Histones	Residues Modified
Acetyltransferase	
HAT1	H4 (K5, K12)
CBP/P300	H3 (K14, K18) H4 (K5, K8) H2A (K5) H2B (K12, K15)
PCAF/GCN5	H3 (K9, K14, K18)
TIP60	H4 (K5, K8, K12, K16) H3 K14
HB01 (ScESA1, SpMST1)	H4 (K5, K8, K12)
ScSAS3	H3 (K14, K23)
ScSAS2 (SpMST2)	H4 K16
ScRTT109	H3 K56
Deacetylases	
SirT2 (ScSir2)	H4 K16
Arginine Methlytransferases	
CARM1	H3 (R2, R17, R26)
PRMT4	H4R3
PRMT5	H3R8, H4R3
Serine/Thrionine Kinases	
Haspin	H3T3
MSK1	H3S28
MSK2	H3S28
CKII	H4S1
Mst1	H2BS14
Lysine Demethylases	
LSD1/BHC110	H3K4
JHDM1a	H3K36
JHDM1b	H3K36
JHDM2a	H3K9
JHDM2b	H3K9
JMJD2A/JHDM3A	H3K9, H3K36
JMJD2B	H3K9
JMJD2C/GASC1	H3K9, H3K36
JMJD2D	H3K9

Table 2. *Continued*

Lysine Methyltransferase	
SUV39H1	H3K9
SUV39H2	H3K9
G9a	H3K9
ESET/SETDB1	H3K9
EuHMTase/GLP	H3K9
CLL8	H3K9
SpClr4	H3K9
MLL1	H3K4
MLL2	H3K4
MLL3	H3K4
MLL4	H3K4
MLL5	H3K4
SET1A	H3K4
SET1B	H3K4
ASH1	H3K4
Sc/Sp SET1	H3K4
SET2 (Sc/Sp SET2)	H3K36
NSD1	H3K36
SYMD2	H3K36
DOT1	H3K79
Sc/Sp DOT1	H3K79
Pr-SET 7/8	H4K20
SUV4 20H1	H4K20
SUV420H2	H4K20
SpSet 9	H4K20
EZH2	H3K27
RIZ1	H3K9
Ubiquitilases	
Bmi/Ring1A	H2AK119
RNF20/RNF40	H2BK120
Proline Isomerases	
ScFPR4	H3P30, H3P38

indicated for each enzyme is that suggested by different studies. However, it has become clear that the specificity is not only dictated by a particular residue; since these enzyme can participate in the formation of different complexes, which can alter their residue selection, the context in which they recognize it or the extent to which they will modify it (mono-, di- or tri-). Additionally, canonical histones in a nucleosome can be replaced by histone variants through a replication independent deposition mechanism [reviewed by (Kamakaka and Biggins, 2005)].

1.3.3 Chromatin remodeling enzymes.

Histone modifications are believed to alter chromatin structure by directly affecting its level of compaction. This is probably achieved by affecting the contact between different histones in adjacent nucleosomes or the interaction of histones with DNA. Additionally, histone modifications can either prevent the docking of certain non-histone proteins onto chromatin or specifically recruit chromatin remodeling enzymes. Such enzymes can further modify chromatin through their different enzymatic activities.

A wide variety of nucleosome remodeling complexes have been described to date : the SWI/SNF family (which includes SWI/SNF and RSC), the ISWI family, the CHD family and the INO80 and SWR1 family [Reviewed by (Gangaraju and Bartholomew, 2007)]. Proteins are recruited and bind to histone modifications via specific domains. Methylation appears to be recognized by chromo-like domains of the Royal family (chromo, tudor, MBT) as well as nonrelated PHD domains, acetylation is recognized by bromodomains and phosphorylation is recognized by a domain within 14-3-3 proteins

(Daniel et al., 2005). Different groups have found that the chromodomain of HP1 specifically recognizes methylated H3-K9 and that its specific nuclear localization is dependent on Suv39h catalytic activity (Bannister et al., 2001; Lachner et al., 2001). Subsequently it was shown that HP1 and Suv39h interact and that the interaction is necessary for the targeting of HP1 to chromatin (Stewart et al., 2005).

It has been recently demonstrated that members of the Polycomb group (PcG) and trithorax group (trxG) of proteins contain intrinsic histone methyltransferase (HMTase) [Reviewed by (Cao and Zhang, 2004)]. Polycomb group (PcG) proteins are known to play essential roles in development and in the epigenetic maintenance of lineage-specific gene repression (Ringrose and Paro, 2004). They are required for ES cell pluripotency and are markedly downregulated upon differentiation (Valk-Lingbeek et al., 2004). Polycomb repressive complex 2 (PRC2) catalyzes H3K27 methylation, while PRC1 binds methylated H3K27 and mediates chromatin compaction (Margueron et al., 2005; Ringrose and Paro, 2004). Further contributions of PcG and trxG proteins in chromatin remodeling will certainly emerge.

1.3.4 Chromatin changes in senescence

Senescent human fibroblasts stained with DAPI exhibit a unique pattern of punctate DNA foci. Work from our lab identified these foci as a distinctive type of facultative heterochromatin—designated SAHF (Senescence Associated Heterochromatic Foci)—that accumulates in senescent cells (Narita et al., 2003). SAHFs contain the heterochromatin-associated proteins K9M-H3 and HP1, exclude histone modifications

found in euchromatin (e.g., K9Ac-H3 and K4M-H3), and are not sites of active transcription. SAHFs are distinct from pericentric heterochromatin, and their appearance is accompanied by an increase in HP1 incorporation into senescent chromatin and an enhanced resistance of senescent DNA to nuclease digestion. SAHF formation requires an intact Rb pathway and we showed that heterochromatin-associated proteins and the Rb tumor suppressor can accumulate on the E2F-responsive promoters in senescent but not quiescent cells, and that these changes are associated with more stable repression of E2F responsive genes. Taken together our results suggest that SAHFs causally contribute to cellular senescence, at least in part, by controlling the stability of the arrest.

Following the initial studies in our laboratory, Peter Adams and co-workers showed that SAHF are enriched for the histone variant macroH2A (Zhang et al., 2005). Their work also indicates that a known chromatin regulator, HIRA, enters PML nuclear bodies, where it transiently colocalizes with HP1 proteins, prior to incorporation of HP1 proteins into SAHF. They further show that HIRA and another chromatin regulator, ASF1a, physically interact and that overexpression of these proteins can induce senescence and SAHF formation. Through the use of a short hairpin RNA targeting ASF1a they showed that the protein is required for formation of SAHF. In a follow up study they showed that HIRA's translocation to PML bodies occurs in response to a variety of senescence triggers and that it precedes other markers of senescence, including the appearance of (SAHF) and cell cycle exit (Ye et al., 2007). Through the use of T Antigen they suggest that translocation of HIRA to PML bodies occurs in the absence of functional pRB and p53 tumor suppressor pathways. They report however that downstream of HIRA's localization to PML bodies, the HIRA/ASF1a pathway cooperates

with pRB and p53 to make SAHF, with the HIRA/ASF1a and pRB pathways acting in parallel. They propose that the convergence of the HIRA/ASF1a and pRB pathways occurs through a DNAJ-domain protein, DNAJA2.

In yet another follow up study, Adams showed that the ability of ASF1a to induce senescence and SAHF formation depends on its physical interaction with its deposition substrate, histone H3, in addition to its co-chaperone, HIRA (Zhang et al., 2007). They also showed that in cells entering senescence, HP1γ, but not the related proteins HP1α and HP1β, becomes phosphorylated on serine 93 and that this phosphorylation might affect incorporation of HP1γ into SAHF. Interestingly, reduction in the amount of chromatin-bound HP1 proteins does not detectably affect chromosome condensation into SAHF, accumulation in SAHF of histone H3 methylated on lysine 9, the recruitment of macroH2A proteins, nor other hallmarks of senescence, such as the expression of SAβ-gal activity and senescence-associated cell cycle exit.

In order to further characterize chromatin changes that contribute to cellular senescence, our laboratory examined the protein composition of chromatin preparations from quiescent (reversibly arrested) and senescent normal diploid IMR90 fibroblasts (Narita et al., 2006). Microsequencing by mass spectrometry of bands that accumulated on chromatin of senescent but not quiescent cells led to the identification of HMGA1 and HMGA2 as proteins that specifically accumulate on chromatin in senescent cells, where they promote and maintain proliferative arrest. HMGA proteins act in a mutually reinforcing manner with p16INK4a to promote SAHF formation and contribute independently to the stable repression of E2F target genes. Accordingly, suppression of both HMGA and p16INK4a in growing cells acts synergistically to enable cells to bypass

senescence and leads to the reactivation of some E2F target genes and S phase re-entry in senescent cells. Thus, HMGA proteins act as part of the program that initiates and maintains the senescent state. How this works together with the HP1 proteins and histone modifications remains unclear.

Among other chromatin changes that accompany senescence is the loss of linker histone H1 from chromatin (Funayama et al., 2006). Interestingly, the authors report that the extent of histone H1 reduction is directly correlated with the frequency of SAHF-positive cells. Their hypothesis is that HMGA2 substitutes for H1 during senescence (since both proteins appear to compete with each other for the same binding site (Catez et al., 2004)), and that this substitution is required for SAHF formation.

Numerous reports resulting from studies in a variety of organisms speak to a re-localization of silenced genes to regions of heterochromatin. It is not yet clear if and how this re-localization to repressed regions of the genome is involved in gene silencing. Work from the lab of Peter Adams and Fuyuki Ishikawa suggests that each SAHF represents a single chromosome (Funayama et al., 2006; Zhang et al., 2007). Both groups based their results on the analysis of optical section rather than 3-D renderings of the senescent nuclei. Even when it should be easier to prove colocalization of the chromosome paint with a single SAHF at a single focal plane, their data does not seem to support their conclusion. Further, more refined experiments will be needed before we understand what is contained in SAHF.

Despite the general heterochromatinization observed in senescent fibroblasts, a variety of genes are upregulated during senescence. A study by Zhang and coworkers reported clustering of 150 of the 376 genes they found to be specifically upregulated

during senescence in human fibroblasts (Zhang et al., 2003). The 150 clustered up-regulated genes were present in a total of 60 groupings of 2 to 6 genes. Their finding led to the suggestion that the opening of certain chromatin domains (i.e., conversion of heterochromatin to euchromatin) may be a feature of replicative senescence. This possibility remains to be explored.

1.3.5 Rb and heterochromatin

Rb is known to functionally interact with a number of factors that are involved in heterochromatin biology including HP1, Suv39h1, DNMT1, and components of the SWI/SNF chromatin remodeling complex (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Nielsen et al., 2001). Classically, Rb is believed to function as a tumor suppressor through its ability to block cell cycle progression. These interactions were therefore viewed as Rb's recruitment of heterochromatinizing activities for the silencing of specific promoters.

Recently reports have emerged correlating Rb loss with global heterochromatic defects. MEFS where all Rb family members (Rb, p130, p107) were knocked out display genomic instability, which coincides with decreased DNA methylation, increased acetylation of histone H3 and decreased tri-methylation of histone H4 at lysine 20 (H4K20). H4K20 tri-methylation was specifically decreased at pericentric and telomeric chromatin (Gonzalo et al., 2005).

As mentioned earlier, constitutive heterochromatin is characterized by hypermethylation of DNA, and hypoacetylation and hypermethylation of histones. It has

been shown that H3K9 tri-methylation by the Suv39h HMTases occurs at telomeres and centromeres (Garcia-Cao et al., 2004; Peters et al., 2001). Tri-methylation of H4K20 by Suv4-20h HMTases also marks pericentric chromatin and requires previous H3K9 tri-methylation by the Suv39h enzymes (Kourmouli et al., 2004; Schotta et al., 2004). Interestingly, cells deficient in the Suv39h HMTases (Suv39 dn cells) share many of the phenotypes described here by Gonzalo and colleagues for Rb family knockout cells, such as chromosome segregation errors leading to hypotetraploidy and to non-segregated chromosomes and deregulated telomere length.

Subsequently, a study utilizing mouse adult fibroblasts (MAFs) derived from RbloxP/loxP mice to acutely knockout RB showed that histone H4 lysine 20 trimethylation was absent from heterochromatin domains following loss of Rb (Siddiqui et al., 2007). The original studies reported no defects in the Rb knock out cells, but perhaps this was due to developmental compensation by Rb family members, something that does not happen in the acute knock out MAF model. Through a generation of a mutant strain of mice carrying three amino acid substitutions in the LXCXE binding site of the pocket domain of Rb Fred Dick's group showed the interaction of LXCXE-containing chromatin regulators with pRb are essential for H4-K20 trimethylation at pericentric heterochromatin (Isaac et al., 2006). Disruption of pericentric heterochromatin structure leads to centromere fusions in, chromosome missegregation and genomic instability. Together, these reports illustrate that loss of chromatin regulation by pRb may affect processes that are seemingly unrelated, or indirectly related, to changes at the G1-to-S-phase transition.

The role of Rb in the maintenance of senescence-associated heterochromatic foci will be extensively discussed in Chapter 3.

1.4 Discussion

Current literature point to cellular senescence being a carefully orchestrated cellular program that parallels apoptosis as a cellular response to stress. The key difference between apoptosis and senescence is that the apoptosis results in the elimination of the cell that executed the program, while senescence re-directs the cell into a specialized form of terminal differentiation that prevents it from further dividing. This means that senescence need not only to be establishes but also maintained in order to be an effective anti-tumor response. A great deal of work has focused on situations in which senescence can be bypassed, as this is emerging as a key even during tumorigenesis. Mutations that allow for the reversal of senescence rather than its bypass could also promote tumor progression and contribute to drug resistance, yet this topic has remained largely unexplored. The main goal of my thesis work has been to characterize the molecular mechanisms responsible for maintaining cellular senescence, which we believe, is essential for the potent tumor suppressive effect of this process.

At its conception the goal of my project was to assess the ability of the adenoviral E1A oncoprotein to reverse senescence in normal human diploid fibroblasts. It has been long known that E1A can override Ras induced senescence in human fibroblasts, but it is not known whether E1A can reverse the arrest once it has been established (Ruley, 1983).

The ultimate goal was to use E1A as a guide to identify cellular activities also capable of reversing senescence; which, in turn, would provide insight into the mechanism of senescence maintenance. Despite the initial encouraging results, the cells only managed to re-enter the cell cycle but died before they could divide. As a lot of troubleshooting took place to evaluate whether the death was preventable (i.e. the result of a technical problem) one result stood from the rest - the senescent cells that re-entered the cell cycle lost the condensed DAPI foci that are present in the nuclei of senescent fibroblasts.

At the time I joined the lab, Masashi Narita had started characterizing this condensed DAPI foci and had preliminary data indicating they contained features of heterochromatin such as HP1 and tri-methyl K9 H3. As I continued my efforts to reverse senescence, a significant amount of my attention was diverted to the characterization of SAHF under the guidance of Dr. Narita. This work led to the publication of our first article, in which we show that senescent cells form a novel type of facultative heterochromatin that we termed Senescence Associated Heterochromatic Foci (SAHFs) (see section 1.3.1) One of the observations that came out of this work is that the p16/Rb pathway is required for the formation of SAHF. Since it is known that E1A binds Rb and interferes with its function, I hypothesized that inhibition of Rb might, like E1A does, reverse SAHF. Additionally, despite some controls that suggested otherwise, we had not ruled out the possibility that the death of the cells that re-entered the cell cycle as a result of E1A infection was due to the oncoproteins' ability to sensitize to apoptosis. If that was the case, inhibition of Rb could allow for re-entry into the cell cycle. By this time I had tested multiple lentiviral systems and established a protocol to infect senescent human fibroblasts with high efficiency. Unfortunately the results obtained with lentiviral E1A

recapitulated those obtained with adenoviral E1A, formally ruling out adenoviral toxicity as the cause of death during the reversal of senescence.

The lentiviral system proved useful however, since it allowed us to clone in and transduce any DNA sequences into senescent cells. At the time, work from the Hannon lab showed that short hairpin RNAs could induce sequence-specific silencing in mammalian cells (Paddison et al., 2002). The shRNAs could be generated exogenously or its transcription could be driven endogenously from a RNA polymerase III promoter. By cloning the hairpin sequences under a Pol III promoter into the lentiviral vector it became possible to suppress the expression of any gene in senescent cells. I was therefore able to test the effect of Rb knockdown in senescent cells. Like enforced E1A expression, Rb knockdown resulted in the re-entry of cells into the cell cycle. The kinetics of the reversal was much slower yet the results were ultimately the same and the cells died prior to dividing, in a manner that could not be prevented by Bcl-2 expression or use of pan-caspase inhibitors. Once again SAHF dissolved prior to re-entry into the cell cycle and E2F targets were re-expressed. The nuclease resistant fraction in these cells was diminished, consistent with a loss in heterochromatin. Analysis of chromatin bound proteins indicated a shift into a more euchromatic profile. (Hetrochromatic marks were specifically loss and euchromatic marks were gained at E2F-target promoter following Rb knockdown.)

Chapter 2 of this dissertation describes the efforts devoted to the initial characterization of SAHF, including the development of assays to measure the presence of SAHF in bulk populations. A number of experiments included in this chapter were done by Masashi Narita, and that is indicated in the corresponding figure legends. Also

included in that chapter is the work done on reversal of SAHF-negative senescence. Chapter 3 contains the core of the thesis, the data concerning the reversal of SAHF and cell cycle re-entry of senescent cells as a result of E1A expression and Rb knockdown.

The field of senescence has made outstanding progress during the years I have worked on this dissertation, and my work was constantly re-directed to incorporate the new information. I initially undertook a project to reverse senescence, but ended up with a project in which I described and attempted to characterize the reversal of the facultative heterochromatin associated with senescence. One problem was that we were concomitantly trying to characterize these same structures. This in itself presented a challenge, since we found ourselves as part of the heterochromatin field (certainly not our area of expertise) at a time when the notion of a "histone code" was just being conceived and our understanding of chromatin regulation increased daily.

Added to this, the canonical function of Rb as a mere cell cycle regulator was being challenged. Reports emerged suggesting p107 and p130, but not Rb, are associated with E2F-responsive genes during the cell cycle and quiescence (Rayman et al., 2002; Takahashi et al., 2000). We confirmed this data and showed Rb associates with E2F-responsive promoters preferentially during senescence. Recent investigations of RB function suggest that it works as a fundamental regulator to coordinate pathways of cellular growth and differentiation. Rb has been attributed a role in differentiation, lineage specification of stem cells, DNA repair, cell cycle checkpoints and heterochromatin maintenance (Du and Pogoriler, 2006; Galderisi et al., 2006; Genovese et al., 2006). Adding to these newly defined roles for Rb, the work in this dissertation

identifies a role for Rb in both the establishment and the maintenance of senescence-associated heterochromatic foci.

1.5 References

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Chapter 2:

Characterization of Rb-mediated chromatin changes during cellular senescence and evaluation of their effect on senescence stability.

2.1 Summary

Cellular senescence is an extremely stable form of cell cycle arrest that limits the proliferation of damaged cells and may act as a natural barrier to cancer progression. Here we describe a distinct type of facultative heterochromatin that accumulates in senescent human fibroblasts, which we have designated senescence-associated heterochromatic foci (SAHF). SAHF formation coincides with the recruitment of heterochromatin proteins and the retinoblastoma (Rb) tumor suppressor to E2F-responsive promoters and is associated with the stable repression of E2F target genes. Both SAHF formation and the silencing of E2F target genes depend on the integrity of the Rb pathway and do not occur in reversibly arrested cells. Through a variety of genetic manipulations intended to interfere with the p16/Rb pathway, we show that cells that acquire morphological features of senescence but fail to form SAHFs can resume growth upon inactivation of p53. These results provide a molecular explanation for the stability of the senescent state, as well as new insights into the action of Rb as a tumor suppressor.

2.2 Introduction

Cellular senescence was originally described as the process of cell cycle arrest that accompanies the exhaustion of replicative potential in cultured human fibroblasts (Hayflick and Moorhead, 1961). Subsequently a phenotypically indistinguishable arrest termed "premature senescence" was shown to be induced by a variety of cellular stresses (reviewed in section 1.1.2). Senescent cells remain metabolically active; display characteristic changes in cell morphology, physiology, and gene expression; and typically upregulate a senescence-associated β-galactosidase (SA-β-gal) activity (see section 1.1.1). Unlike quiescent cells, senescent cells are unable to express genes required for proliferation, even as a result of strong mitogenic stimulation (Dimri et al., 1994).

Work over the last few decades has earned senescence a place next to apoptosis as a cellular response to stress that limits the proliferation of damaged cells (see Chapter 1 for details). Despite this, the molecular mechanisms responsible for the execution and maintenance of the program remain poorly characterized. Reports in the literature point to Rb and p53 as being the main tumor suppressors controlling cellular senescence, and mutations in these genes compromises senescence (see section 1.2.2).

The majority of the literature has focused on the effect expression or loss of these tumor suppressor genes has on the establishment of the senescent arrest. In human fibroblasts, DNA tumor virus oncoproteins that interfere with Rb and p53 function can bypass senescence. For example, SV40 large T antigen binds both Rb and p53 and overcomes replicative senescence, whereas large T mutants defective in binding either protein are less able to do so (Shay et al., 1991). Similarly, adenovirus E1A targets the Rb

family and interferes with p53-mediated arrest and prevents senescence induced by oncogenic ras and DNA damaging agents (Deng et al., 2005; Serrano et al., 1997).

In many instances, p53 and Rb are activated to promote senescence by products of the INK4a/ARF locus (Lowe and Sherr, 2003). This locus encodes two tumor suppressors, p16INK4a and p14ARF (p19ARF in mice), expressed from partially overlapping nucleotide sequences read in alternative reading frames. p16INK4a engages the Rb pathway by inhibiting cyclin D-dependent kinases that would otherwise phosphorylate and inactivate Rb. In contrast, p14ARF increases the growth suppressive functions of p53 by interfering with its negative regulator, Mdm2. Both p16INK4a and p14ARF accumulate in senescent cells and can promote senescence when overexpressed (Lundberg et al., 2000).

The magnitude of p16INK4a induction at the onset of senescence appears to vary by cell type. p16INK4a is poorly induced in senescent BJ fibroblasts (Beausejour et al., 2003b) and appears dispensable for senescence in MEFs (Lowe and Sherr, 2003). IMR90 and WI38 human fibroblasts have a robust p16INK4a response during senescence (see results section). Interestingly, p53 inactivation is sufficient to reverse the senescent arrest in both MEFs and BJ fibroblasts (Beausejour et al., 2003b; Dirac and Bernards, 2003) but not IMR90 or WI38. These results highlight the importance of the p16/Rb pathway in controlling the stability of the arrest. It is worth noting that the Rb family contributes to senescence in MEFs, since cells lacking Rb along with the related p107 and p130 proteins fail to senesce in culture (Sage et al., 2000), and acute inactivation of Rb can reverse senescence (Sage et al., 2003).

The mechanism by which Rb promotes senescence is not known. Rb family proteins are corepressors of the E2F transcription factors, and their combined activities are required for many aspects of cell cycle progression [reviewed by (Trimarchi and Lees, 2002)]. Rb-family members are thought to recruit histone deacetylases (HDACs) to E2F-dependant promoters, thereby deacetylating nearby histones and repressing gene expression. As cells approach S phase, cyclin D and E-dependent kinases phosphorylate Rb and free E2F, allowing it to act with histone acetyltransferases (HATs) to open chromatin structure and transactivate E2F-responsive genes important for G1 to S phase transition. The modification of histones by HATs and HDACs is dynamic and readily explains the reversibility of cell cycle arrest in quiescent cells. Recent studies suggest that p107 and p130, but not Rb, are associated with E2F-responsive genes during the cell cycle and quiescence (Rayman et al., 2002; Takahashi et al., 2000). Rb is known to interact with a number of factors that are involved in heterochromatin biology (see section 1.3.5). The relative contribution of these interactions to cell cycle regulation and Rb tumor suppressor functions has yet to be determined.

Although the molecular mechanisms underlying the irreversibility of cellular senescence remain poorly understood, these processes are extremely efficient. In fact, human fibroblasts almost never spontaneously escape replicative senescence and cannot be transformed unless the process is disabled (Campisi, 2001). In this chapter, we investigate the molecular basis for the stability of the senescent arrest. We show that in senescent cells Rb mediates the formation of a novel type of facultative heterochromatin. Moreover, formation of the heterochromatic structures correlates with the recruitment of

heterochromatin proteins and the retinoblastoma (Rb) tumor suppressor to E2F-responsive promoters, and is associated with the stable repression of E2F target genes. We propose that these processes contribute to the tumor-suppressive properties of the senescence program. Accordingly inhibition of the Rb pathway, and consequently of the accompanying chromatin changes, results in a reversible senescent arrest.

2.3 Results

Changes in nuclear morphology and chromatin structure accompany senescence

IMR90 human diploid fibroblasts undergoing senescence display a characteristic nuclear morphology involving changes to the nucleolus and the organization of DNA. IMR90 cells induced to senesce by exogenous expression of oncogenic Ras, expression of activated MEK, treatment with the chemotherapeutic drug etoposide, enforced expression of p16INK4a, or extensive passaging (i.e., replicative senescence), typically displayed one large nucleolus and punctate DNA foci as visualized by DAPI staining (Figure 2.1; data not shown). In contrast, exponentially growing IMR90 cells, as well as cells made quiescent by serum withdrawal or confluence, usually display several small nucleoli and a more uniform 4',6-diamidino-2-phenylindole (DAPI) staining pattern (Figure 2.1; data not shown). IMR90 cells expressing E1A, which readily bypass senescence, also did not develop pronounced nucleoli or DNA foci in response to Ras (Figure 2.1). Finally, senescent WI38 cells, another normal fibroblast strain, acquired prominent DNA foci that appeared indistinguishable from senescent IMR90 cells (data not shown). Therefore, the changes to nuclear architecture are not unique to IMR90 cells, nor are they a necessary consequence of cell cycle arrest or oncogenic ras expression. Rather, these changes are specific for the senescent state.

Figure 2.1

Changes in nuclear morphology and chromatin structure accompany senescence

IMR90 cells containing empty vector, H-rasV12 (Ras), or E1A12S/H-rasV12 (E1A/Ras) were stained at post-selection day 6 for SA-β-gal activity, a classical marker of senescence, followed by DAPI staining. Replicative senescent cells (Late passage) are also shown for comparison. Enlarged images of DAPI staining are shown in the lower panels.

(Masashi Narita)

E1A/Ras
Low serum
Late passage
Ras
Vector
SA-β-Gal
DAPI

Acute induction of senescence by H-RasV12

Ras-induced senescence was our system of choice to characterize the senescence-associated DNA foci, since oncogenic ras acutely and reproducibly induces senescence in IMR90 cells over several days (Serrano et al., 1997). IMR90 cells were infected with retroviruses co-expressing oncogenic ras (H-RasV12) and a selectable marker and selected with antibiotic 2 days later, to eliminate uninfected cells (Figure 2.2A). Cell populations were analyzed at various times for Ras, $p16^{INK4a}$ and Rb expression, cellular proliferation (BrdU incorporation) and the onset of senescence (SA-β-gal staining). Cells expressing oncogenic ras accumulated p16INK4a and hypophosphorylated Rb between 3 and 5 days post selection (Figure 2.2B), a time when these cells stopped incorporating BrdU and became positive for SA-β-gal (Figure 2.2C).

Visualization and kinetics of the formation of DNA foci in senescent cells

The appearance of DNA foci in ras-expressing cells coincided precisely with cell cycle exit and the onset of senescence (Figure 2.3A). Moreover, BrdU-positive nuclei containing DNA foci were never observed, implying that DNA synthesis and DNA focus formation were mutually exclusive processes (not shown). Control cells continued to proliferate and did not display senescence-related changes. The formation of the DNA foci occurred gradually over time, following induction of senescence by activated Ras overexpression (Figure 2.3B).

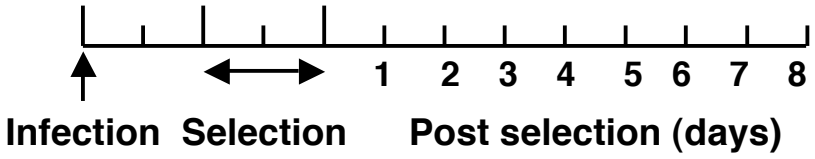
Figure 2.2

Features of Ras-induced senescence

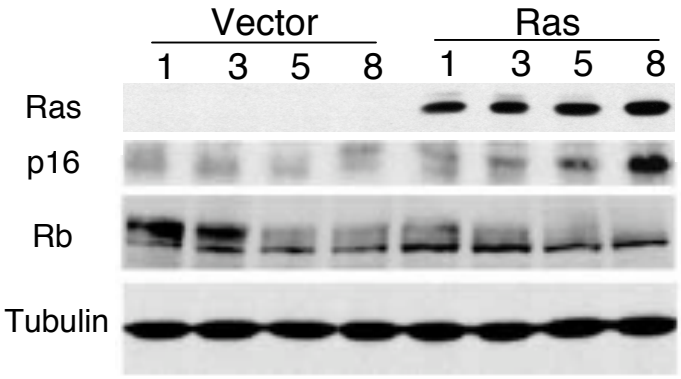
(A) Experimental design. Cells were subjected to retroviral-mediated gene transfer and selected with the appropriate antibiotic/s 2 days later. For cells that were serially selected, day zero is set 2 days after the first selection. (B) Protein expression of Ras, p16INK4a and Rb was assessed by Western blotting at the indicated days post-infection in cells expressing either the empty vector or H-rasV12 (Ras). Tubulin serves as a loading control.(C) IMR90 cells containing empty vector or H-rasV12 (Ras) were scored for percentage of cells with SA-β-gal activity (left image) and BrdU incorporation (right image), at the indicated days post selection.

(Masashi Narita)

A


1 2 3 4 5 6 7 8
Infection Selection Post selection (days)

B


Vector
Ras
1 3 5 8 1 3 5 8
Ras
p16
Rb
Tubulin

C

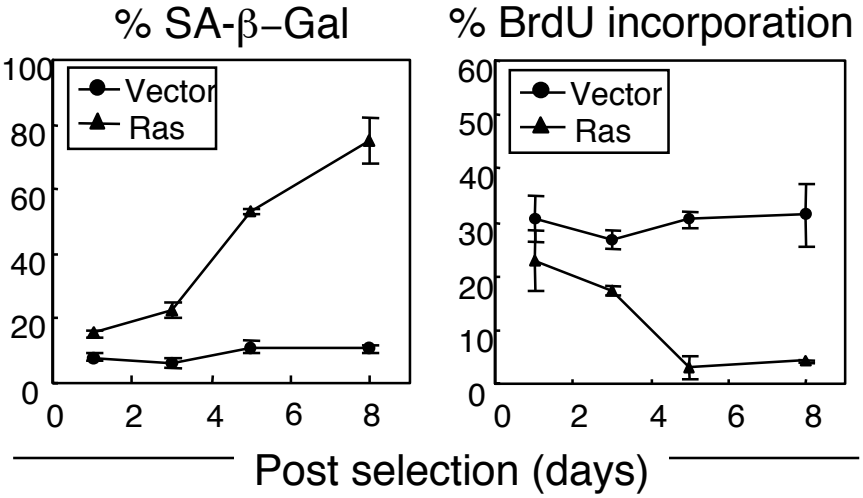

% SA-β−Gal
% BrdU incorporation
Vector
Ras
100
80
60
40
20
0
60
50
40
30
20
10
0
0 2 4 6 8
0 2 4 6 8
Post selection (days)

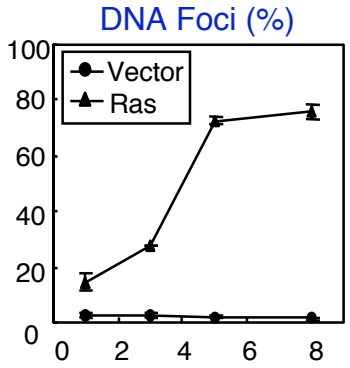
Figure 2.3

Visualization and kinetics of the formation of DNA foci in senescent cells

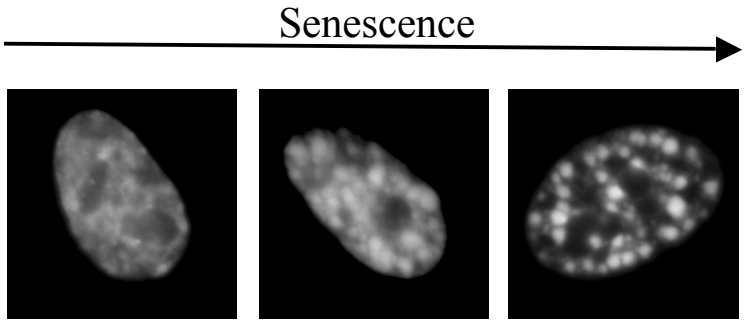
(A) IMR90 cells containing empty vector or H-rasV12 (Ras) were scored for the presence of DNA foci at the indicated days post selection. (B) Representative image of a DAPI stained nuclei 0, 3 and 7 days post-selection following infection with H-rasV12. DNA foci appear gradually over time. (C) Top panels show electron microscopy images of vector control (Growing) and Ras-senescent IMR90 cells (senescent). "N" is nucleolus. Arrows show the higher electron density regions in the senescent nuclei. The bottom panels show high magnification images of a nucleus from low serum quiescent cells and a senescent cell for which DNA was detected using a monoclonal anti-DNA antibody and gold-coupled secondary antibody. Note the concentration of DNA labeling in the electron-dense regions of senescent nuclei.

(Figures A and C were done by Masashi Narita. Figure C was done by M.N. in collaboration with Stephen Hearn)

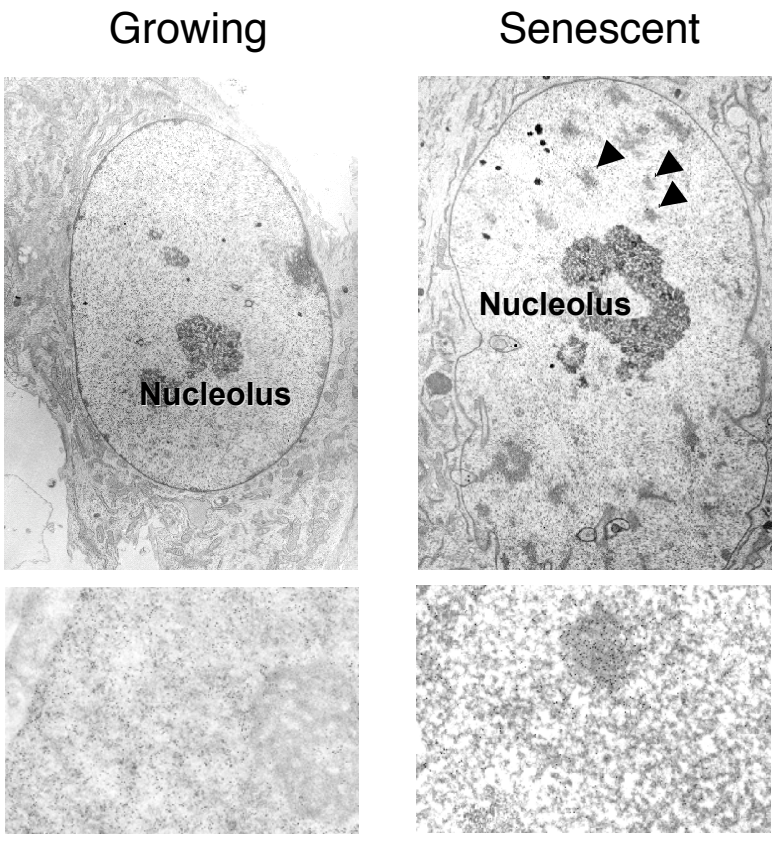
A


DNA Foci (%)
100
80
60
40
20
0
Vector
Ras
0
2
4
6
8

B


Senescence

C


Growing
Senescent
Nucleolus
Nucleolus

DAPI is a fluorescent stain that binds strongly to DNA. When excited with ultraviolet light DAPI, which is bound to double-stranded DNA, will emit light in the 460 nm range, allowing the visualization of DNA by fluorescent microscopy. Due to fluorescence distortion, the regions of DNA revealed by DAPI staining might appear larger than they really are. Therefore, to further characterize the nuclear changes that accompany senescence, normal and senescent IMR90 cells were examined by electron microscopy. In contrast to exponentially growing or quiescent controls (Figure 2.3C; data not shown), senescent nuclei showed prominent nucleoli, an irregular nuclear envelope, and electron-dense regions interspersed throughout the nucleoplasm (Figure 2.3C). To test whether these electron-dense regions correspond to the DNA foci observed by DAPI staining, we immunolabeled quiescent and senescent cells with an anti-DNA antibody and a secondary antibody conjugated to colloidal gold. In contrast to the diffuse pattern observed in quiescent cells, DNA labeling was concentrated in the electron-dense regions of senescent nuclei (Figure 2.3C, bottom panels).

Senescence-Associated DNA Foci Are Not Sites of Active Transcription

To assess global RNA localization, senescent cells were labeled with gold-conjugated RNase, which binds to and localizes to RNA following electron microscopy. RNA was mostly excluded from the interior of the large DNA foci (marked by arrows) and was instead interspersed throughout the nucleoplasm and concentrated in small foci (Figure 2.4A) To localize specific gene transcription, we performed RNA fluorescence in

situ hybridization (RNA FISH) using bacterial artificial chromosome (BAC) clones harboring the cyclin A and INK4a genes as probes. Cyclin A is expressed in dividing cells and downregulated during senescence. As expected, two cyclin A signals were observed in exponentially growing cells (Figure 2.4, vector). In cells undergoing senescence, these two signals were often observed at the periphery of DNA foci and subsequently extinguished (Figure 2.4, compare Ras PS1 to Ras PS7). In contrast, INK4a is silent in dividing cells but upregulated during senescence. Concordantly, no INK4a RNA FISH signal was observed in growing cells, while two signals appeared in most senescent cells (Figure 2.4, compare vector to Ras PS7). In all cases, the positive signals were either at the periphery or outside of the DNA foci. Hence, senescence-associated DNA foci are condensed regions of DNA that correlate with transcriptionally inactive sites.

The DNA foci of Senescent Cells contain Features of Heterochromatin

The senescence-associated DNA foci are reminiscent of heterochromatin. At the molecular level, heterochromatic regions often lack histone H3 that is acetylated on lysine 9 (K9Ac-H3) and methylated on lysine 4 (K4M-H3). In contrast, these regions are usually enriched for histone H3 methylated on lysine 9 (K9M-H3). Methylated lysine 9 provides a docking site for heterochromatin protein 1 (HP1) family of proteins (Bannister et al., 2001; Lachner et al., 2001), a family of adaptor molecules that are required for heterochromatin assembly and are involved in epigenetic gene regulation.

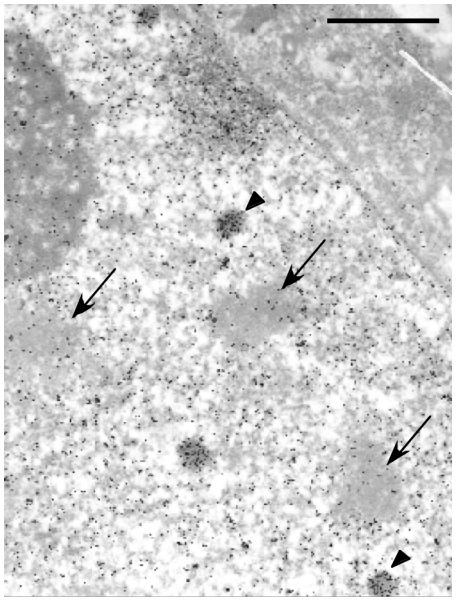
Figure 2.4

Senescence-associated DNA foci are not sites of active transcription

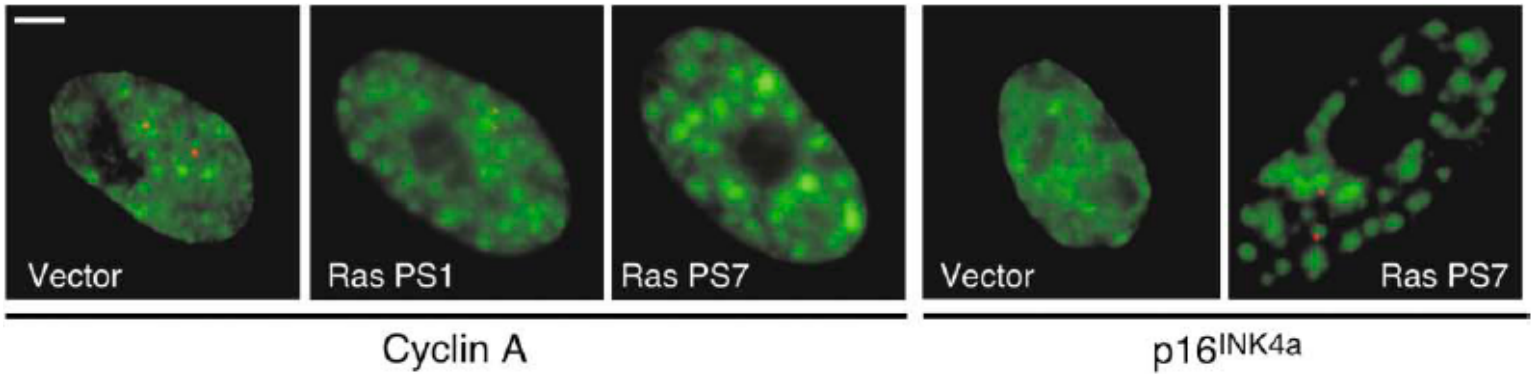
(A) Senescent nucleus showing RNA labeled with gold-coupled RNase T1 following electron microscopy. Arrows indicate the higher electron density regions, arrowheads point to small RNA foci (B) RNA FISH for Cyclin A and INK4a (p16INK4a) was performed on vector control and Ras-senescent IMR90 cells (Ras) at the indicated days post-selection (PS). Intense signals of INK4a in Ras-senescent cells were localized at the periphery or outside of the DNA foci. DNA was counterstained by DAPI, which was pseudocolored green. Scale bar is equal to 5 μm.

(Figure A was done by Masashi Narita in collaboration with Stephen Hearn. Figure B was done by Masashi Narita in collaboration with Edith Heard.)

A



B



To determine whether the senescence-associated DNA foci are related to heterochromatin, we conducted confocal fluorescence microscopy on growing, quiescent, and senescent cells using modification-specific antibodies against histone H3 or the HP1 proteins α, β, and γ. Growing and quiescent cells expressed histones with all modifications examined, and these appeared distributed throughout the nucleoplasm (Figure 2.5A, vector and low serum). In marked contrast, senescent cells showed a more distinctive localization of modified histones. Consistent with their preference for euchromatic regions, K9Ac-H3 and K4M-H3 were largely excluded from DNA foci. Conversely, K9M-H3 was concentrated in the DNA foci of senescent cells (Figure 2.5A, Ras and Late passage). Similarly, all three HP1 proteins were dispersed throughout the nucleoplasm in normal and quiescent cells but were concentrated to varying degrees in the DNA foci of senescent cells (Figure 2.5B). This distinctive pattern was not due to variable antibody accessibility, since IMR90 cells expressing a green fluorescence protein (GFP)-HP1β fusion protein also showed a GFP pattern that was dispersed in normal cells but concentrated in DNA foci of senescent cells (Figure 3.6). Consequently, senescence-associated DNA foci contain heterochromatin and were defined as "senescence-associated heterochromatic foci" (SAHF).

SAHFs do not represent a redistribution of constitutive heterochromatin

In principle, the SAHF might result from a redistribution of preexisting heterochromatin (e.g., owing to changes in nuclear structure) rather than from new

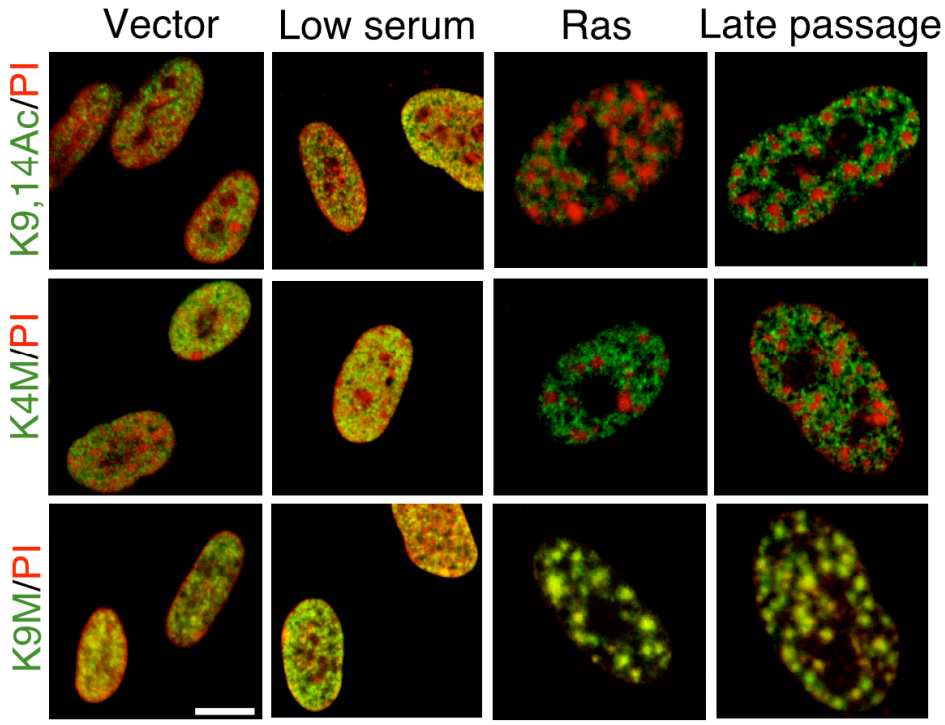
Figure 2.5

The DNA foci of senescent cells contain features of heterochromatin

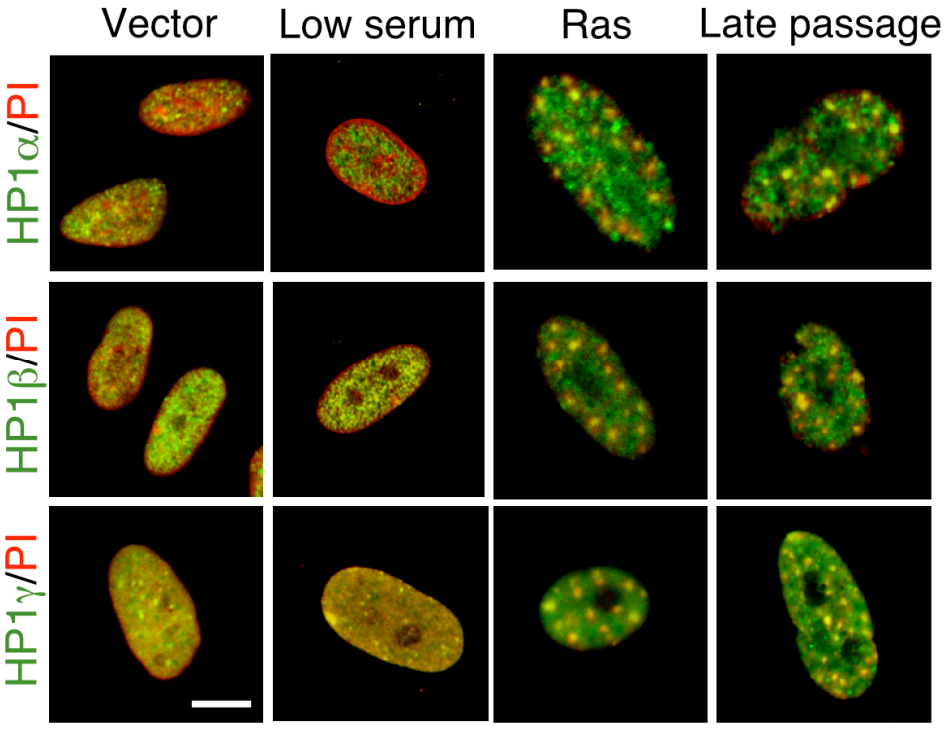
(A) Confocal images of indirect immunofluorescence of acetylated histone H3 on Lysine 9/14 (K9/14Ac), methylation on lysine 4 (K4M), and methylation on lysine 9 (K9M) (green) in normal growing (vector), quiescent (low serum), H-rasV12 (Ras) senescent, or replicative senescent IMR90 cells (late passage). The DNA was counterstained by propidium iodide (PI) (red). Merged images are shown.(B) Localization of endogenous HP1 proteins (green) was determined by indirect immunofluorescence using the indicated antibodies in the cells described in (A). DNA was stained with propidium iodide (PI) (red). Merged images are shown.

(Masashi Narita)

A


Vector
Low serum
Ras
Late passage
K9,14Ac/PI
K4M/PI
K9M/PI

B


Vector
Low serum
Ras
Late passage
HP1α/PI
HP1β/PI
HP1γ/PI

heterochromatin that accumulates in senescent cells. Constitutive heterochromatin consists of large regions of heterochromatin found near the centromeres and telomeres of mammalian chromosomes. We therefore tested for co-localization of SAHF and centromere and/or telomeres (Figure 2.6). Immunofluorescence using autoserum that recognizes centromeres or an antibody directed against the telomere-associated protein TRF2 indicated no substantial co-localization between SAHF and centromeres or telomeres.

Increased resistance of DNA of senescent cells to limited nuclease digestion

Micrococcal nuclease is known to preferentially cleave chromatin within linker DNA. Further digestion results in cleavage at a set of sites within the nucleosome (Axel, 1975). Additionally, there is a direct relationship between the degree of compaction of chromatin and the resistance of linker DNA to digestion by micrococcal nuclease (Leuba et al., 1994). In order to verify higher sensitivity to nuclease treatment of euchromatic regions in our system, we fixed cells after 5 minutes of MNase digestion and stained them with DAPI. The DAPI-dense areas at the nuclear periphery of quiescent cells and the DNA foci in senescent cells are more resistant to nuclease attack than the diffuse DNA staining throughout the nucleoplasm (Figure 2.7B).

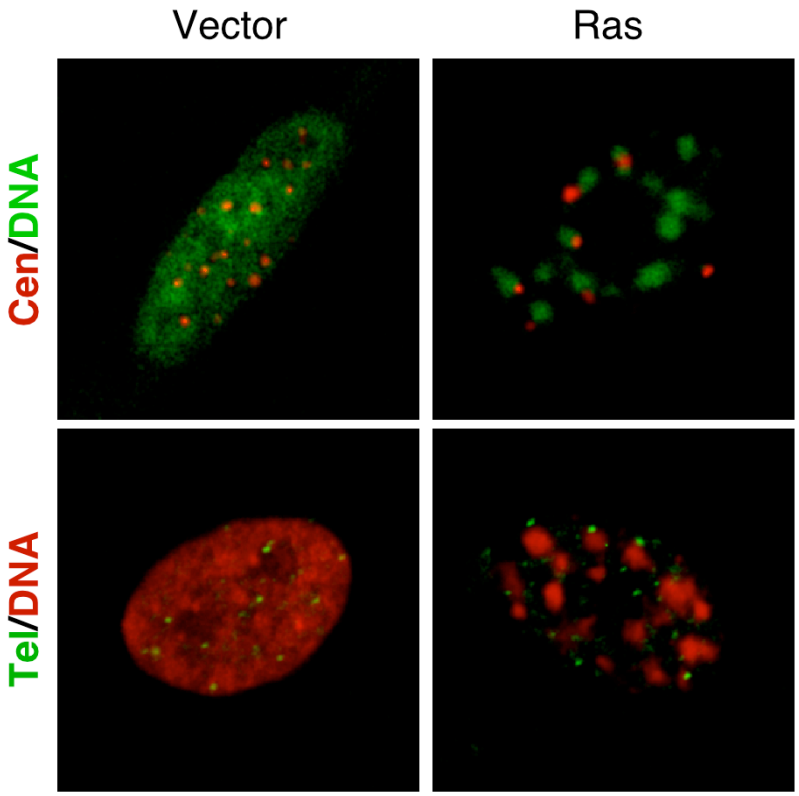
We then subjected growing, Ras senescent and E1A/Ras immortalized cells to in vivo MNase digestion over a range of times. DNA was isolated from these cells and resolved on an agarose gel. Consistent with the known resistance of heterochromatin to

Figure 2.6

SAHFs do not represent a redistribution of constitutive heterochromatin

(A) Confocal images of IMR90 cells expressing an empty vector or H-rasV12 (Ras) stained with autoserum that recognizes the centromeres (Cen) or an anti-TRF2 antibody (Upstate) that recognizes telomeres. The DNA was counterstained by either YO-PRO™-1 (Molecular Probes) (green) or propidium iodide (red). Merged images are shown.

(Masashi Narita)


Vector
Ras
Cen/DNA
Tel/DNA

limited nuclease digestion, DNA from senescent cells was more resistant to micrococcal nuclease compared to normal cells and E1A-expressing cells that had bypassed ras-induced senescence (Figure 2.7C). The nuclease resistance pattern exhibited by growing and quiescent cells is indistinguishable (Figure 3.15), indicating this increase in the nuclease-resistant fraction is not a feature of cell cycle exit but rather of senescence. This result also implies that formation of a distinct heterochromatic structure accompanies the senescence process.

Some E2F-target promoters acquire features of heterochromatin during senescence

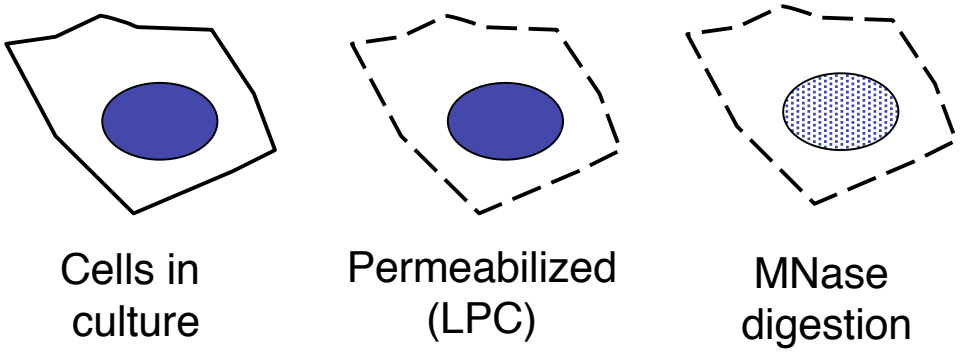
Chromatin organization is tightly linked to gene expression. The changes in euchromatic and heterochromatic organization that accompany SAHF formation might therefore produce the senescence-specific changes in gene expression. Since heterochromatin has been linked to gene silencing, we reasoned that SAHFs might contribute to the stable cell cycle arrest that is a hallmark of senescence. If true, components of euchromatin or heterochromatin would be associated with genes that are induced or repressed during senescence, respectively. To test this, we examined the association of K9/K14 acetylation of histone 3 (K9/14Ac-H3), K9 di/tri-methylation of histone 3 (K9M-H3), and HP1γ to the promoters of several genes in senescent cells in vivo using chromatin immunoprecipitation. We focused on E2F-target promoters because these genes are essential for cell cycle progression and are negatively regulated by the Rb

Figure 2.7

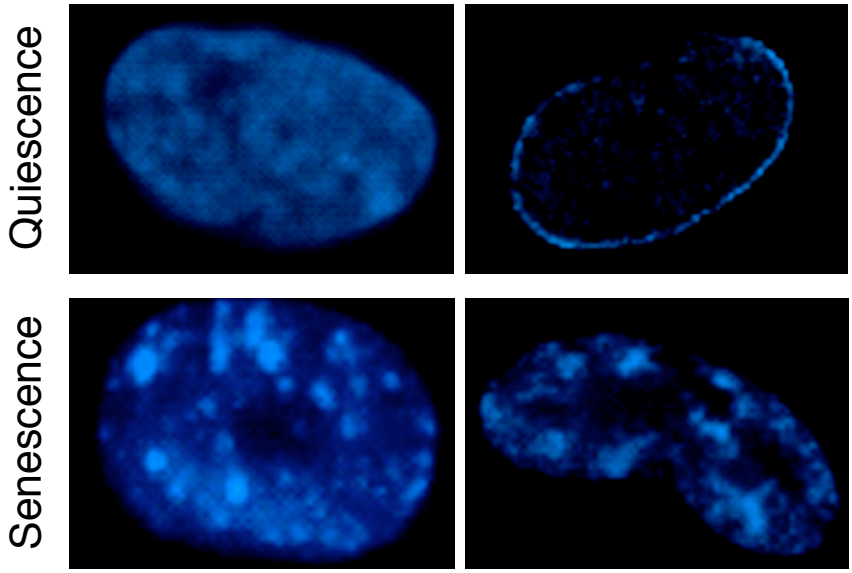
Increased resistance of DNA of senescent cells to limited nuclease digestion

In vivo micrococcal nuclease (MNase) digestion. (A) Diagram summarizing the procedure utilized for nuclease digestion. Cells were permeabilized on the plate and subjected to limited MNase digestion. DNA was isolated from the cells and analyzed as indicated (B) Detergent-permeabilized quiescent and senescent cells were subjected to MNase digestion for 5 min. Cells were fixed in 4% formaldehyde and stained with DAPI. (C) Micrococcal nuclease digestion of detergent-permeabilized cells from growing (Vector), senescent (Ras), and Ras/E1A transformed cells. DNA was isolated from cells after digestion for the indicated time (min) and subjected to agarose gel electrophoresis.

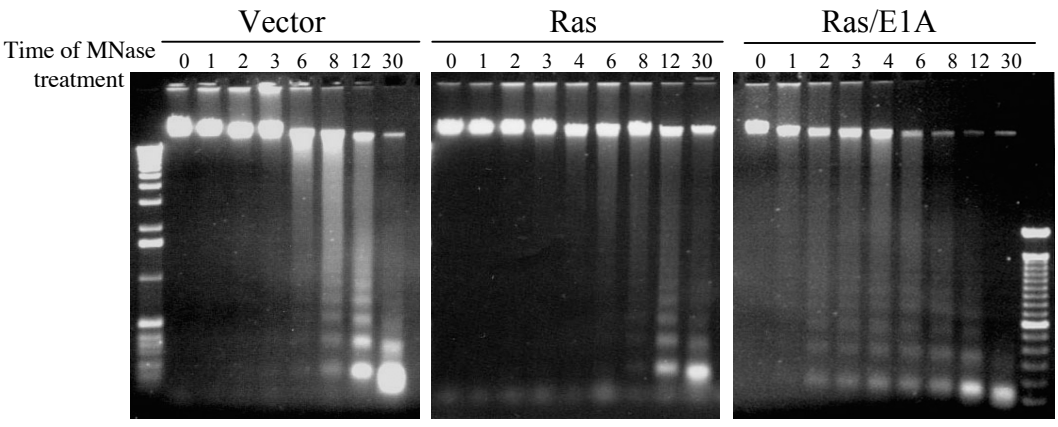
A


Cells in culture
Permeabilized (LPC)
MNase digestion

B


Quiescence
Senescence

C


Vector
Ras
Ras/E1A
Time of MNase treatment
0 1 2 3 6 8 12 30
0 1 2 3 4 6 8 12 30
0 1 2 3 4 6 8 12 30

family, which is known to influence senescence. These genes are also constitutively induced by E1A, which inactivates Rb and prevents SAHF formation and senescence. We chose to analyze senescent cells produced by oncogenic ras, because these cells undergo a relatively synchronous arrest.

We first examined the binding of K9/14Ac-H3, a marker of euchromatin, for its presence on genes associated with senescence. Stromelysin-1 and INK4a are upregulated during senescence, whereas cyclin A and PCNA are E2F-target genes that are repressed. Compared to normal cells, the amount of K9/14Ac-H3 bound to the stromelysin-1 promoter increased in senescent cells, although no change was observed on the INK4a promoter (Figure 2.8A, compare lanes 2 and 3). In contrast, the amount of K9/14Ac-H3 that associated with cyclin A and PCNA declined during senescence. Interestingly, E1A abolished the increase in K9/14Ac-H3 binding to the stromelysin-1 promoter, but produced an increase in K9/14Ac-H3 association with the cyclin A and INK4a promoters (Figure 2.8A, compare lanes 3 and 4). These latter results are consistent with the known E2F responsiveness of cyclin A, and the observation that INK4a levels often increase following Rb inactivation (Stott et al., 1998). The amount of K9/14Ac-H3 bound to the stromelysin-1 promoter did not increase in quiescent cells (produced by serum depletion or confluence), indicating that this effect was specific for senescence (Figure 2.8B, compare lane 2 to lanes 3 and 4). However, the amount of K9/14Ac-H3 bound to the cyclin A and PCNA promoters also declined in quiescent cells, indicating that hypoacetylation of histones on E2F target promoters is not unique to senescence.

We next examined the occupancy of E2F target promoters by K9M-H3 and HP1γ: two proteins that are enriched in the SAHFs and are known to be involved in

heterochromatin formation (Bannister et al., 2001; Lachner et al., 2001). In marked contrast to acetylated histone H3, the amount K9M-H3 associated with the cyclin A and PCNA promoters increased in senescent cells relative to quiescent cells (Figure 2.8C, compare lanes 1 and 2). Although the amount of K9M-H3 detected between experiments was variable, similar results were produced with two anti-K9M-H3 antibodies. Similarly, HP1γ, which binds K9M-H3 in the context of heterochromatin, also associated with the PCNA and cyclin A promoters in senescent but not quiescent cells (Figure 2.8C, compare lanes 3 and 4). Importantly, E1A, which prevents senescence and SAHF formation, also prevented ras-induced HP1γ and K9M-H3 accumulation on the two E2F target promoters (Figure 2.8D, compare lanes 1–2 and 7–8). Therefore, the accumulation of K9M H3 and HP1γ on the promoters examined is not detected when cells exit the cell cycle into a reversible quiescent state, but accompanies the more stable senescence-like arrest.

The nuclease resistant fraction of senescent cells is enriched for E2F-targets

Our results indicate the existence of a novel type of facultative heterochromatin in senescent cells and specifically identify genes that acquire features of heterochromatin in senescent cells. These results would predict that some E2F target genes should co-localize with SAHFs. To examine this, we performed DNA fluorescence in situ hybridization (FISH) for several E2F target genes in senescent and quiescent cells. Although we could detect two signals in growing cells, we were unable to find

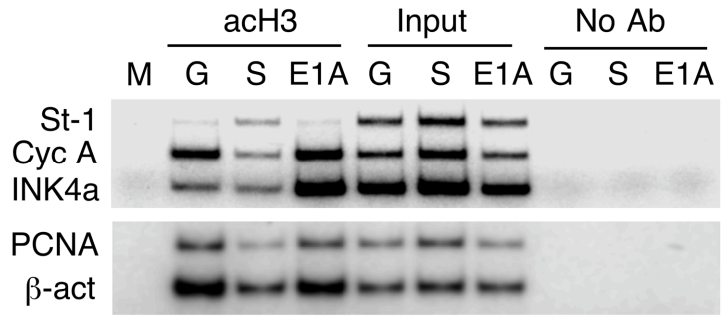
Figure 2.8

Chromatin immunoprecipitation analysis of E2F-target promoters in quiescent and senescent cells

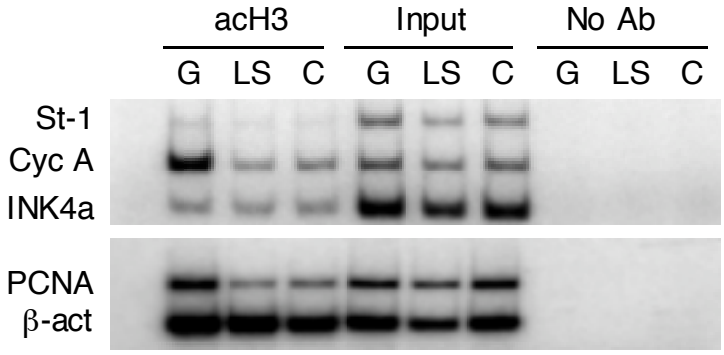
(A) Chromatin immunoprecipitation assay (ChIP) using either an antibody against acetylated histone H3 (acH3), no antibody (No Ab) or nuclear extract (input). DNA fragments were amplified by PCR from the promoter regions of stromelysin-1 (St-1), cyclin A (Cyc A), INK4a, PCNA, and β-actin (β-act). Buffer without nuclear extract served as Mock (M) control. Normal growing IMR90 cells (G), Ras-senescent cells (S), and E1A/Ras transformed cells (E1A) were used in (A); normal growing cells (G), low serum (LS), and confluent quiescent cells (C) were used in (B). (C) and (D) ChIP assays were performed using HP1γ and K9M-H3 antibodies on extracts from quiescent (Q), Ras-senescent (S), and E1A/Ras-expressing (E1A) IMR90 cells. DNA fragments were amplified by PCR with the same primers as in A.

(Masashi Narita)

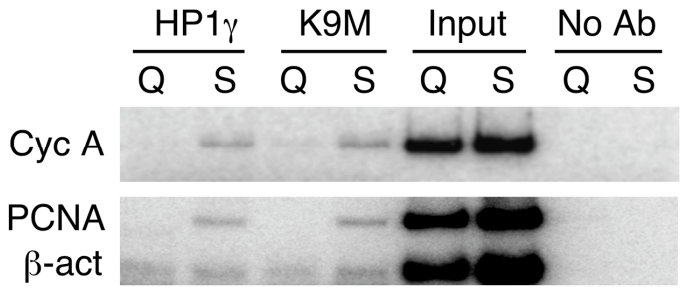
A


acH3
Input
No Ab
M G S E1A G S E1A G S E1A
St-1
Cyc A
INK4a
PCNA
β-act

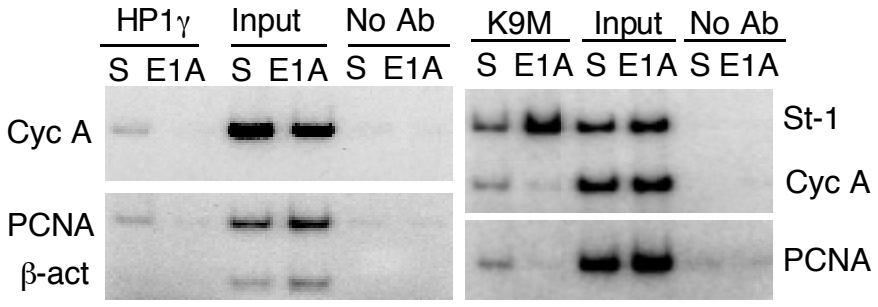
B


acH3
Input
No Ab
G LS C G LS C G LS C
St-1
Cyc A
INK4a
PCNA
β-act

C


HP1γ
K9M
Input
No Ab
Q S Q S Q S Q S
Cyc A
PCNA
β-act

D


HP1γ
Input
No Ab
K9M
Input
No Ab
S E1A S E1A S E1A S E1A S E1A S E1A
Cyc A
PCNA
β-act
St-1
Cyc A
PCNA

denaturation conditions that allowed us to detect two signals in senescent cells without destroying the structure of the SAHFs (data not shown). We occasionally detected one signal that was located either in or at the periphery of the SAHF. We suspect that this is because these genes are "buried" in condensed DNA and more difficult to detect, but cannot rule out other explanations.

To get around the accessibility problems we experienced with FISH, we tried an indirect assessment of our theory. If SAHF confer the increased nuclease resistance of senescent chromatin, then E2F-targets should be found within the nuclease-resistant fraction of senescent but not growing or quiescent cells. To test this we tried a variety of approaches. First we transferred the DNA from the micrococcal nuclease assay (Figure 2.7C) onto a nylon membrane and subjected it to Southern blot using either PCNA or a $p16^{INK4a}$ probe (Figure 2.9A). The E2F-target gene PCNA is preferentially protected from nuclease degradation in senescent cells compared to growing cells. Conversely the INK4a locus, which is repressed in growing cells and de-repressed in senescent cells, is preferentially found in the nuclease-resistant fraction of growing cells as compared to senescent cells.

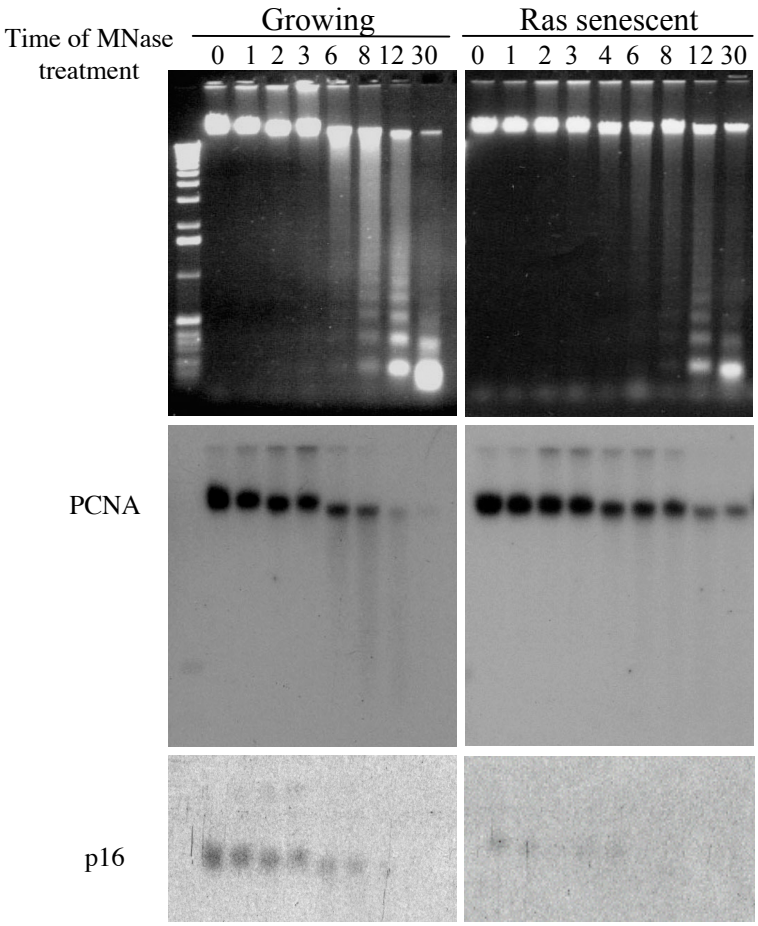
Alternatively, we excised the high molecular weight DNA (defined as the >12 kb) from the agarose gel shown in figure 2.7C and subjected the samples to real time Q-PCR using primers specific to the genomic sequence of PCNA, MCM3, and β-actin promoters. PCNA and Mcm3 were both preferentially protected from nuclease digestion in senescent cells as compared to growing cells (Figure 2.10). All values are plotted as the percentage of signal relative to the input (time 0) and normalized to the β-actin signal.

Figure 2.9

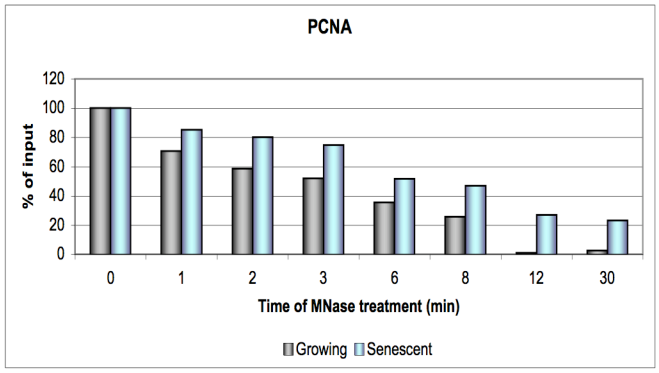
The nuclease resistant fraction of senescent cells is enriched for the PCNA gene.

In vivo micrococcal nuclease (MNase) digestion followed by Southern blotting. (A) Micrococcal nuclease digestion of detergent-permeabilized cells from growing and Ras-senescent cells. DNA was isolated from cells after digestion for the indicated time (min) of micrococcal nuclease digestion and subjected to agarose gel electrophoresis. DNA was then transferred to a nylon membrane and subjected to Southern blot using a PCNA or a p16 probe. (B) Quantification of the Southern blot signal.

A


Time of MNase treatment
Growing
Ras senescent
0 1 2 3 6 8 12 30
0 1 2 3 4 6 8 12 30
PCNA
p16

B


PCNA
% of input
120
100
80
60
40
20
0
0 1 2 3 6 8 12 30
Time of MNase treatment (min)
Growing Senescent

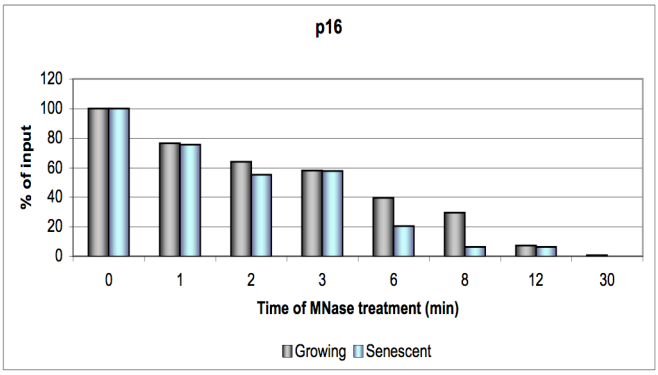
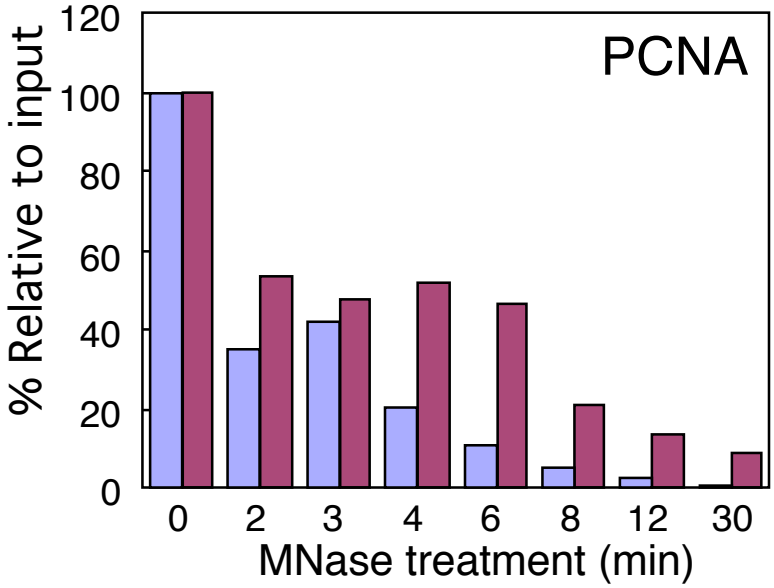
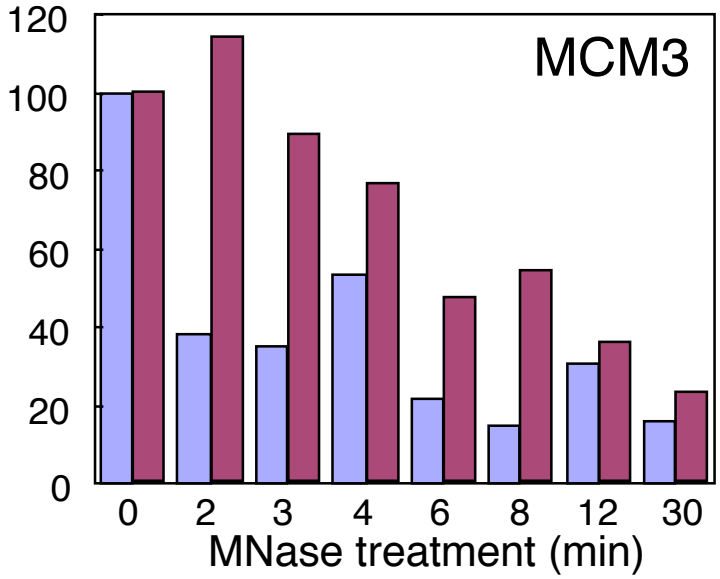

p16
% of input
120
100
80
60
40
20
0
0 1 2 3 6 8 12 30
Time of MNase treatment (min)
Growing Senescent

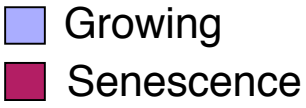
Figure 2.10

The nuclease resistant fraction of senescent cells is enriched for the PCNA and Mcm3 genes.

High molecular weight DNA (defined as the >12 kb band seen in Figure 2.7C) was excised from the gel for each MNase sample and subjected to real time Q-PCR using primers specific to genomic sequence of PCNA, MCM3, and β-actin promoters. The data are relative to time zero (input), with the β-actin signal used as an internal control.


PCNA
% Relative to input
120
100
80
60
40
20
0
0 2 3 4 6 8 12 30
MNase treatment (min)


MCM3
120
100
80
60
40
20
0
0 2 3 4 6 8 12 30
MNase treatment (min)


Growing
Senescence

We then modified the nuclease assay to employ DNaseI rather than micrococcal nuclease. Unlike micrococcal nuclease DNaseI doe not preferentially cut at any particular place relative to the position of the nucleosome. It does however preferentially degrade euchromatin over heterochromatin. In vivo DNaseI digestion was performed on growing and Ras-senescent IMR90s. DNA was recovered at several different times and subjected to quantitative PCR analysis using primers specific to the genomic sequences of PCNA. Mcm4 and cyclin A promoters as well as the chromosome 1 satellite 2 repeat. All three E2F-target genes are enriched in the DNaseI resistant fraction of senescent cells relative to growing cells (Figure 2.11). All values are plotted as the percentage of signal relative to the input (time 0) and normalized to the chromosome 1 satellite 2 signal, which is a known region of constitutive heterochromatin.

Taken together these results indicate that some E2F-target genes are found in the nuclease resistant fraction of senescent cells, which we believe represents the heterochromatic structures that accumulate in these cells.

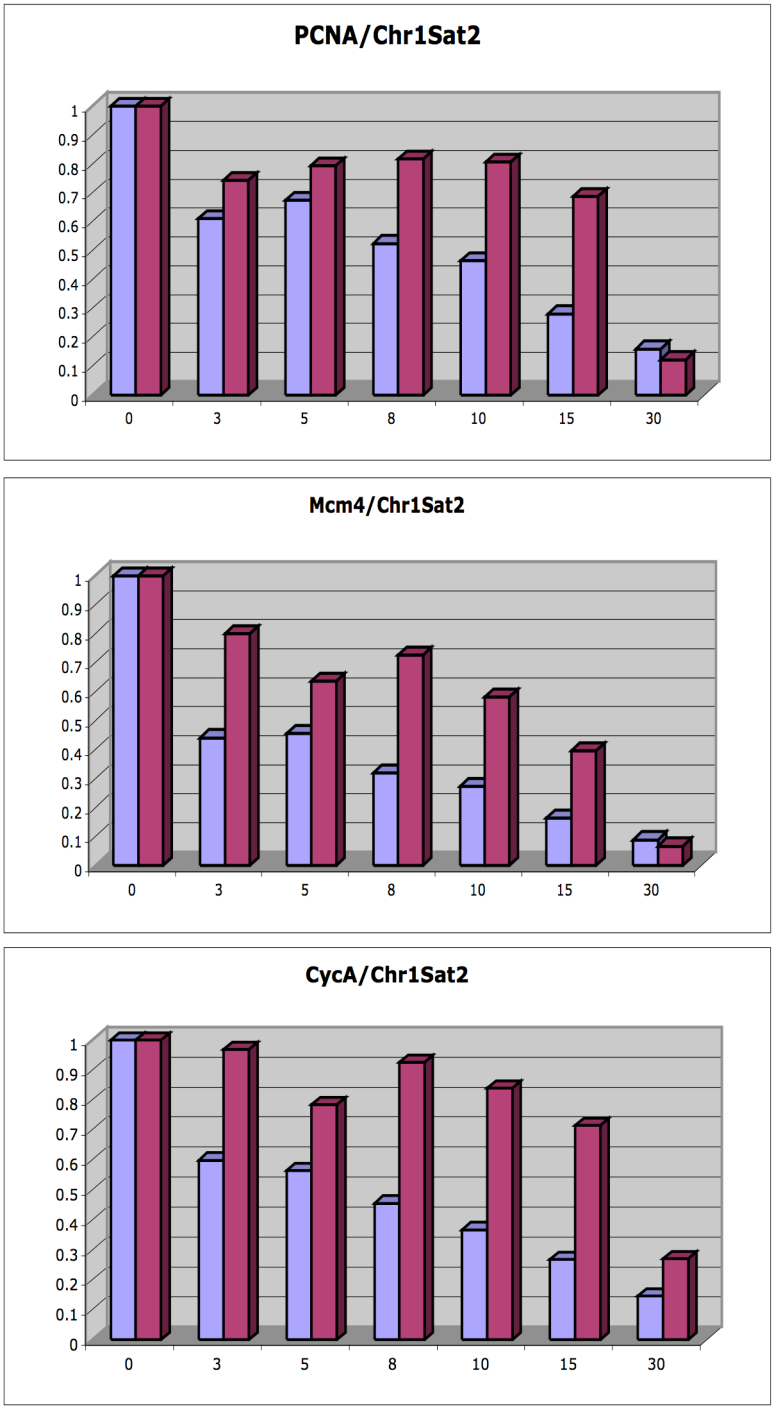
Senescent cells have a distinct pattern of chromatin-associated proteins

Formation of SAHF is likely to be reflected in the pattern of proteins that associate with chromatin. We therefore examined the protein composition of chromatin preparations from normal growing (G), serum-derived quiescent (Q) and Ras-senescent fibroblasts (Ras). Nuclei were isolated and subjected to a no-salt extraction procedure that enriches for chromatin-associated proteins (Mendez and Stillman, 2000). The

Figure 2.11

The nuclease resistant fraction of senescent cells is enriched for PCNA, Mcm4 and cyclin A genes.

In vivo DNaseI digestion. IMR90 growing and Ras-senescent cells were permeabilized on the plate and subjected to limited DNaseI digestion. DNA was isolated from the cells at the indicated time (min) and subjected to real time Q-PCR using primers specific to the genomic sequence of PCNA, MCM4 and cyclin A promoters and the chromosome 1 satellite 2 repeat (Chr1Sat2) . The data are relative to time zero (input), with the Chr1Sat2 signal used as an internal control.


PCNA/Chr1Sat2
Mcm4/Chr1Sat2
CycA/Chr1Sat2
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
3
5
8
10
15
30
Growing
Senescence

isolated proteins were resolved on an acrylamide gel, with histones H3, H2B, H2A, and H4 serving as loading controls. Coomassie staining of the gel revealed that growing and quiescent cells display a pattern of chromatin-associated proteins that, at this level of resolution, are indistinguishable from each other. There is however an overall difference in the pattern of chromatin bound proteins between senescent and growing and quiescent cells (Figure 2.12A).

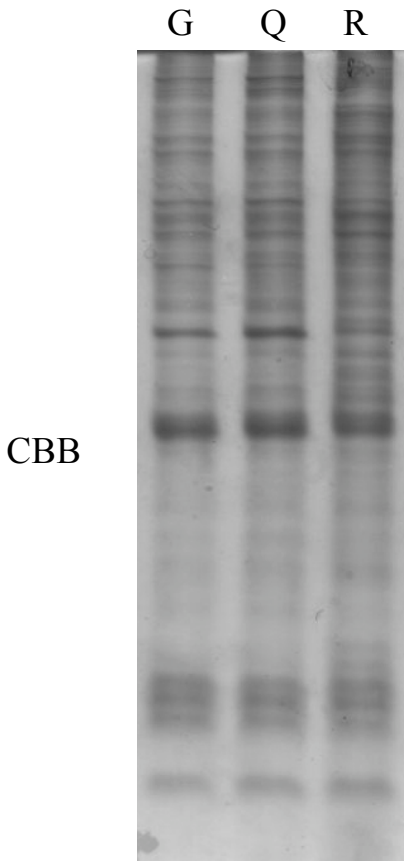
Western blot analysis indicated that Ras-senescent cells display a substantial increase in the total amount of chromatin-bound HP1γ relative to normal proliferating and quiescent cells (Figure 2.12B). There is a more moderate increase in the levels of chromatin-bound HP1α of senescent cells and although we have seen this for HP1β also, the result is not always reproducible. Senescent cells exhibit a decrease in chromatin-bound Ac-K9/14H3, consistent with the reported loss of this euchromatic mark. Similarly, levels of the cell cycle promoting E2F1 are markedly decreased in the chromatin fraction of senescent cells compared to growing and quiescent. This reduction does not merely reflect cell cycle exit, since E2F1 in the chromatin fraction of quiescent cells is not reduced to the same level. There seems to be no significant enrichment of di- and tri-methyl K9 or di-tri methyl K27 of histone 3 in the chromatin fraction of senescent cells as compared to growing and quiescent cells (Figure 2.12B). The lack of enrichment of these heterochromatic marks suggests that the apparently strong anti-D/TMk27, DMK9 and TMK9 H3 signals at SAHF reflect the condensation of DNA. We do however show gain of methylation on Lysine 9 of histone three at some E2F target promoters. This assay might lack the sensitivity to distinguish discrete enrichment of the indicated posttranslational histone modifications.

Figure 2.12

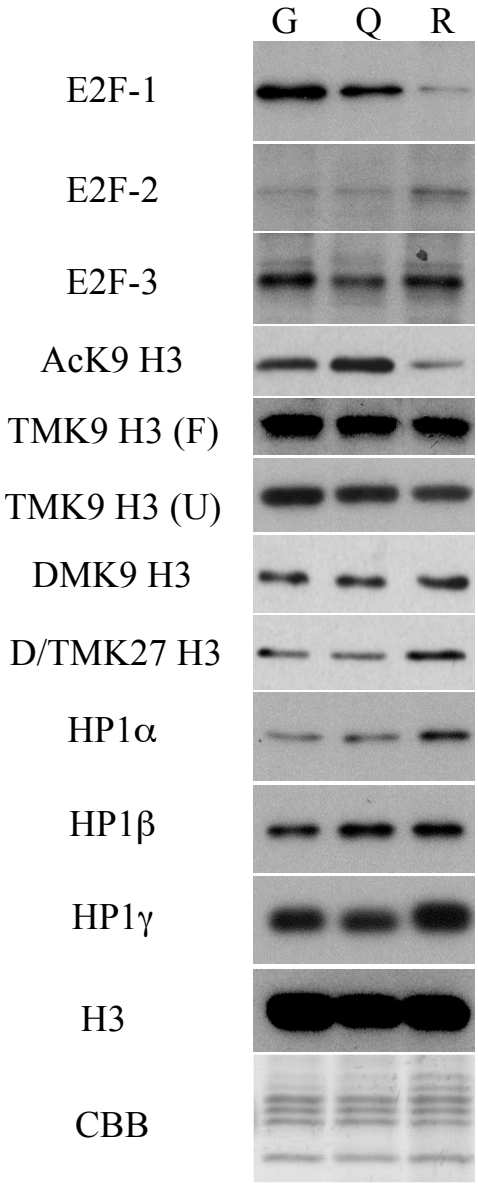
Senescent cells have a distinct pattern of chromatin-associated proteins

The chromatin fraction from normal growing (G), quiescent (Q) and ras-induced senescent IMR90 (Ras) cells was resolved by SDS-PAGE followed by Coomassie brillian blue staining (CBB) to ensure equal loading of core histones (A and bottom panel of B). (B) Proteins were transferred to an Immobilon-P membrane and immunoblotted with the indicated antibodies.

A


G
Q
R
CBB

B


G
Q
R
E2F-1
E2F-2
E2F-3
AcK9 H3
TMK9 H3 (F)
TMK9 H3 (U)
DMK9 H3
D/TMK27 H3
HP1α
HP1β
HP1γ
H3
CBB

Rb preferentially associates with E2F-target promoters in senescent cells

Rb is known to regulate E2F-responsive genes and, as previously discussed, plays an important role in cellular senescence. Recent reports indicate that Rb-family members p107 and p130 rather than Rb are predominantly bound to E2F responsive promoters in quiescent and G1 cells (Rayman et al., 2002; Takahashi et al., 2000). Interestingly, in addition to the E2Fs, Rb can also associate with HP1 and histone methyltransferases such as SUV39H1, raising the possibility that Rb helps direct the process of histone methylation and HP1 recruitment to E2F responsive promoters during senescence. Consistent with this possibility, Rb showed a limited co-localization with SAHF in the nuclei of senescent cells, which was greater than that observed for p107 and p130 (Figure 2.13A). Additionally, a higher level of Rb is found in the chromatin-enriched fraction of senescent cells as compared to growing and quiescent (Figure 2.13B). This difference in chromatin-bound Rb levels does not mirror total Rb protein levels observed in these cells, which are similar in all settings but differ in phosphorylation status (see figure 2.2B). It is possible that Rb plays a more predominant role regulating protein complexes off chromatin in growing and quiescent cells than in senescent cells, where it exhibits a higher association with promoters. Such a possibility remains to be explored.

To determine whether Rb might occupy E2F target gene promoters during senescence, we examined the association of all three pocket proteins with the cyclin A and PCNA promoters in quiescent (confluent) and senescent (ras) cells using chromatin immunoprecipitation. As expected, p107 and p130 were readily detected on the cyclin A and PCNA promoters in quiescent cells (Figure 2.13C, lanes 1 and 3). While the

association between p107 and these promoters typically declined in senescent cells, p130 was retained to varying degrees (Figure 2.13C, lanes 2 and 4; data not shown). In contrast, Rb was difficult to detect on the cyclin A and PCNA promoters in quiescent cells, but was detected on these promoters in senescent cells (Figure 2.13C, compare lanes 5 and 6). Although the Rb signal in senescent cells was often weak (perhaps owing to antibody inaccessibility to the Rb localized to compacted chromatin), we observed similar results in multiple experiments (data not shown). These results indicate that Rb can physically associate with some E2F target promoters in senescent cells and raise the possibility that Rb plays a special role in this process.

The $p16^{INK4a}$/Rb pathway is crucial for SAHF formation and gene silencing

We know E1A abrogates ras-induced senescence, and that E1A-expressing cells continue to proliferate, do not accumulate SA-β -gal activity or SAHFs, and express high levels of E2F-responsive genes. Work by Masashi Narita in the lab showed that an E1A deletion mutant (E1AΔN), which preserves the LXCXE motif and targets the Rb family but not the p300 and p400 proteins, was not as effective as full-length E1A at preventing cell cycle arrest or SA-β-gal accumulation, but retained its ability to prevent SAHF formation and repression of E2F targets (See appendix 1). This result indicates that activation of one or more Rb-family proteins is necessary, and perhaps sufficient, for SAHF formation and the stable repression of E2F target genes.

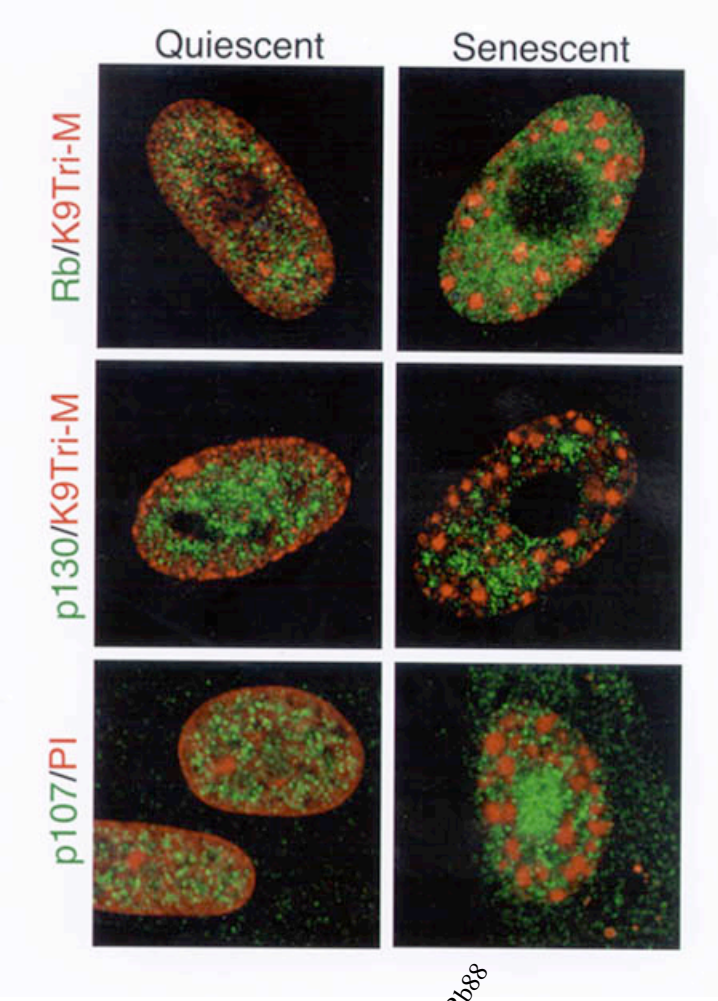
Figure 2.13

Rb preferentially associates with E2F-target promoters in senescent cells

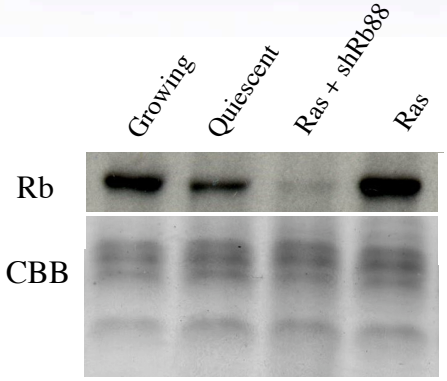
(A) Quiescent (low serum) and Senescent (ras) cells were costained with antibodies directed against Rb or p130 and an antibody directed against K9 trimethyl H3 and analyzed by confocal microscopy. The lower panel shows cells stained with an antibody directed against p107, where DNA was counter stained with PI. Merged images are shown. A fraction of Rb colocalizes to the SAHFs, whereas p130 and p107 are largely excluded. (B) The chromatin fraction from normal growing, quiescent, Ras-induced senescence in the presence of a short hairpin agaist Rb (Ras + shRb88) and Ras-induced senescent IMR90 (Ras) cells was resolved by SDS-PAGE followed by Coomassie brillian blue staining (CBB) to ensure equal loading of core histones (bottom panel). Proteins were transferred to an Immobilon-P membrane and immunoblotted with an antibody against Rb. (C) ChIP assays were performed using p107, p130, or Rb antibodies on extracts from quiescent (Q) and Ras-senescent (S) cells. DNA fragments were amplified by PCR from the promoter regions of cyclin A (Cyc A), PCNA and β-actin (β-act).

(A and C were done by Masashi Narita)

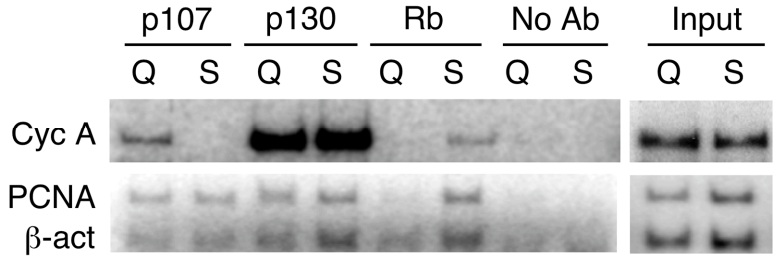
A


Quiescent
Senescent
Rb/K9Tri-M
p130/K9Tri-M
p107/PI

B


Growing
Quiescent
Ras + shRb88
Ras
Rb
CBB

C


p107
p130
Rb
No Ab
Input
Q S Q S Q S Q S Q S
Cyc A
PCNA
β-act

We generated short hairpin RNAs (shRNAs) targeting different sequences in the p16 and Rb mRNAs. IMR90 cells were infected with retroviruses expressing a shRNA directed against p16INK4a (sh-p16) or Rb (sh-Rb), in combination with a ras-expressing retrovirus to induce senescence. Both p16INK4a and Rb were stably repressed by their respective shRNA relative to the control vector (Figure 2.14A, compare lanes 4 to 5 for p16; compare lanes 4–6 for Rb). Interestingly, like E1A ΔN, neither sh-p16 nor sh-Rb prevented eventual cell cycle exit, but both reduced SAHF formation (Figure 2.14B) and prevented the silencing of E2F targets (Figure 2.14A, compare lane 4–5 and 6 for MCM3, cyclin A, and PCNA).

When examined at single cell level, a close correlation exists between SAHF formation and the repression of the E2F target genes (not shown, see appendix 1).

The DNA of SAHF-negative cells exhibits increased nuclease sensitivity

If senescent-specific chromatin changes are responsible for the increased resistance of senescent chromatin to limited nuclease digestion, inhibiting SAHF formation should increase nuclease sensitivity. Accordingly IMR90 cells infected with a short hairpin RNA targeting Rb or $p16^{INK4a}$ and Ras display a higher nuclease sensitivity that cells made to senesce by Ras infection alone (Figure 2.15 and Figure 3.15).

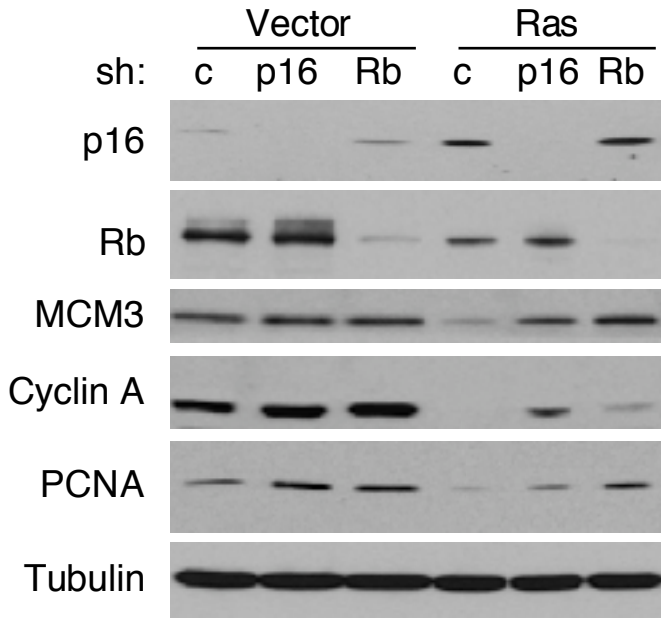
Figure 2.14

The $p16^{INK4a}$/Rb pathway is crutial for SAHF formation and gene silencing

(A) The expression of the indicated protein was determined by immunoblotting of extracts from IMR90 cells expressing empty vector or H-rasV12 (Ras) in combination with short hairpin RNAs (sh) against p16INK4a or Rb, as well as control vector (c) at day 5 post-infection. (B) The cells were also assessed for SAHF formation and BrdU incorporation at the same time point as in (A).

(Masashi Narita in collaboration with Sabrina Nuñez)

A


Vector
Ras
sh:
c
p16
Rb
c
p16
Rb
p16
Rb
MCM3
Cyclin A
PCNA
Tubulin

B

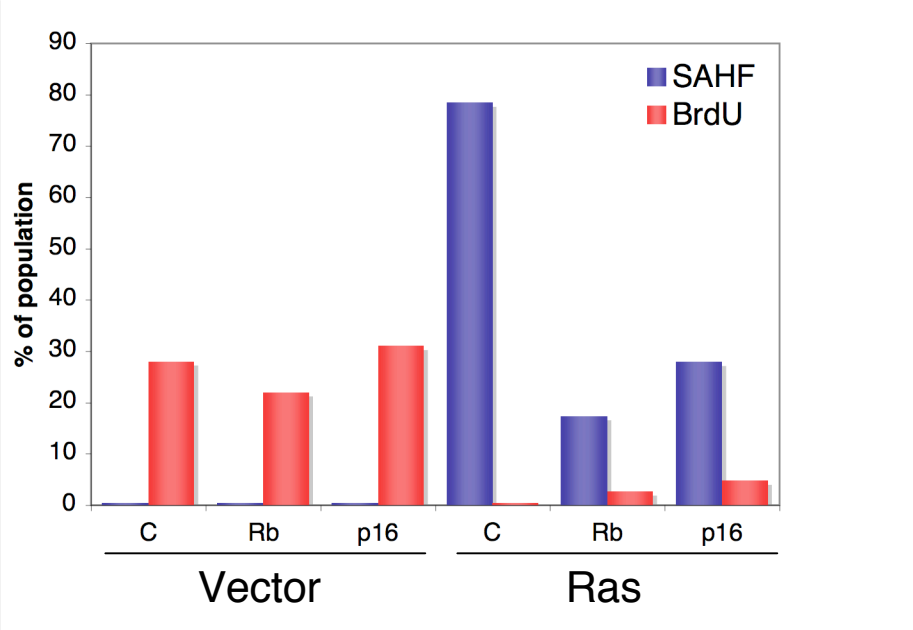
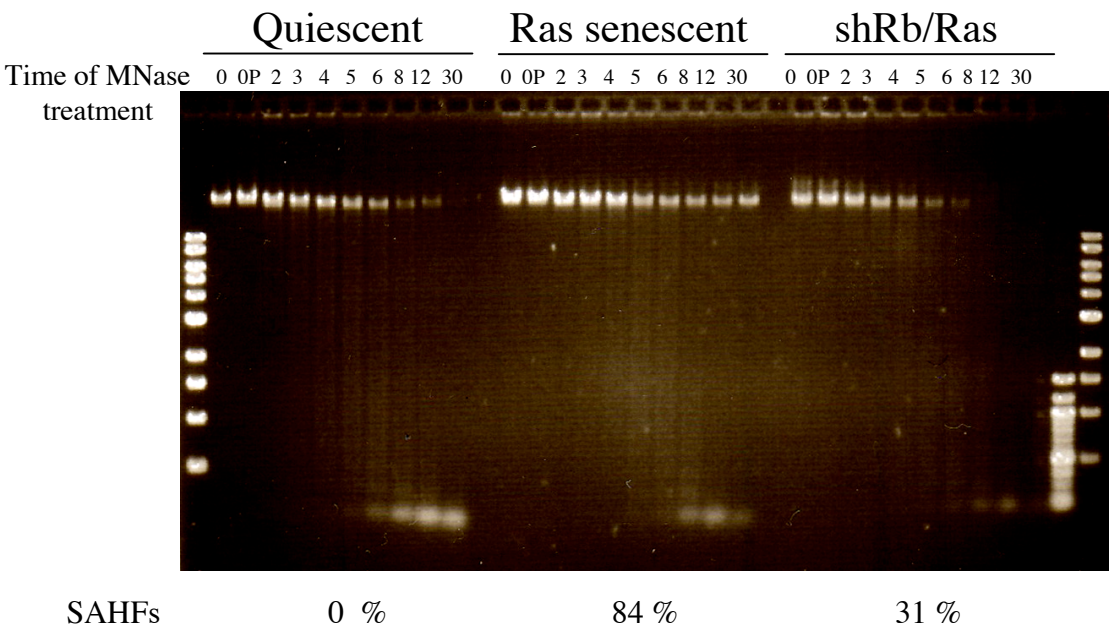

90
80
70
60
50
40
30
20
10
0
% of population
SAHF
BrdU
C
Rb
p16
C
Rb
p16
Vector
Ras

Figure 2.15

SAHF-negative cells display increased nuclease sensitivity

In vivo micrococcal nuclease digestion of detergent-permeabilized quiescent, Ras-senescent and Ras-senescent cells expressing a short hairpin RNA targeting Rb (shRb/Ras). DNA was isolated from cells after digestion for the indicated time (min) and resolved on an agarose gel. The percentage of SAHF exhibited by each cell population at the time of the assay is indicated at the bottom of the gel.


Quiescent
Ras senescent
shRb/Ras
Time of MNase treatment
0 0P 2 3 4 5 6 8 12 30
0 0P 2 3 4 5 6 8 12 30
0 0P 2 3 4 5 6 8 12 30
SAHFs
0 %
84 %
31 %

The levels of SAHF directly correlate with the levels of p16^{INK4a} expression upon senescence

Work from the lab of Judith Campisi has shown that p16^{INK4a} is poorly induced in senescent BJ fibroblasts (Beausejour et al., 2003b). We observed that BJ cells purchased from ATCC were able to upregulate p16 quite well and 70 percent of the population displayed SAHF (Figure 2.16A). We obtained BJ cells from the Campisi lab (BJc) and verified that they exhibited poor upregulation of p16 upon induction of senescence (Figure 2.16, lane compare lanes 4 and 6). Comparison of ras-senescent IMR90s to the two different BJ populations revealed a strict correlation between the level of p16 induction and the percentage of cells that form SAHF (Figure 2.16A). Of note, as we passaged the BJ cells from ATCC we noted that they progressively lost their ability to induce p16 upon senescence induction, and therefore, exhibited reduced SAHF formation (not shown).

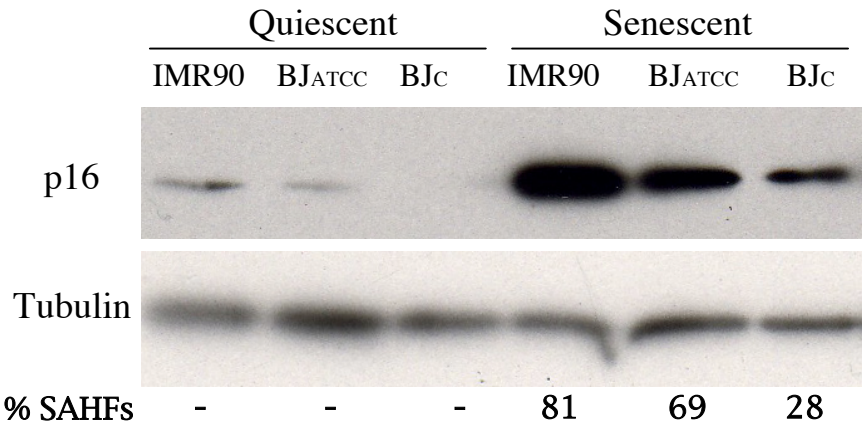
Re-introduction of p16^{INK4a} along with H-RasV12 into BJ cells restored SAHF formation to levels similar to those seen in IMR90 fibroblasts (Figure 2.16B and 2.17). Conversely, abrogation of p16^{INK4a} levels in Ras-senescent IMR90 cells by use of a short hairpin RNA targeting p16^{INK4a} reduced their ability to form SAHF. Indirect manipulation of p16 levels confirms these results, since co-expression of HMGA2 (which upregulates p16 levels, figure 2.18A and (Narita et al., 2006)) and H-RasV12 restores SAHF formation in BJ fibroblasts while co-expression of Bmi1 [which suppresses p16 levels, figure 2.18B and (Jacobs et al., 1999)] and H-RasV12 suppresses SAHF formation in IMR90 (Figure 2.18).

Figure 2.16

The levels of SAHF directly correlate with the levels of $p16^{INK4a}$ expression upon senescence

(A) The expression of $p16^{INK4a}$ was determined by immunoblotting of extracts from IMR90 and 2 different lots of BJ cells that were made quiescent by low serum or senescent by expression of H-rasV12. Tubulin served as a loading control. The percentage of SAHF for each cell population is indicated below the gel. (B) SAHF quantitation for growing (G) and Ras-senescent (Ras) IMR90 and BJ fibroblasts, as well as BJ made to undergo Ras senescence in the presence of $p16^{INK4a}$ overexpression (BJ p16+Ras) and IMR90 that underwent Ras-induced senescence with low levels of p16 due to expression of a hairpin targeting $p16^{INK4a}$ (IMR90 shp16+Ras).

A


Quiescent
Senescent
IMR90
BJATCC
BJC
IMR90
BJATCC
BJC
p16
Tubulin
% SAHFs
-
-
-
81
69
28

B

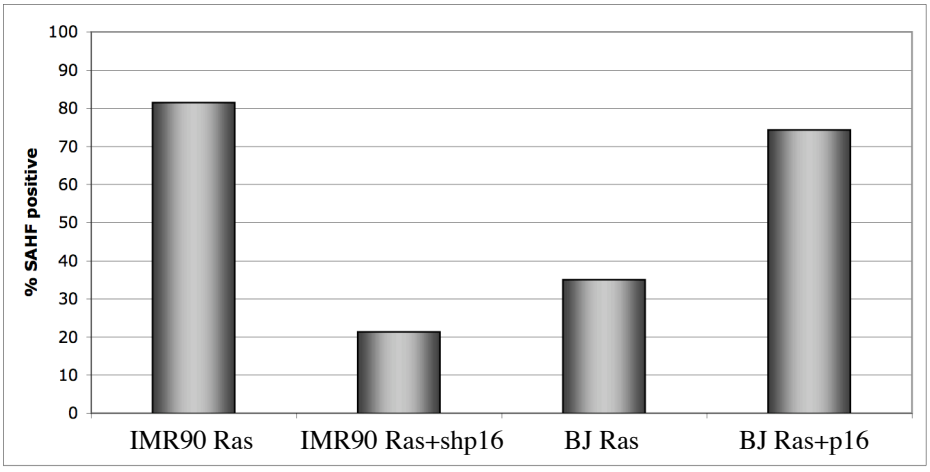

% SAHF positive
100
90
80
70
60
50
40
30
20
10
0
IMR90 Ras
IMR90 Ras+shp16
BJ Ras
BJ Ras+p16

Figure 2.17

Formation of SAHF, as visualized by TMK9H3 immunofluorescence, depends on the level of expression of $p16^{INK4}$

Growing (G) and Ras-senescent (Ras) IMR90 and BJ fibroblasts, as well as BJ infected with Ras and $p16^{INK4a}$ (BJ p16+Ras) and IMR90 infected with Ras and a short hairpin targeting $p16^{INK4a}$ (IMR90 shp16+Ras) were subjected to immunofluorescence with an antibody directed against K9 trimethyl H3 and analyzed by confocal microscopy. DNA was counterstained by propidium iodide (PI). The presence of SAHF correlates with the level of expression of p16INK4a.

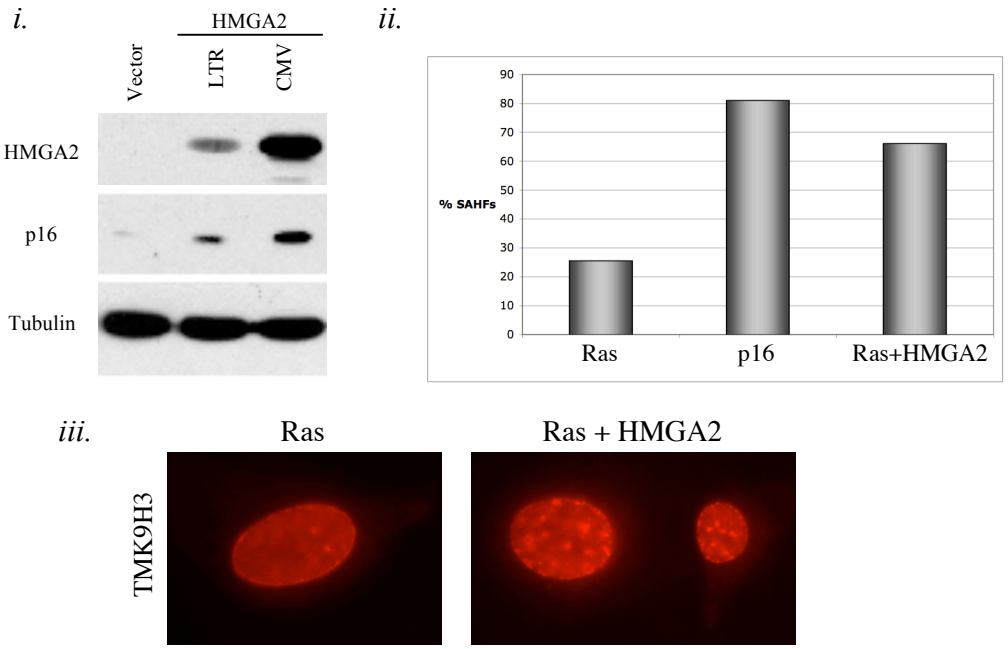
Q
Ras
p16+Ras
Q
Ras
shp16 + Ras
BJ
IMR90
PI
TMK9

Figure 2.18

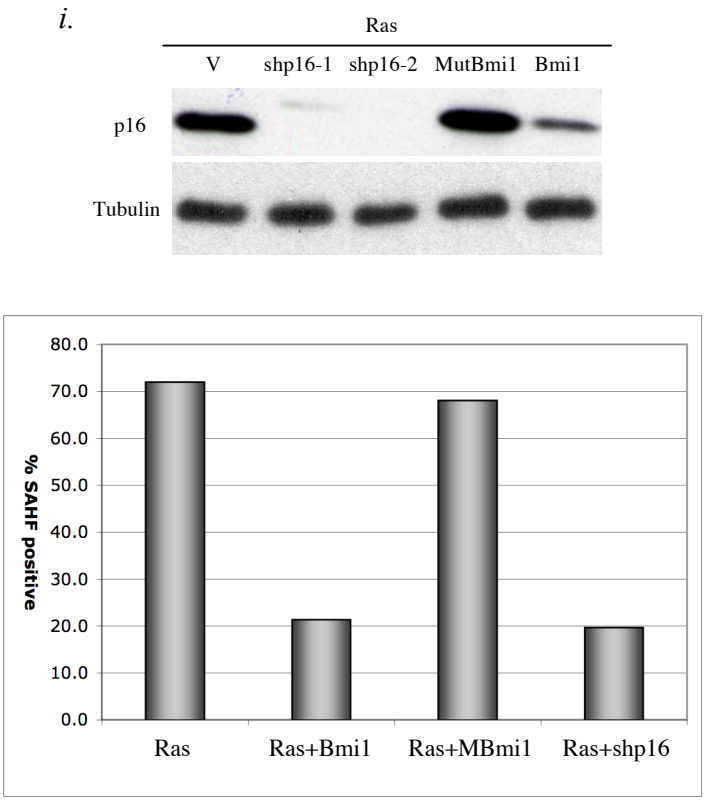
HMGA2 restores SAHF formation in senescent BJ fibroblasts and Bmi1 suppresses SAHF in senescent IMR90.

(A) (i)BJ cells were infected with a vector control or HMGA2, (driven either by the LTR or the CMV promoter, which results in different levels of protein overexpression). Protein extracts were analyzed by Western Blotting with antibodies against HMGA2, p16 and tubulin. (ii) SAHF quantitation in BJ fibroblasts infected with H-RasV12, p16INK4a or HMGA2 together with H-RasV12. (iii) Representative image of the nucleus of BJ cells infected with H-RasV12 or H-RasV12+HMGA2 following immunofluorescence with anti-TMK9 H3 antibody. (B) (i) IMR90 cells were infected with H-RasV12 along with either vector, one of two short hairpin RNAs targeting p16 (shp16-1 and shp16-2), Bmi1 or a mutant form of Bmi1 that cannot inhibit p16 (MuthBmi1). Protein extracts from these cells were analyzed by Western blot with antibodies against p16 and tubulin. (ii) SAHF quantitation in cells from (B)(i).

A


i.
HMGA2
Vector
LTR
CMV
HMGA2
p16
Tubulin
ii.
% SAHFs
90
80
70
60
50
40
30
20
10
0
Ras
p16
Ras+HMGA2
iii.
Ras
Ras + HMGA2
TMK9H3

B


i.
Ras
V
shp16-1
shp16-2
MutBmi1
Bmi1
p16
Tubulin
ii.
% SAHF positive
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Ras
Ras+Bmi1
Ras+MBmi1
Ras+shp16

Interestingly, manipulation of the levels of $p16^{INK4a}$ expressed in Ras-senescent BJ and IMR90 fibroblasts did not affect their overall senescent morphology as visualized by phase contrast (Figure 2.19). Senescence-associated beta-galactosidase activity also appears to be independent of SAHF formation (Figure 2.19). The cellular morphology and SA-βgal staining are therefore not related to $p16^{INK4a}$ levels or SAHF formation.

The chromatin-bound protein profile differs between SAHF-positive and SAHF-negative cells

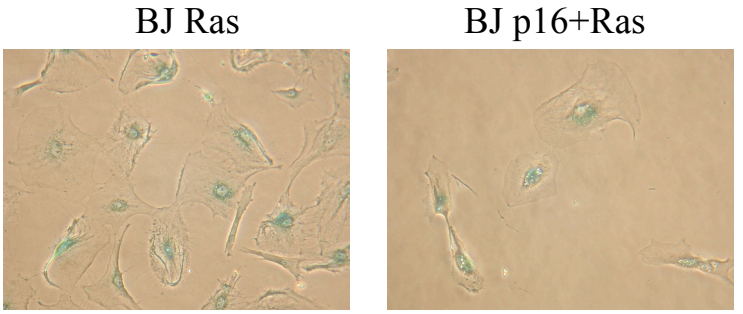
We then analyzed the chromatin-bound protein profile of ras-senescent IMR90 cells that failed to form SAHF due to expression of a short hairpin targeting p16. There is no apparent difference in this profile relative to that of Ras-senescent IMR90 (Figure 2.20A, compare lanes 2 and 3). In contrast, the chromatin-bound protein profile of ras-senescent BJ cells is quite different to that of Ras-senescent IMR90 (Figure 2.20A, compare lanes 2 and 5). In particular HMGA2 (marked by a green asterisk) does not accumulate on he chromatin of senescent BJ (Figures 2.20 A and B). We have recently shown that HMGA proteins cooperate with the p16INK4a tumor suppressor to promote SAHF formation and proliferative arrest and stabilize senescence by contributing to the repression of proliferation-associated genes (Narita et al., 2006). Re introduction of p16 into BJ cells restores some HMGA2 association to chromatin (Figure 2.20A lanes 6 and 7) and BJ cells co-infected with HMGA2 and ras show chromatin association of the

Figure 2.19

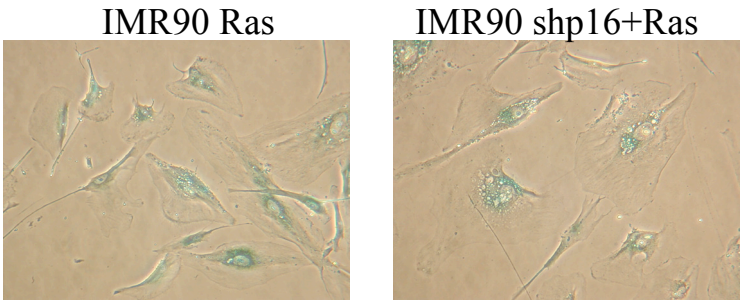
The senescent morphology and SA β-gal staining are independent of SAHF formation

IMR90 cells were infected with either H-RasV12 (Ras) or a short hairpin targeting p16 and H-RasV12 (shp16+Ras). BJ were infected with either H-RasV12 or p16 and H-RasV12. (A) Phase contrast image of the senescent fibroblasts stained for SA β-gal activity. (B) Quantitation of SA β-gal positive cells in samples shown in (A).

A

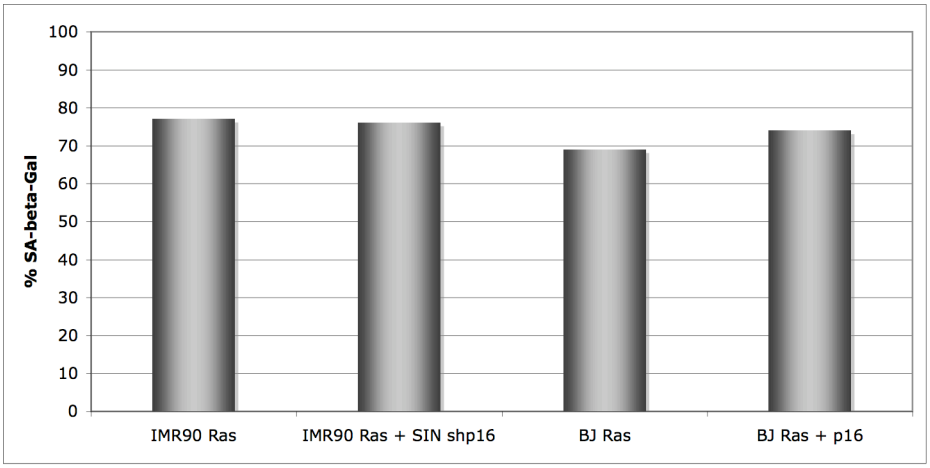

BJ Ras
BJ p16+Ras

SAHF + +++


IMR90 Ras
IMR90 shp16+Ras

SAHF +++ +

B


% SA-beta-Gal
100
90
80
70
60
50
40
30
20
10
0
IMR90 Ras
IMR90 Ras + SIN shp16
BJ Ras
BJ Ras + p16

tagged HMGA2 (marked by a green asterisk in figure 2.20A, lane 8) but not the endogenous HMGA2. The significance of these observations remains to be elucidated.

Consistent with their failure to form SAHF the total amount of chromatin-bound HP1γ fails to increase in ras-senescent BJ fibroblasts relative to normal proliferating and quiescent cells (Figure 2.20B). Also unlike senescent IMR90, senescent BJ do not exhibit a decrease in chromatin-bound Ac-K9H3 relative to growing and quiescent cells (Figure 2.20B). Intriguingly the amount of histone H3 trimethylated at lysine 9 increases in senescent BJ but not IMR90 relative to its growing and quiescent counterparts (Figure 2.20B). Similarly senescent IMR90 in which p16 expression has been knocked down through the use of a short hairpin RNA (and are therefore less able to form SAHF) have less HP1γ and more AcK9H3 associated with their chromatin than their ras-senescent counterparts (Figure 2.20C compare lanes 4 and 5).

Replicative senescent BJ fibroblasts resume growth upon p53 inactivation

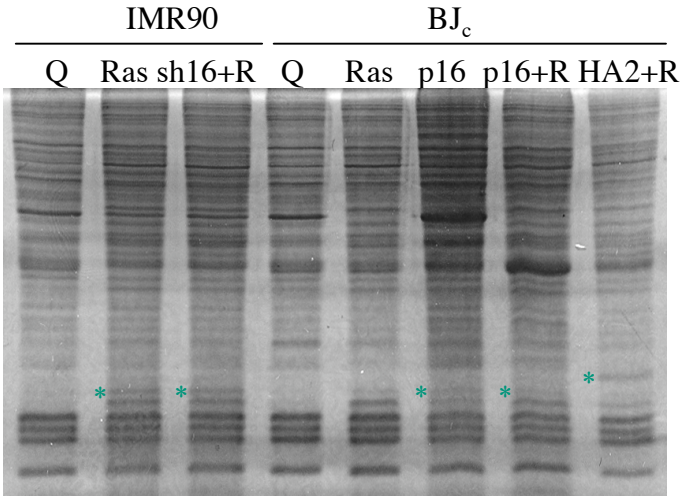
Data from the lab of Judith Campisi indicates that inactivation of p53 is enough to allow for senescence reversal in cells that have low levels of p16 at the onset of senescence (Beausejour et al., 2003b). We recapitulated these results in replicative senescent BJ fibroblasts by infecting them with a lentivirus expressing LgTK1 (a LgT mutant that can bind and inactivate p53 but not Rb) or GSE 22 (a p53 genetic suppressor element which inactivates p53 (Ossovskaya et al., 1996)) (Figure 2.21)

Figure 2.20

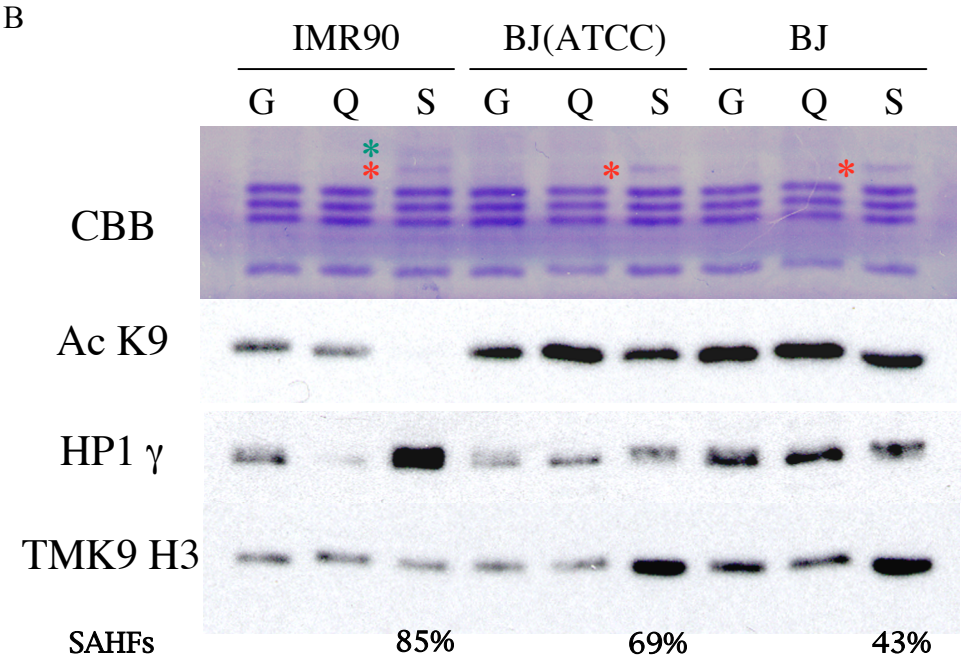
The chromatin-bound protein profile differs between SAHF-positive and SAHF-negative cells

A) Chromatin-bound protein profile of quiescent (Q), Ras-senescent (Ras) and shp16 and Ras senescent (sh16+R) IMR90 as well as quiescent (Q), Ras-senescent (Ras), p16-senescent, p16 plus Ras-senescent (p16+R) and HMGA2 plus Ras-senescent (HA2+R) BJ fibroblasts. The chromatin-bound protein profile of Ras-senescent BJ cells differs from that of IMR90 that senesce with low levels of p16. The green asterisk marks the HMGA2 protein (B) Chromatin bound protein profile of IMR90, early passage BJ from ATCC(BJatcc) and BJ from the lab of Judith Campisi (BJ) that were either growing (G), made quiescent by low serum (Q) or mase senescent by H-RasV12 (S). Proteins were transferred to Immobilon P membranes and blotted with antibodies against histone H3 acetylated at K9 (AcK9H3), HP1γ, histone H3 tri-methylated at K9 (TMK9H3). Commassie blue staining (CBB) was used to confirm equal loading by core histones. The percentage of senescent cells that exhibited SAHF is indicated at the bottom of the gel. Red asterisks mark HMGA1, the green asterisk marks HMGA2. (C) Chromatin-bound protein profile of quiescent (Q), growing (G), synchronized growing (Gs), Ras-senescent (Ras) and cells made Ras-senescent in the presence of a short hairpin RNA targeting p16 (Ras+shp16). Commassie blue staining shows equal loading of core histones. Proteins were transferred to an Immobilon P membrane and blotted for HP1γ and AcK9H3.

A


IMR90
BJc
Q
Ras
sh16+R
Q
Ras
p16
p16+R
HA2+R

B


IMR90
BJ(ATCC)
BJ
G
Q
S
G
Q
S
G
Q
S
CBB
Ac K9
HP1 γ
TMK9 H3
SAHFs
85%
69%
43%

C

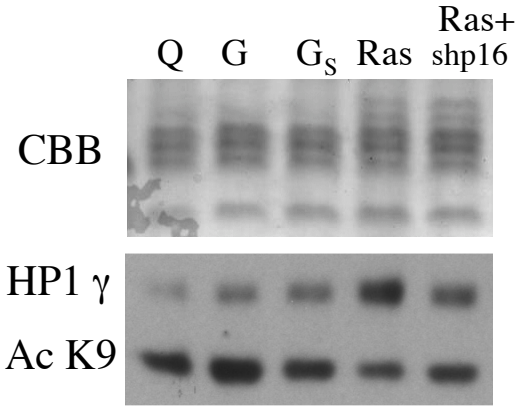
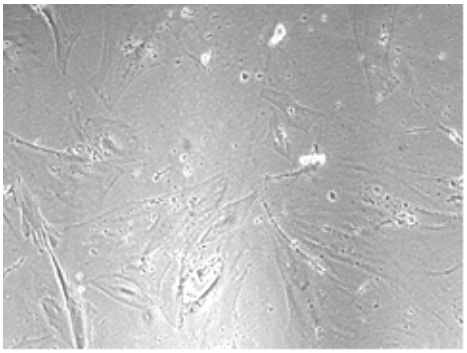

Q
G
GS
Ras
Ras+
shp16
CBB
HP1 γ
Ac K9

Figure 2.21

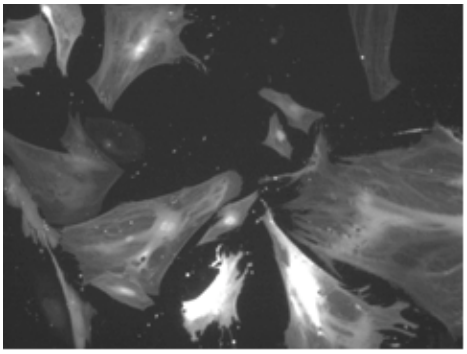
Replicative senescent BJ fibroblasts resume growth upon p53 inactivation

Replicative senescent fibroblasts were infected with GFP, largeTK1 or GSE22 lentivirus. A representative phase contrast image and the corresponding GFP image are shown for cells infected with lentiviral GFP and phase contrast images of growing cells following infection with LgTK1 and GSE22.

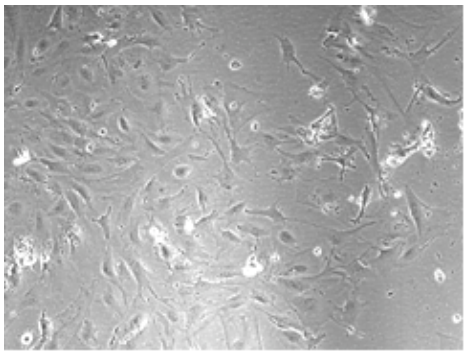
L-GFP brightfield



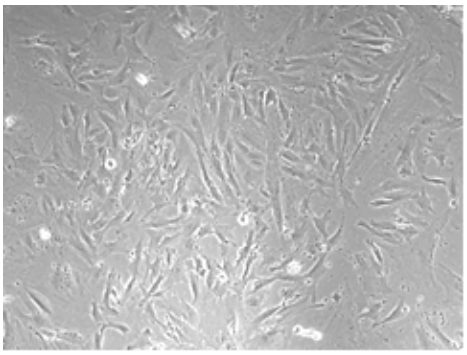
L-GFP



L-LgTK1



L-GSE22



Inactivation of p53 reverses senescence in SAHF-negative senescent fibroblasts.

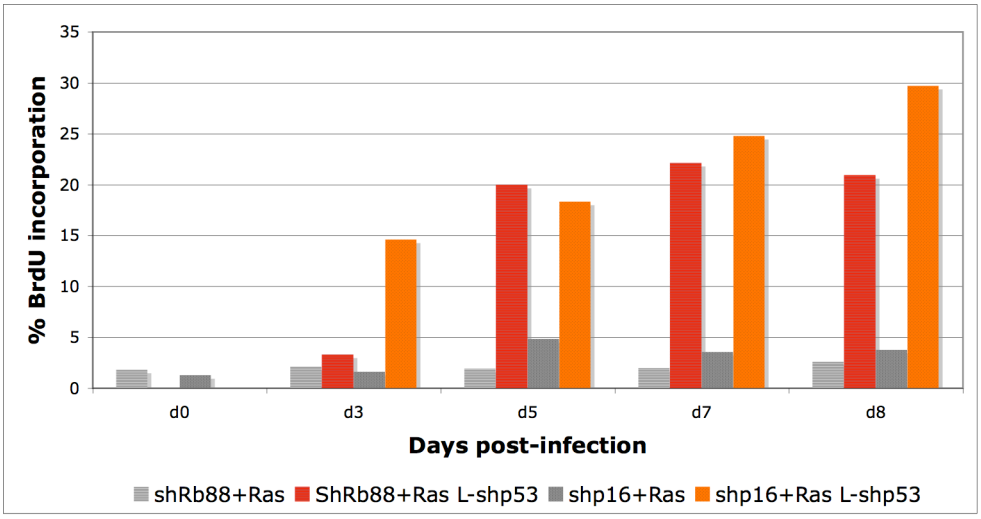
Our results indicate that p16INK4a upregulation during senescence engages the Rb pathway to produce a permanent arrest by altering the chromatin state of growth regulatory genes. We therefore hypothesized that the failure of these processes in cells sustaining INK4a or Rb mutations is what allowed for the reversal of senescence by p53 inactivation. To test this we made lentiviral short hairpin RNAs targeting p53 and tested its ability to reverse senescence in SAHF-negative senescent IMR90. p53 knockdown in IMR90 ras senescent cells that express either a short hairpin RNA against p16 or Rb allows them to re-enter the cell cycle (Figure 2.22A and B). The SAHF positive cells do not resume growth (not shown) but rather, as the proliferating cells take over the cultures, the percentage of SAHF-positive cells decreases (Figure 2.22C). These results lend support to our model in which the failure to silence E2F target genes would reduce the probability that a damaged cell undergoes senescence or, alternatively, makes the arrest more difficult to sustain.

Figure 2.22

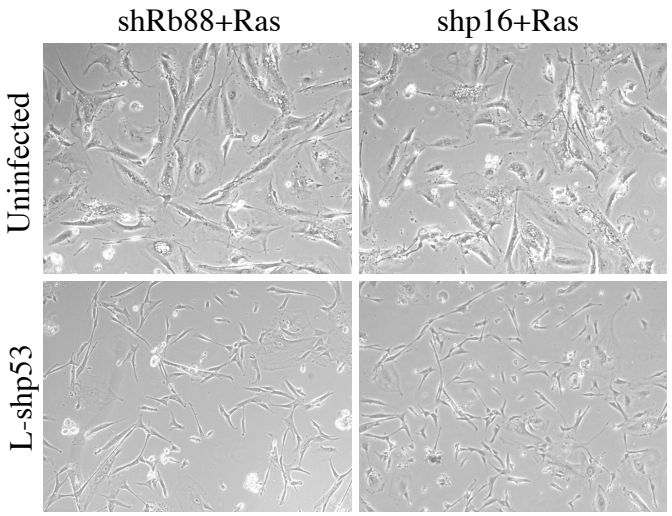
Inactivation of p53 reverses senescence in SAHF-negative senescent fibroblasts

IMR90 fibroblasts were infected with H-RasV12 together with a short hairpin RNA targeting Rb (shRb88) or p16 (shp16). At post-selection day 7 the senescent cells were infected at with lentiviral short hairpin RNA targeting p53 (L-shp53). (A) Quantitation of the percentage of cells described above that incorporate BrdU (6 hour pulse) at the indicated time-points following infection with L-shp53. (B) phase contrast image of the cells describes above 5 days post-infection with L-shp53. (C) Quantitation of the percentage of cells described above that exhibit SAHF at the indicated time-points following infection with L-shp53.

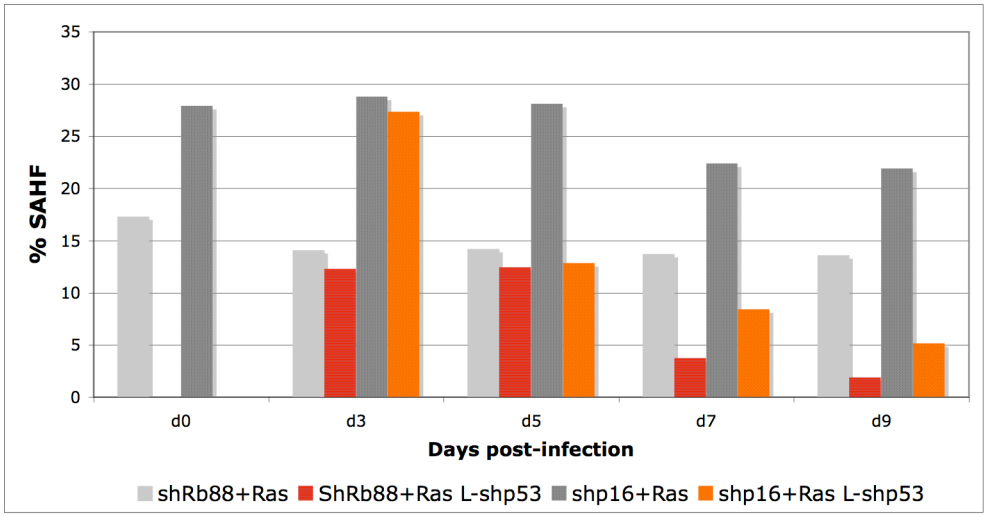
A


% BrdU incorporation
35
30
25
20
15
10
5
0
d0
d3
d5
d7
d8
Days post-infection
shRb88+Ras
ShRb88+Ras L-shp53
shp16+Ras
shp16+Ras L-shp53

B


shRb88+Ras
shp16+Ras
Uninfected
L-shp53

C


% SAHF
35
30
25
20
15
10
5
0
d0
d3
d5
d7
d9
Days post-infection
shRb88+Ras
ShRb88+Ras L-shp53
shp16+Ras
shp16+Ras L-shp53

2.4 Discussion

Unlike constitutive heterochromatin, which is important for chromosome segregation and the silencing of repetitive elements, facultative heterochromatin is developmentally controlled and contributes to gene regulation during differentiation (Verma, 1988). Here, we identify a distinctive type of facultative heterochromatin - designated SAHF - that accumulates in senescent cells. SAHF are observed in interphase nuclei and contain the heterochromatin-associated proteins K9M-H3 and HP1, exclude histone modifications found in euchromatin (e.g., K9Ac-H3 and K4M-H3), and are not sites of active transcription. We have shown that SAHF are distinct from pericentric heterochromatin. Indeed, an independent evaluation of post-translational histone modifications associated with SAHF in Ras-senescent WI38 cells confirmed that SAHFs are a type of chromatin condensation that is distinct from those found in mitotic chromosomes or apoptotic cells (Funayama et al., 2006). Moreover, the appearance of SAHFs is accompanied by an increase in HP1 incorporation into chromatin in senescent cells. These observations, along with the enhanced resistance of DNA to nuclease digestion, suggest that SAHFs do not represent a redistribution of pre-existing heterochromatin but constitute a novel type of facultative heterochromatin that is associated with senescence.

SAHF formation requires an intact Rb pathway, since expression of E1A, or inactivation of either p16INK4a or Rb, can prevent their appearance. Little is known about the signals that increase p16 expression. p16 induction has been proposed to be a response to the stress of standard culture conditions (Sherr and DePinho, 2000; Wright and Shay, 2000). Even if this is true, in vivo data indicate that cell culture stress is

activating a mechanism that can likewise be activated by other forms of stress (see section 1.1.3). p16 is only known to be upregulated in senescent settings and its overexpression is enough to induce senescence in human fibroblasts. Additionally, p16 loss is associated with a variety of tumors (Baylin et al., 1998).

According to the current cell cycle paradigm p16 inhibits cyclin dependent kinases 4 and 6, preventing them from phosphorylating Rb. The hypophosphorylated Rb can remain associated to E2F and DP1 on E2F-target promoters and recruit HDACs to transcriptionally repress these genes, thus preventing S-phase entry. Recent reports have challenged this paradigm. In two different studies the authors were unable to detect Rb on E2F target promoters in G1 and quiescent cells (Rayman et al., 2002; Takahashi et al., 2000). In contrast, p107 and p130 were readily detected. While we confirm these reports, we also show that Rb appears on some E2F target promoters in senescent cells. Thus, of the Rb family members Rb itself appears to plays a dominant role in senescence regulation. It is tempting to speculate that this is the reason why Rb but not p107 or p130 is considered a tumor suppressor.

The mechanism by which Rb controls SAHF formation remains an unknown. We show that Rb is specifically associated with some E2F targets in IMR90 senescent cells. On those same promoters histone H3 acetylation of lysine 9 is replaced by methylation, a modification linked to gene silencing and assembly of heterochromatin. E2F-responsive promoters in quiescent cells lose histone H3 acetylation on lysine 9, but do not gain the methyl mark (see Figure 2.8). Methylated lysine 9 forms a docking site for HP1 proteins and, accordingly, HP1 γ preferentially associates with E2F target promoters in senescent

cells. These modifications are predicted to form a "lock" on the transcription of E2F responsive promoters, making them less accessible to the transcription machinery.

We propose that during the initial phases of senescence Rb might control the nucleation of heterochromatin at specific sites throughout the genome, which then spreads by the action of histone methyltransferases and recruitment of HP1 proteins (Nielsen et al., 2001b). HP1 proteins have the capacity to dimerize and may interact to form higher order chromatin structures once a critical mass has been reached (Brasher et al., 2000; Nielsen et al., 2001a). A similar pattern of nucleation and spreading occurs during silencing of the mating type locus in S. pombe, position effect variegation in Drosophila, and X inactivation in mammalian cells (Hall et al., 2002; Heard et al., 2001; Peters et al., 2002). A prediction would therefore be that an Rb variant incapable of interacting with these chromatin factors would fail to induce SAHF formation and stable silencing of E2F-target genes upon the onset of senescence. Such an experiment remains to be done.

According to our proposed model, E2F-target genes should colocalize with SAHFs in senescent cells. As discussed in the results section, we were unable to find a denaturing condition that would allow us to detect E2F-targets by FISH without destroying the SAHFs themselves. A large volume of literature supports the notion that the nuclear resistant fraction represents heterochromatic regions of the genome. We believe that in the case of senescent cells the observed increase in the nuclease-resistant fraction (figure 2.7C) is the result of acquired changes in the content of heterochromatin. DNA within SAHFs should therefore be represented in the nuclease-resistant fraction. Consistent with this notion, we show that the nuclease resistant fraction of senescent cells

is enriched for E2F-targets compared to growing or quiescent cells (figures 2.9-2.11). This indirect assessment suggests E2F-target genes are part of the heterochromatic foci of senescent cells.

The observation that Rb-/- embryos do not survive past embryonic day (E) B13.5–14.5 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992) suggests that, besides its role in G1-/S-phase control, Rb might play a role in development. A variety of recent studies indicate that Rb is involved in the control of cellular differentiation and lineage specification [reviewed by (Skapek et al., 2006)]. If we view senescence as a terminal differentiation state of non-division, we can speculate that Rb contributes to the establishment of senescence through the recruitment of the same cellular functions it engages during cellular differentiation. A better understanding of Rb pathways will allow us to dissect the relative importance of the cell cycle regulatory functions and the cell-type-specific developmental functions of Rb in its tumor suppression.

According to our model expression of p16INK4a should result in the activation of Rb, which directs SAHF formation. SAHFs appear to causally contribute to cellular senescence, at least in part, by controlling the stability of the arrest. Indeed there appears to be a direct correlation between SAHF formation directed by the p16/Rb pathway and the prevention of senescence reversal by p53 inactivation.

Both the p16/Rb and the p53 pathway must contribute to the morphological changes associated with senescence as well as the cell cycle exit, since IMR90 cells lacking p16, Rb or p53 exit the cell cycle and acquire morphological features of senescence. Before the publishing of our work, these different states of senescence were morphologically indistinguishable and were assumed to be the same. The presence or

absence of SAHFs now allows for a distinction between different types of senescence and is predictive of the stability of the arrested state.

Data on the senescence of human mammary epithelial cells appears to support our conclusions. Senescence in HMECs has been distinguished from that in fibroblasts in that HMECs spontaneously emerge from a growth plateau (termed M0) to continue growing until reaching a second plateau (termed M1), which is characterized by cell crisis. Numerous groups have shown that emergence from the first plateau is dependent on loss of p16 (Brenner et al., 1998). In a more detailed study it was observed that M0 is equivalent to the fibroblast senescent arrest and that the cells that emerge from this arrest are those that never had p16 (Romanov et al., 2001). These cells eventually reach a growth plateau termed M1, exhibit eroding telomeric sequences and ultimately enter telomere-based crisis. This crisis generates the types of chromosomal abnormalities seen in the earliest lesions of breast cancer, suggesting that growth past senescence barriers may be a pivotal event in the earliest steps of carcinogenesis. These data argue for an important role for p16 in senescence, as cells without p16 can bypass the senescent arrest. Indeed the same result is observed in fibroblasts (Beausejour et al., 2003a; Beausejour et al., 2003b). Therefore, despite the traditional belief that senescence in fibroblasts is different from senescence in HMECs, these data suggest they might be the same. What might differ instead is the predisposition of HMECs to inactivate p16 in culture.

We attempted to re-introduce p16, either by itself or along with H-RasV12, into MCF10A and MCF7 mammary epithelial cells in the hopes that, as was the case with BJ fibroblasts, it would restore SAHF formation. The cells acquired a senescent morphology in response to both p16 and p16 plus H-RasV12 expression, but did not display SAHFs.

Both of these cell lines are “post-M0” or “post-stasis”, meaning they might have acquired other changes besides loss of p16 to allow them to escape the M0 arrest. We do not rule out the possibility that pre-stasis mammary epithelial cells do form SAHFs. Since all mammary epithelial cells follow this biphasic growth in culture, no pre-stasis cell lines exist. These cells can only be isolated from mammary tissue, and grow in culture for only a few passages before reaching M0. The validity of our observations and conclusions remain to be tested in this system.

Beausejour et al. have shown that BJ fibroblasts express low levels of p16 upon induction of senescence as compared to IMR90 or WI38. We thought this difference might reflect the fact that BJs are neonatal foreskin fibroblasts and IMR90 and WI38 are embryonic fibroblasts. To evaluate this possibility we assayed p16 upregulation and SAHF formation in CCD-1112Sk (ATCC CRL-2429), a neonatal foreskin fibroblast line. These cells upregulate p16 and form SAHF following Ras-induced senescence as well as IMR90 and WI38. On closer examination of the ATCC record for BJ fibroblasts we found that these cells are reported to have a normal diploid karyotype at population doubling 61 but an abnormal karyotype at population doubling 82. The BJ cell line is also reported to have a long lifespan compared to other normal human fibroblast cell lines. We have observed that the chromatin-bound protein profile of Ras-senescent BJ differs substantially from that of Ras-senescent IMR90 that lack SAHFs due to the expression of a short hairpin targeting p16 (Figure 2.20). This would suggest there are other differences between BJ and IMR90 fibroblasts besides their differential ability to upregulate p16 during senescence. It is probably not accurate to describe BJ cells as normal fibroblasts.

Our data suggest that failure to silence E2F-target genes during the establishment of senescence increases the probability that a damaged cell will bypass the senescent arrest or, alternatively, makes the arrest more difficult to sustain. Mutations in the maintenance part of the pathway can therefore allow hyperproliferative cells that are initially controlled by senescence to resume growth, thus contributing to cell immortalization and cancer. Analysis of the genetic makeup of tumors might be predictive of their likelihood to resume growth both in benign tumors that are controlled by senescence (e.g. melanocytic nevi) as well as tumors that respond to chemotherapeutic treatment by induction of senescence (Chang et al., 1999; Schmitt et al., 2002; te Poele et al., 2002).

The data in this chapter provides some of the first mechanistic insights into the effector mechanisms of senescence. Further studies will certainly continue to aid in the identification of processes that control cancer progression and response to therapy.

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Chapter 3:

Rb is required for the maintenance of senescence-associated heterochromatic foci and the stable silencing of E2F-target genes

3.1 Summary

Cellular senescence is an extremely stable form of cell cycle arrest that limits the proliferation of damaged cells. Mechanistically the senescence program can be separated into an 'initiation' phase (in which the cell cycle arrest is established), and a 'maintenance' phase (which provides the cellular memory that prevents the cells from resuming growth). Work from our lab and others indicate that p53, p16 and Rb all act in the initiation phase of senescence. p53 appears to play a dominant role in the maintenance of senescence only when the arrest is established in the absence of p16 or Rb. We have shown that p16 and Rb are required for the formation of senescence associated heterochromatic foci (SAHF) during the initiation phase of senescence. It is not clear what role they play in maintaining senescence. The studies in this chapter focus on the mechanisms maintaining senescence in SAHF-positive cells. We show that the adenoviral oncoprotein E1A, previously known to prevent the establishment of senescence, can force SAHF-positive fibroblasts into S phase. SAHF dissolution precedes the onset of DNA synthesis. The cells fail to divide and die despite the disassembly of heterochromatin and the re-expression of E2F target genes, thus uncovering an additional

layer of complexity in the maintenance of the senescent arrest. The same result is obtained when a short hairpin RNA against Rb is used, indicating that Rb is the cellular target of E1A relevant to the maintenance of SAHF and the arrest. These findings identify Rb as being required for both the formation and the maintenance of heterochromatin in senescent cells, furthering our understanding of the molecular mechanisms responsible for the maintenance of the senescent arrest.

3.2 Introduction

E1A is a viral oncogene that encodes a multi-functional protein capable of promoting both proliferation and apoptosis in normal cells (Rao et al., 1992). E1A renders quiescent cells permissive for adenovirus replication by promoting entry into S phase [Reviewed by (Brockmann and Esche, 1995)]. The *E1A* gene expresses several alternatively spliced transcripts, including the 12S and 13S messages encoding 243 (243R) and 289 (289R) amino acid oncoproteins, respectively [Reviewed by (Shenk and Flint, 1991)]. E1A 243R, which is the isoform used in this project, associates with a number of cellular proteins, including the retinoblastoma gene product (Rb), the Rb-related proteins p107 and p130, the p300 and CREB binding protein (CBP) transcriptional co-activators, cyclin A, certain cyclin-dependent kinases (cdk) and p400/TRRAP (Deleu et al., 2001; Whyte et al., 1988b; Whyte et al., 1989). Mutations in defined regions can be used to identify cellular activities important for E1A action [e.g. (Fuchs et al., 2001; Samuelson and Lowe, 1997)].

Houweling and colleagues were the first to report E1A's ability to immortalize primary cells (Houweling et al., 1980). Studies by Earl Ruley showed that while H-RasV12 was unable to transform baby rat kidney cells when expressed by itself, a combination of activated Ras and E1A cooperated to transform this primary cell line (Ruley, 1983). Work done by Manuel Serrano and colleagues in Scott Lowe's lab showed that H-RasV12 actually induces premature senescence in primary mouse and rodent cells and that E1A is extremely effective at preventing this arrest (Serrano et al., 1997). Presumably, the ability of E1A to circumvent ras-induced senescence is what enables it to cooperate with ras in oncogenic transformation.

Three different transforming proteins from three different classes of DNA tumor viruses (including E1A) have been shown to form complexes with Rb [Reviewed by (Dyson et al., 1989)]. Mutational analysis of these viral proteins indicates that the regions needed for interaction with Rb are also required for transformation [Reviewed by (Buchkovich et al., 1990)]. This suggests that Rb is a crucial cellular target for transformation by these viral oncoproteins. Supporting this notion is the observation that the two non-contiguous regions of Rb essential for complex formation with adenovirus E1A or SV40 large T antigen overlap with the positions of naturally occurring, inactivating mutations of the Rb gene (Hu et al., 1990). These results suggest that these viral oncoproteins are targeting a protein domain that is important for the normal functions of Rb. Indeed the crystal structure of the pRb pocket domain in complex with conserved region 1 (CR1) of Ad5-E1A reveals that E1A-CR1 binds at the interface of the A and B cyclin folds of the pRb pocket domain, and that both E1A-CR1 and the E2F

transactivation domain use similar conserved nonpolar residues to engage overlapping sites on pRb (Liu and Marmorstein, 2007).

A few studies have evaluated the role of Rb in the maintenance of senescent-like arrest. The first study employed a temperature-sensitive pRb mutant protein (tsRb) that induces a G1 arrest and morphological features of senescence in Saos-2 cells at the permissive temperature. Serial activation and inactivation of tsRb in SAOS-2 cells lead to the apoptotic death of the cells (Tiemann and Hinds, 1998). In this experimental setting Bcl-2 and E1B 19K were both able to prevent cell death upon the temperature shift, leading to the appearance of small, proliferating cells. In a follow up study this group found that inactivation of Rb resulted in a rapid E2F reactivation and subsequent S-phase re-entry (Alexander et al., 2003). The death that would follow could be prevented by inhibition of cyclin-dependent kinase 2 activity or expression of a dominant-negative DP1, both of which inhibited S-phase re-entry. These results indicate that death following Rb inactivation in senescent cells is triggered as the cells progress through S-phase. The authors employed different drugs to block the cells at various stages of the cell cycle and determined that the cells died before the G2-M checkpoint but after G1 and early S-phase. Since SAOS-2 cells are p53-null, the authors evaluated the role of p73 in the observed cell death. They found that blocking p73 function prevented the death following temperature shift of the arrested cells to the non-permissive temperature. The cells maintained their senescent morphology and did not divide.

In a second series of experiments, Tyler Jacks' group used a conditional allele of the mouse Rb gene and showed that acute loss of Rb in replicative and H-RasV12 senescent MEFs lead to the reversal of the cellular senescence program (Sage et al.,

2003). Previously they had shown that MEFs in which all three Rb-family members were deleted (TKO) do not undergo senescence in culture and possess some characteristics of transformed cells (Sage et al., 2000). $Rb^{-/-}$ MEFs undergo senescence in culture, suggesting either a functional overlap or developmental compensation within this gene family.

Other reports in the literature point to p53 rather than Rb as the gene required to maintain senescence. Rene Bernards' group employed a lentiviral vector that directs the synthesis of a p53-specific short hairpin RNA to show that stable suppression of p53 expression is sufficient to induce cell cycle re-entry in senescent MEFs (Dirac and Bernards, 2003). The rapid cell cycle re-entry is associated with loss of expression of senescence-associated genes and leads to cellular immortalization. Another study showed that microinjection of monoclonal antibodies specific for the N terminus of p53 (PAb1801 and DO-1) allowed senescent HCA2 neonatal foreskin fibroblasts to re-enter the cell cycle and divide (Gire and Wynford-Thomas, 1998). A contradicting report indicates senescent HCA2 fibroblasts fail to resume growth following infection with an Rb-binding-defective mutant of T (LgTK1), which can bind and inactivate p53 but not Rb (Hara et al., 1996).

In normal diploid human fibroblasts, Beausejour et al. showed that p53 inactivation can reverse senescence in fibroblasts that do not induce p16 during the establishment of the senescence, such as BJ fibroblasts (Beausejour et al., 2003). We have confirmed these results and extended them to show that failure to form SAHF at the onset of senescence allows for reversal of the senescent arrest by p53 inactivation (see Chapter 2). However, HCA2 fibroblasts upregulate p16 during senescence (Bond et al.,

1999). It is known however that some cell lines lose the ability to activate p16 as they are passaged in culture. That appears to be the case with BJ fibroblasts (see figure 2.2.16). The results obtained by Gire el al. should be verified using other fibroblast lines.

Cellular senescence is a cellular program that parallels apoptosis as a cellular response to stress (Campisi, 2001). Unlike apoptotic cells, which are eliminated from the organism as a result of the execution of the program, senescence re-directs the cell into a specialized form of terminal differentiation that prevents it from further dividing. As a result the cell persists in the organism and the senescent arrest needs to be maintained to prevent the damaged cells from resuming growth. A great deal of work has focused on situations in which senescence can be bypassed, as this is emerging as a key even during tumorigenesis. Mutations that allow for the reversal of senescence rather than its bypass could also promote tumor progression and contribute to drug resistance, yet this topic has remained largely unexplored. The main goal of my thesis work has been to characterize the molecular mechanisms responsible for maintaining cellular senescence, which we believe, is essential for the potent tumor suppressive effect of this process.

E1A is a viral oncogene that can override Ras-induced senescence in human fibroblasts, but it is not known whether E1A can reverse the arrest once it has been established. We have shown that p16 and Rb are required for the formation of SAHF during the initiation phase of senescence and have correlated these structures to the stability of the senescent arrest (Narita et al., 2003). Here we show that expression of E1A in senescent cells results in a reversal of the chromatin changes associated with senescence. Loss of SAHF correlates with cell cycle re-entry in these cells. We identified Rb as the factor cellular activity responsible for the maintenance of SAHF, since

downregulation of Rb levels in senescent cells also results in loss of SAHF and cell cycle re-entry.

Rb is known to functionally interact with a number of factors that are involved in heterochromatin biology including HP1, Suv39h1, DNMT1, and components of the SWI/SNF chromatin remodeling complex (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Nielsen et al., 2001). According to our model such associations might allow Rb to directly nucleate regions of heterochromatin on E2F target promoters (and perhaps other regions of the genome), leading to the silencing of E2F target genes and perhaps spreading to other euchromatic loci (Narita et al., 2003). The results in this chapter indicate that the heterochromatic changes imposed by the Rb pathway during senescence are not static and, moreover, that Rb is required for their maintenance. The fact that Rb plays such a dominant role in the maintenance of SAHF lends support to our model and expands our knowledge on the mechanism of maintenance of cellular senescence and the role of Rb as a tumor suppressor.

3.3 Results

E1A bypasses Ras-induced senescence in IMR90 cells.

It is known that the adenoviral oncoprotein E1A prevents the establishment of senescence induced by expression of oncogenic Ras (Serrano et al., 1997). Figure 3.1 shows the morphology of IMR90 cells infected with E1A and H-RasV12. The cells are small and do not exhibit SA-βgal activity. Their nuclei display a diffuse DAPI staining pattern, as opposed to the condensed DAPI foci exhibited by Ras-senescent cells. The cells fail to exit the cell cycle and proliferate quite rapidly, as indicated by the high levels of BrdU incorporation in a 5-hour pulse (only 30% of growing IMR90 cells would be labeled by the same pulsing conditions). These results confirm that E1A overrides senescence in our experimental system.

AdE1A induces death in senescent cells that cannot be blocked by Bcl2 expression.

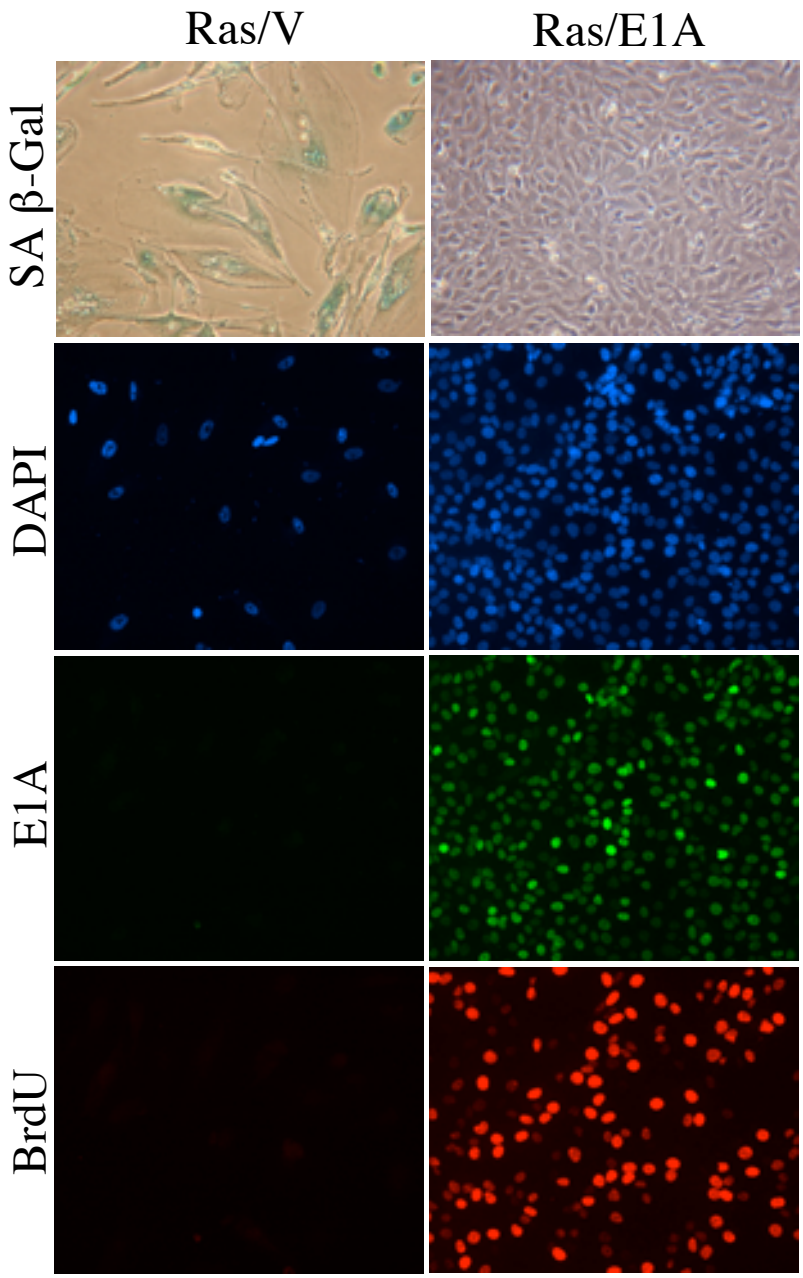
As mentioned above, E1A allows for the bypass of Ras-induced senescence. To determine whether E1A is able to reverse senescence after execution of the program, we chose to use adenoviral E1A (AdE1A). Unlike retroviruses, adenoviruses can infect non-dividing cells, such as senescent cells.

Senescent cells were generated by infection of IMR90 cells with an H-RasV12 expressing retrovirus. Six days after a drug selection to eliminate uninfected cells, the cells were infected with adenoviral E1A at the indicated multiplicity of infection (MOI). Quiescent cells generated by culturing in low serum were used as a control. Our first

Figure 3.1

E1A expression allows for the bypass of Ras-induced senescence

IMR90 fibroblasts were infected with H-RasV12 (Ras) and either vector control (V) or E1A retroviruses. The top panel shows a phase contrast image of senescent Ras/V cells and growing Ras/E1A cells stained for SAβgal activity. Cells were pulsed with BrdU for 6 hours and subjected to immunofluorescence with antibodies against E1A and BrdU. DNA was counterstained with DAPI.


Ras/V
Ras/E1A
SA β-Gal
DAPI
E1A
BrdU

observation was that expression of the viral oncogene induced selective death of senescent but not quiescent cells, as measured by trypan blue exclusion (Figure 3.2A). The death did not seem to result from adenoviral toxicity, since infection with adenovirus LacZ and GFP did not have this effect.

E1A sensitizes cells to apoptosis, and E1B19K (another adenoviral early gene blocks apoptosis during adenoviral infection. Bcl-2 is a human counterpart of this anti-apoptotic factor, and is believed to act by binding to the mitochondria and inhibiting cytochrome c release, thereby blocking caspase activation and the apoptotic process (Kluck et al., 1997). IMR90 cells made to senesce in the presence of Bcl-2 remained viable at 48 h post-infection but ultimately died (Figure 3.2B). About 90 hours after infection with AdE1A most senescent cells had died in an uncharacterized manner. In contrast, AdE1A-infected quiescent cells remained viable, indicating E1A causes preferential death of senescent cells. The same result is obtained in cells expressing E1B19K (not shown).

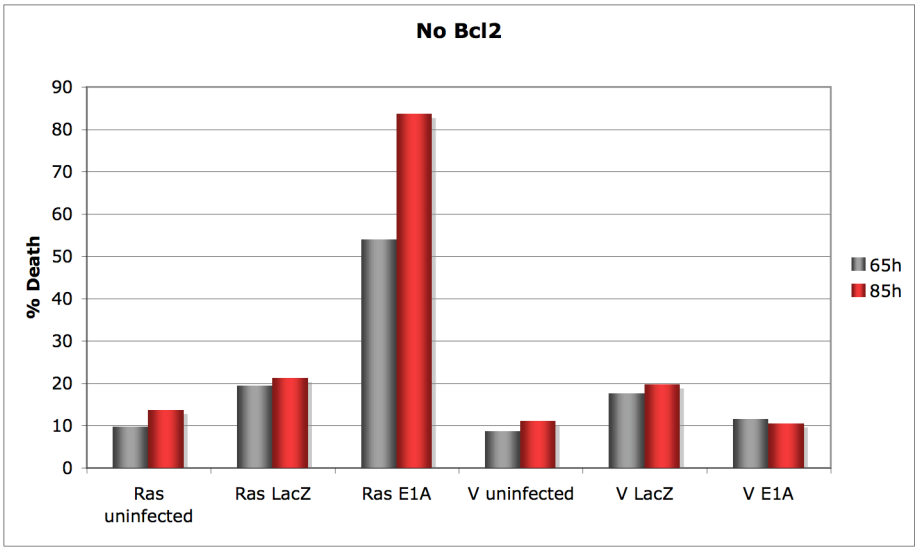
Adenoviral E1A leads to loss of SAHF and BrdU incorporation in senescent cells.

Bcl-2 blocked apoptotic death induced by E1A in senescent fibroblasts long enough to allow studies of S phase re-entry by assaying BrdU incorporation. IMR90 cells were therefore infected with pBabe-Bcl2 and either pWZL or pWZL-H-Ras-V12. The cells expressing the activated Ras senesced and the cells infected with the vector alone were made quiescent by cultivation in low serum media. After 48h of infection with AdE1A (MOI of 60 pfu/cell) BrdU incorporation was observed in both quiescent and senescent cells (Figure 3.3). Addition of serum (S) was not capable of inducing DNA

Figure 3.2

Adenoviral E1A expression induces death in senescent cells

(A) IMR90 cells infected with vector control (V) or made to senesce by infection with H-RasV12(Ras) were subsequently infected with E1A or LacZ adenovirus at a multiplicity of infection (MOI) of 30 plaque-forming units (PFU) per cell. Cell death was assayed by trypan blue 48h and 96 h after adenoviral infection. (B) IMR90 cells infected with either vector and Bcl2 or H-RasV12 and Bcl2 were subjected to the same assay as in (A). Although apoptotic death is initially blocked by Bcl-2, cells eventually die in a manner that can not be prevented by Bcl-2.


No Bcl2
% Death
90
80
70
60
50
40
30
20
10
0
Ras uninfected
Ras LacZ
Ras E1A
V uninfected
V LacZ
V E1A
65h
85h

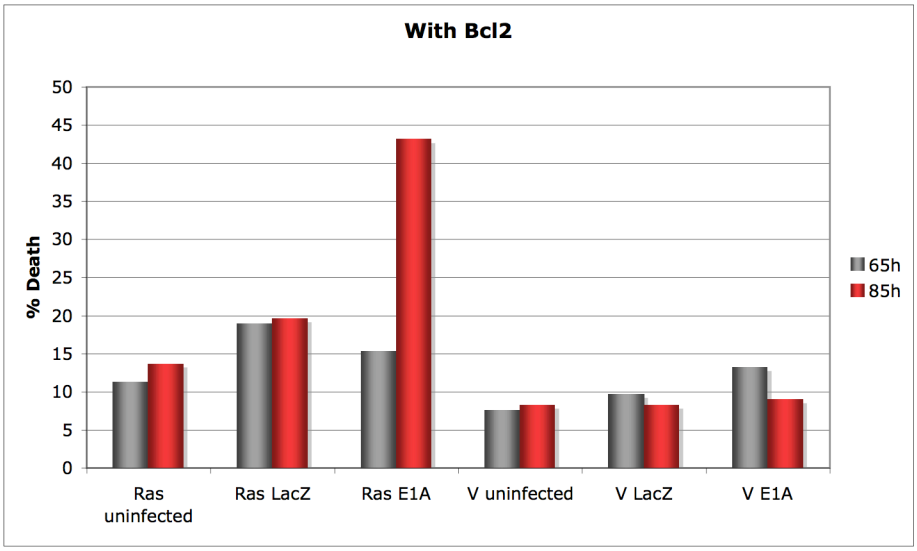
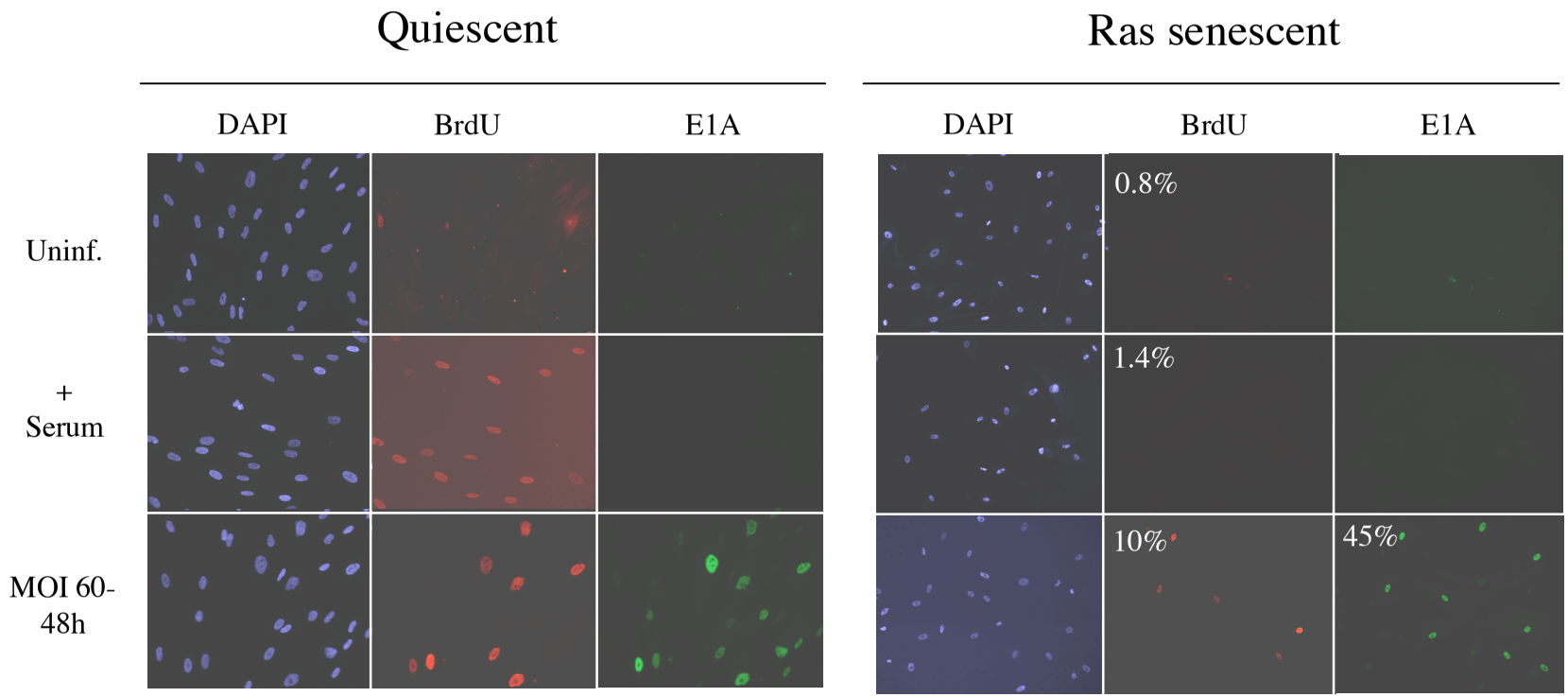

With Bcl2
% Death
50
45
40
35
30
25
20
15
10
5
0
Ras uninfected
Ras LacZ
Ras E1A
V uninfected
V LacZ
V E1A
65h
85h

Figure 3.3

Adenoviral E1A leads to loss of SAHF and BrdU incorporation in senescent cells

IMR90 cells made quiescent by low serum or senescent by expression of H-RasV12 were serum stimulated or infected with adenoviral E1A (AdE1A) at an MOI of 60 pfu/cell. Uninfected cells served as control. 48 hours after treatment the cells were pulsed with BrdU for 5 hours and subjected to immunofluorescence with antibodies against E1A or BrdU. DNA was counterstained with DAPI.


Quiescent
Ras senescent
DAPI
BrdU
E1A
DAPI
BrdU
E1A
Uninf.
+
Serum
MOI 60-
48h
0.8%
1.4%
10%
45%

synthesis in senescent cells but, as expected, allowed quiescent cells to resume proliferation. Neither quiescent nor uninfected senescent cells (U) incorporated BrdU, consistent with their exit from the cell cycle. Immunofluorescence for E1A shows that a comparable number of quiescent and senescent cells were successfully infected.

Notably, senescent cells that incorporated BrdU in response to AdE1A appeared to lack SAHF (Figure 3.4A). Figure 3.4B shows percentages of SAHF-positive cells, BrdU incorporation, and E1A infection as determined by immunofluorescence for quiescent and senescent cells that were either uninfected (U), stimulated with serum, (S) or infected with AdE1A at an MOI of 60 pfu/cell. 45 hours after infection with AdE1A the percentage of SAHF-positive cells was reduced to about 50 percent, with 20 percent of the cells exhibiting BrdU incorporation. 65 hours after the infection less than 10 percent of the cells had SAHF and over 40 percent exhibited BrdU incorporation. Quiescent control cells were infected with similar efficiency as senescent cells and about one third of the population showed BrdU incorporation 65 hours post-infection.

The observation that SAHF disappear as the cells start to replicate their DNA links these heterochromatic structures to the stability of the arrest.

Upregulation of E2F target genes in senescent cells infected with AdE1A.

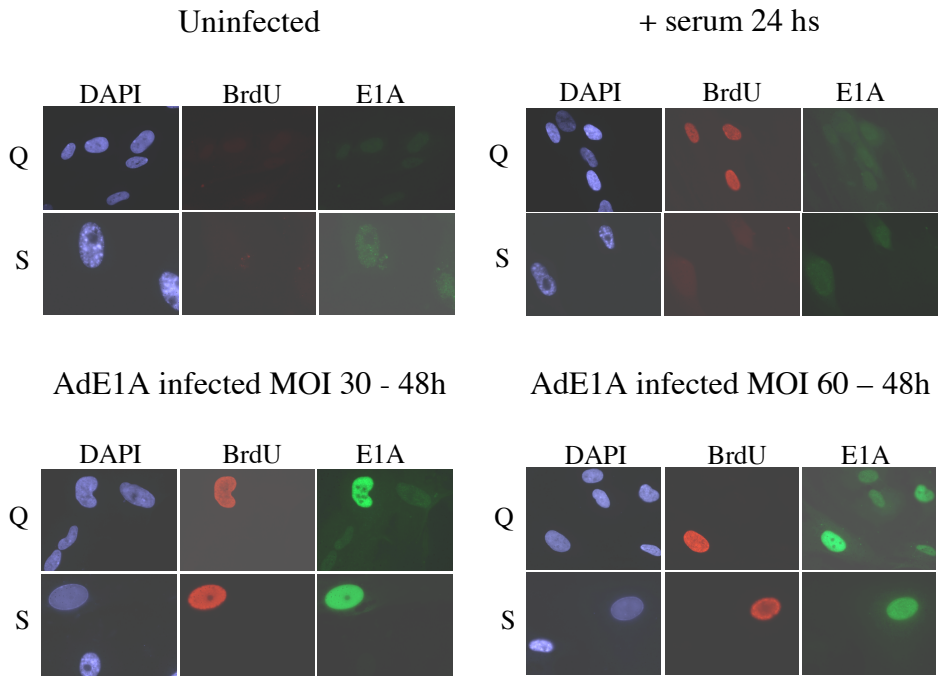
Since senescent cells incorporate BrdU but fail to divide following infection with AdE1A, we chose to examine the expression of some E2F target genes whose expression is increased in proliferating cells. Western Blot analysis was performed 24 and 48 hours

Figure 3.4

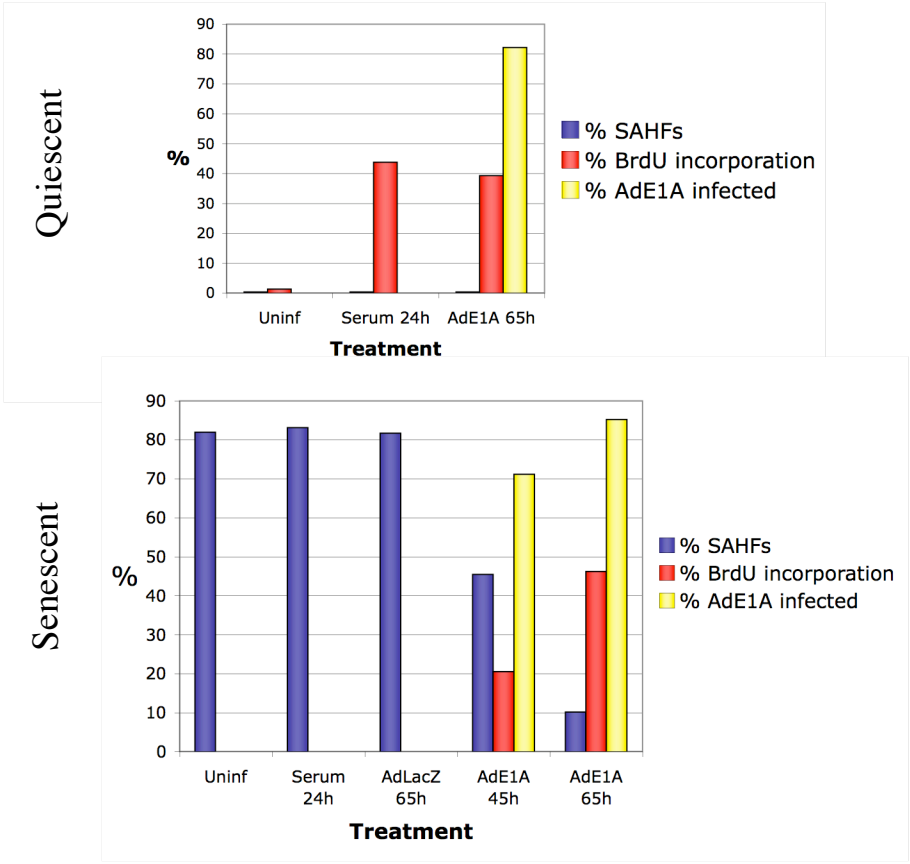
Adenoviral E1A leads to loss of SAHF and BrdU incorporation in senescent cells

IMR90 cells made quiescent by low serum (Q) or senescent by expression of H-RasV12 (S) were serum stimulated or infected with adenoviral E1A (AdE1A) at an MOI of 30 or 60 pfu/cell. Uninfected cells serve a control. (A) At the indicated time-points cells were pulsed with BrdU for 5 hours and subjected to immunofluorescence with antibodies against E1A or BrdU. DNA was counterstained with DAPI. (B) Quantification of SAHF, BrdU and E1A levels in unifected (U), serum stimulated (S) Adenoviral LacZ infected (AdLacZ) and adenoviral E1A infected (AdE1A) quiescent and senescent IMR90. Adenoviral infections were done at an MOI of 60 Pfu/cell and serum stimulation was done for 24 hours.

A


Uninfected
+ serum 24 hs
DAPI
BrdU
E1A
Q
S
AdE1A infected MOI 30 - 48h
AdE1A infected MOI 60 – 48h

B


Quiescent
Senescent
%
90
80
70
60
50
40
30
20
10
0
Uninf
Serum 24h
AdE1A 65h
AdLacZ 65h
AdE1A 45h
Treatment
% SAHFs
% BrdU incorporation
% AdE1A infected

after infection of quiescent and senescent IMR90 with AdE1A at an MOI of 30 or 60 pfu/cell (Figure 3.5). A modest increase in levels of PCNA, Mcm3 and cdc6 was apparent in senescent cells 24 hours after infection. The levels of PCNA detected 48 hours after infection remained low, but high levels of Mcm3, Mcm4 and cdc6 were detectable. As expected, none of the E2F-targets evaluated were induced following serum stimulation of senescent cells.

Uninfected quiescent cells retained some amount of PCNA, Mcm3 and Mcm4 protein expression, although PCNA and Mcm4 levels did increase following serum stimulation and infection with AdE1A. The lack of BrdU incorporation in these cells indicates they were quiescent. We believe the cells were not maintained in low serum long enough for these proteins to be totally degraded. Therefore, in subsequent experiments incubation in low serum was extended for an additional 2 days. Cdc6 levels in these cells were very low, however, and only increased as a result of AdE1A infection. We do not know why serum stimulation failed to induce cdc6. A clear upregulation of PCNA, Mcm4 and cdc6 was observed in quiescent cells 48 hours after infection with AdE1A.

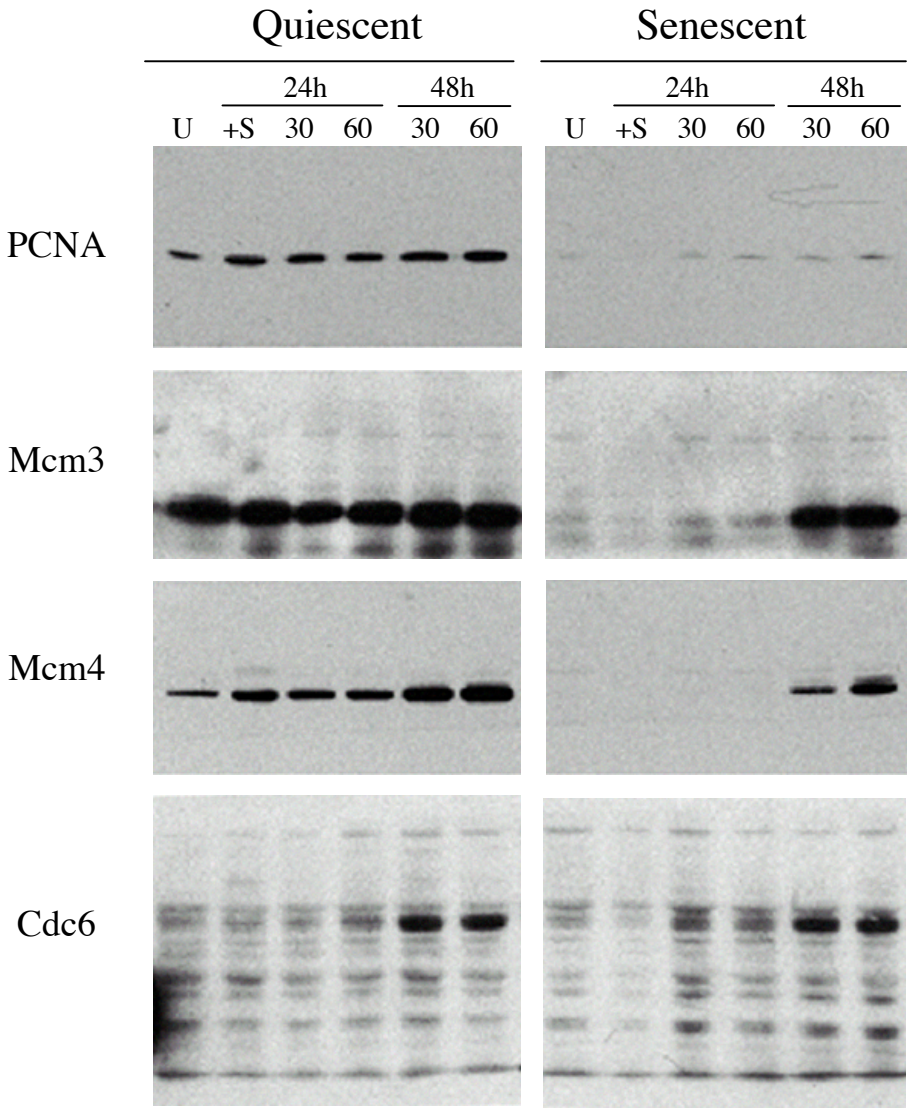
HP1 dissociation precedes SAHF dissolution in senescent cells infected with AdE1A.

We have shown that all three HP1 proteins are dispersed throughout the nucleoplasm in normal and quiescent cells but concentrate to varying degrees in SAHF of senescent cells (Narita et al., 2003). Since HP1 proteins are very abundant it is difficult to

Figure 3.5

E2F-target genes are upregulated in senescent cells infected with AdE1A

IMR90 cells made quiescent by low serum or senescent by expression of retroviral H-RasV12 were either left uninfected (U), serum stimulated (S) or infected with adenoviral E1A (AdE1A) at an MOI of 30 or 60 Pfu/cell protein extracts were prepared at the indicated times. Western blot was performed with antibodies directed against Mcm3, Mcm4, PCNA and cdc6.


Quiescent
Senescent
24h
48h
U
+S
30
60
30
60
U
+S
30
60
30
60
PCNA
Mcm3
Mcm4
Cdc6

assess their loss from SAHF by immunofluorescence without pre-extracting the cells. When we performed immunofluorescence of pre-extracted cells we observed HP1 delocalization from SAHF in Ras-senescent cells infected with adenoviral E1A prior to the dissolution of SAHF, as visualized by DAPI (not shown). To verify that this delocalization was not the result of pre-extraction we constructed a green fluorescence protein (GFP)-HP1β fusion protein, which, when expressed in relative low levels together with H-RasV12, shows a GFP pattern that is concentrated in SAHF (Figure 3.6, top middle panel).

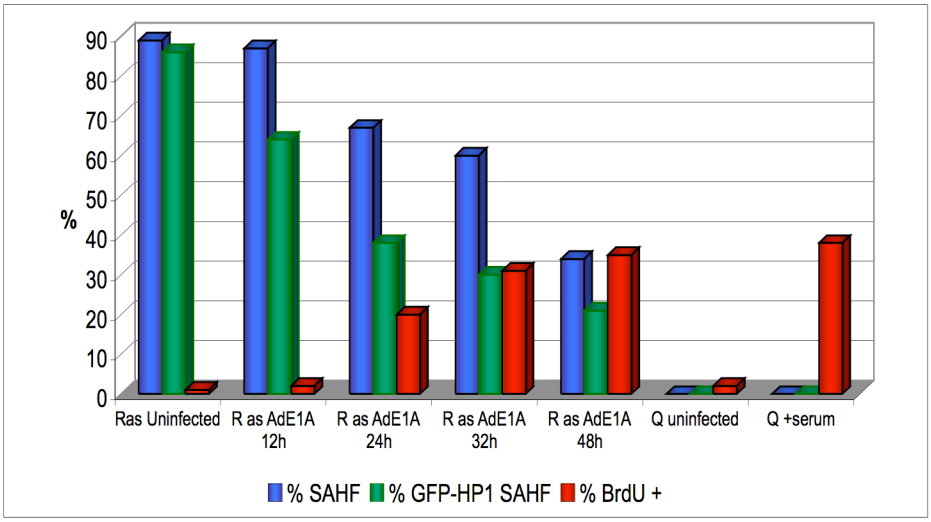
We infected EGFP-HP1β-expressing Ras-senescent cells with AdE1A and evaluated the level of SAHF by GFP and DAPI fluorescence, as well as BrdU incorporation. 12 hours after the infection, the percentage of cells with GFP foci was less than those with DAPI foci, suggesting that HP1β may dissociate from SAHF prior to their dissolution (Figure 3.6A). At this time no cells had incorporated detectable levels of BrdU. 24 hours after infection half the cells had lost GFP signal from SAHF, but only a small proportion of the cells had lost SAHF as assessed by DAPI staining. At this time-point close to 20 percent of cells had incorporated BrdU. Although cells that incorporated BrdU generally corresponded to those that have lost SAHF signal by both DAPI and GFP, a small number of cells with DAPI foci but not GFP foci did incorporate BrdU (Figure 3.6B). In no case did cells with SAHF visible by both signals exhibit BrdU incorporation (figure 3.6B and not shown). 32 hours after the infection about 60 percent of cells had DAPI foci but only half of them had GFP-HP1 foci. About 30 percent of the population exhibited BrdU incorporation, mainly coinciding with those cells that had lost SAHF by both signals. 48 hours after infection BrdU incorporation levels were similar to

Figure 3.6

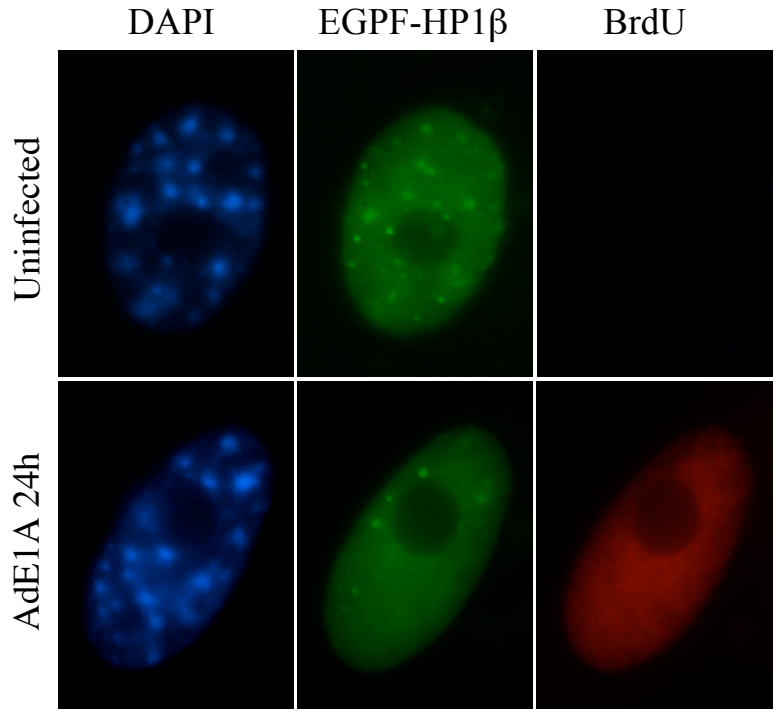
HP1 delocalization from SAHF precedes SAHF dissolution in senescent cells infected with AdE1A

IMR90 cells expressing a GFP-HP1β fusion and H-RasV12 were infected with adenoviral E1A at an MOI of 30 Pfu/cell.(A) Quantitation of SAHF levels and BrdU incorporation. SAHF levels were quantitated by either DAPI or GFP signal 12, 24, 36 and 48 hours after adenoviral infection. (B) Delocalization of HP1 from SAHF can be visualized prior to the dissolution of SAHF as visualized by DAPI. A very small fraction of cells that exhibit loss of GFP foci (HP1) but not DAPI foci incorporate BrdU.

A


90
80
70
60
50
40
30
20
10
0
%
Ras Uninfected
R as AdE1A 12h
R as AdE1A 24h
R as AdE1A 32h
R as AdE1A 48h
Q uninfected
Q +serum
% SAHF
% GFP-HP1 SAHF
% BrdU +

B


DAPI
EGPF-HP1β
BrdU
Uninfected
AdE1A 24h

those seen in quiescent cells re-stimulated to enter the cell cycle by serum addition (Figure 3.6A). The levels of cells with SAHF as visualized by DAPI was similar to that visualized by GFP.

These results suggest that HP1 dissociates from SAHF following infection of senescent cells with AdE1A, with HP1 dissociation preceding SAHF dissolution as visualized by DAPI. In some rare instances cells that lose HP1 from SAHF but still present the structures by DAPI staining appear able to incorporate BrdU (less than 2 percent of BrdU-positive cells). Perhaps loss of HP1 binding to MK9 H3 on E2F-target promoters is sufficient for re-expression of E2F-target genes and cell cycle re-entry. Such a possibility remains to be explored.

Cells that re-enter the cell cycle fail to divide.

As previously mentioned, senescent cells die following infection with AdE1A in a manner that cannot be prevented by Bcl2 or E1B19k expression. There is therefore no apparent potential for growth in these cells. The use of an alpha-tubulin antibody allows for the visualization of the cellular cytoskelelton by immunofluorescence, and when coupled with DAPI staining of DNA, for the visualization of mitotic figures. We chose to employ this immunofluorescence approach to evaluate whether senescent cells infected with AdE1A are able to reach M-phase (which would be indicated by the presence of mitotic figures).

Mitotic figures were easily identified in quiescent cells that were either serum stimulated or infected with AdE1A (Figure 3.7 and not shown). We were unable to find mitotic figures in Ras-senescent IMR90 cells infected with AdE1A, despite scanning dozens of samples at a variety of time-points (representative images are shown in figure 3.7). However, we did observe an overall change in the morphology of senescent cells following AdE1A infection, as cells appeared elongated and regained the shape of growing fibroblasts (figure 3.7). These data indicate that, despite re-entering the cell cycle, senescent cells infected with AdE1A fail to divide, suggesting the cells die before reaching M-phase.

Infection of senescent cells with lentiviral E1A results in loss of SAHF, BrdU incorporation and death of senescent fibroblasts.

Even though Adenoviral E1A killed senescent cells but not quiescent cells, we wanted to rule out adenoviral toxicity as the cause. Lentiviruses allow for the stable infection of non-dividing cells yet have a diminished burden of viral genes, which reduces their toxicity. We therefore screened a variety of lentiviral systems for infection efficiency in primary non-dividing cells. We chose a third generation system from the lab of Luigi Naldini (Dull et al., 1998), which infected Ras-senescent cells at very high efficiency (Figure 3.8A and B).

Infection of Ras-senescent cells with lentiviral E1A resulted in loss of SAHF, BrdU incorporation and death of the cells within 4 days (Figure 3.8). The levels of E1A

Figure 3.7

Cells that re-enter the cell cycle fail to divide

IMR90 were made quiescent by serum withdrawal and subsequently stimulated to re-enter the cells cycle through 30 hours of serum stimulation. The top panel shows a representative image of these cells subjected to immunofluorescence with an antibody against alpha-tubulin. DNA was counterstained with DAPI. The enlarged image shows a cell in metaphase. A cell in anaphase/telophase can also be seen in the lower right part of the image. Such mitotic figures were absent from Ras-senescent cells 40 and 50 hours after infection with AdE1A (bottom panels).

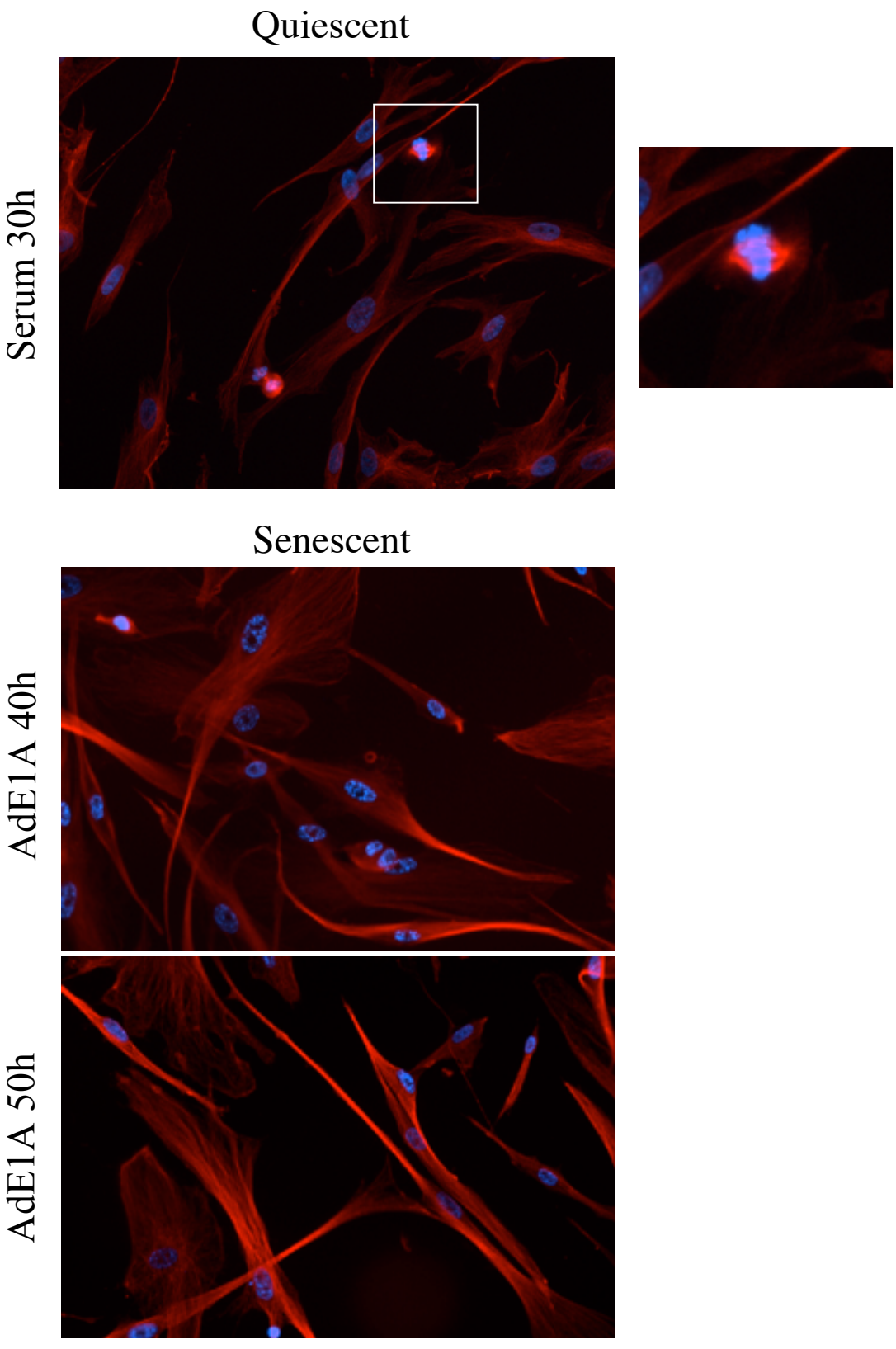
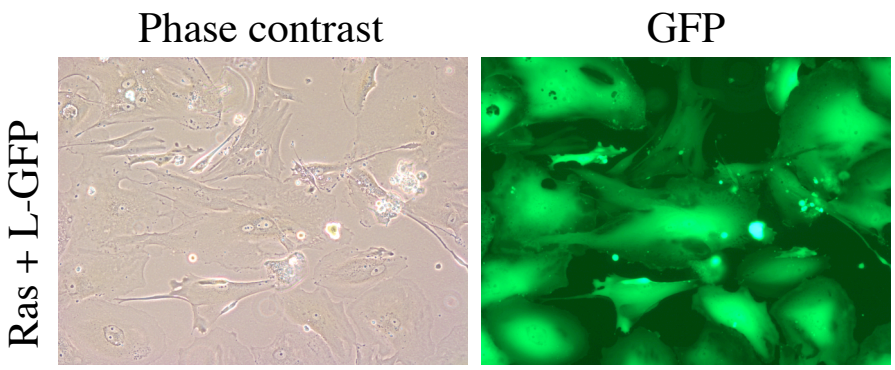

Quiescent
Serum 30h
Senescent
AdE1A 40h
AdE1A 50h

Figure 3.8

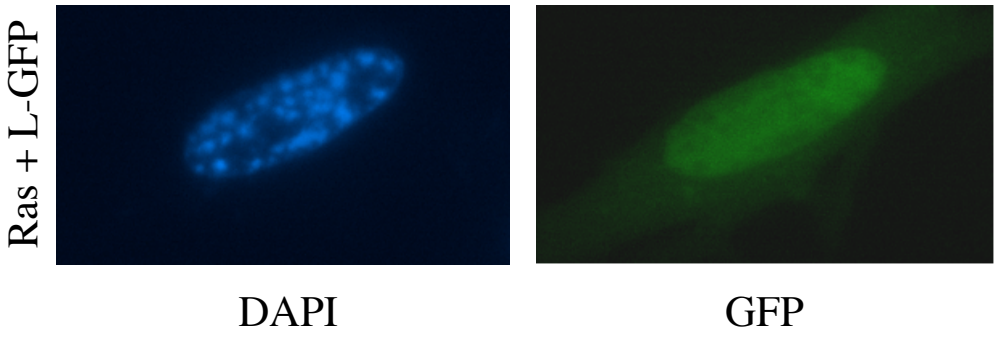
Infection of senescent cells with lentiviral E1A results in loss of SAHF, BrdU incorporation and death of senescent fibroblasts

(A)Phase contrast and GFP images for Ras senescent cells infected with lentiviral GFP indicate that the chosen lentiviral system allows for efficient infection of Ras-senescent IMR90. (B) Immunofluorescence images showing SAHF-positive Ras-senescent cells are efficiently infected with lentiviral GFP. (C) IMR90 cells made quiescent following infection with vector (V) or Ras-senescent cells (Ras) were infected with either lentiviral GFP or lentiviral E1A. 54 hours after the infections these cells along with uninfected controls were assayed by immunofluorescence for percentage of SAHF positive, Brdu positive, E1A positive cells. Cell death was quantified by trypan blue 70 and 96 hours after infection. Results are presented in a graph, as percentages.

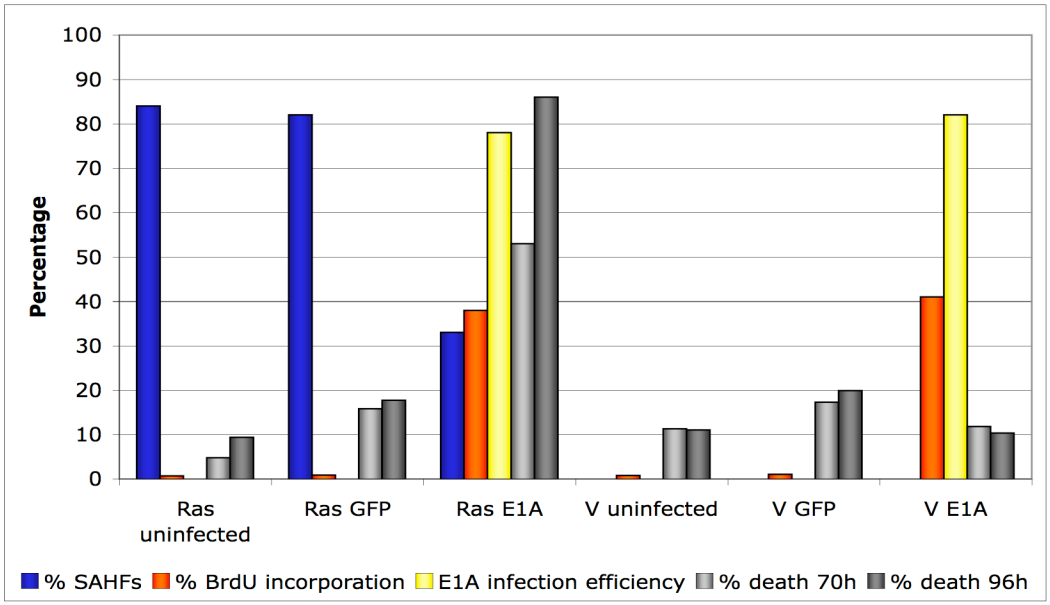
A


Phase contrast
GFP
Ras + L-GFP

B


Ras + L-GFP
DAPI
GFP

C


100
90
80
70
60
50
40
30
20
10
0
Percentage
Ras uninfected
Ras GFP
Ras E1A
V uninfected
V GFP
V E1A
% SAHFs
% BrdU incorporation
E1A infection efficiency
% death 70h
% death 96h

infection with lentiviral E1A were similar to those previously obtained with adenoviral E1A (not shown). Death was not observed in senescent cells infected with GFP control lentivirus or in quiescent cells infected with either control or E1A lentivirus (Figure 3.8). This result indicates that the death of senescent IMR90 cells results from the infection with E1A per se and not adenoviral toxicity.

Rb but not p16 or p53 knockdown results in loss of SAHF and BrdU expression in Ras-senescent fibroblasts.

E1A binds to and interferes with the function of a myriad of cellular proteins, including Rb (Whyte et al., 1988a), p107(Ewen et al., 1991) and p130 (Hannon et al., 1993; Mayol et al., 1993). A classical approach to identify the cellular function targeted by E1A in senescent cells would involve the use of E1A mutants that specifically disrupt known interactions. Since we had already established an essential role for Rb in SAHF formation we opted for a biased approached and designed lentiviral short hairpin RNAs targeting Rb (LshRb). Since p16 acts upstream of Rb in the formation of SAHF, we also made short hairpin RNAs to target p16 (Lshp16). A lentiviral short hairpin targeting p53 was also used (Lshp53, see Chapter 2). Figure 3.9 shows that these short hairpin RNAs efficiently donwregulate target gene expression following lentiviral transduction into Ras-senescent cells.

Ras-senescent cells infected with a control lentivirus (LmiR) or Lshp16 maintained SAHF and did not incorporate BrdU (Figure 3.10). Abrogation of p53 in Ras-

Figure 3.9

Lentiviral hairpin RNAs efficiently downregulate target gene expression in senescent cells

IMR90 fibroblasts were made senescent by infection with H-RasV12, either by itself (Ras) or combined with a short hairpin targeting p16 (shp16+Ras). Ras-senescent cells were infected with lentiviruses expressing short hairpins against p16 (Lshp16), Rb (LshRb and LshRb88) and p53 (Lshp53) as well as an empty vector control (LmiR). Shp16+Ras cells were either left uninfected or infected with L-shp53 . Extracts from these cells were analyzed by Western blotting using antibodies against p53, p16 and Rb. Actin served as a loading control. All lentiviral hairpins efficiently downregulate their target gene expression.

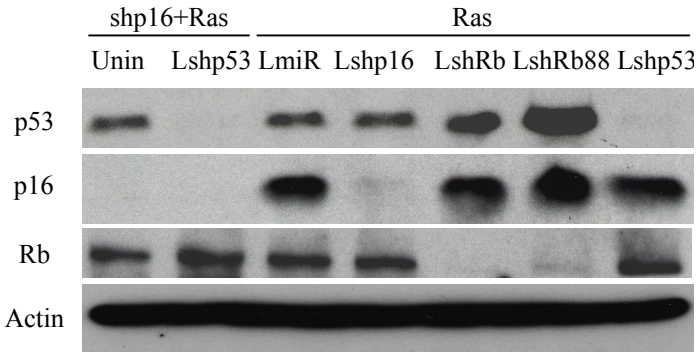
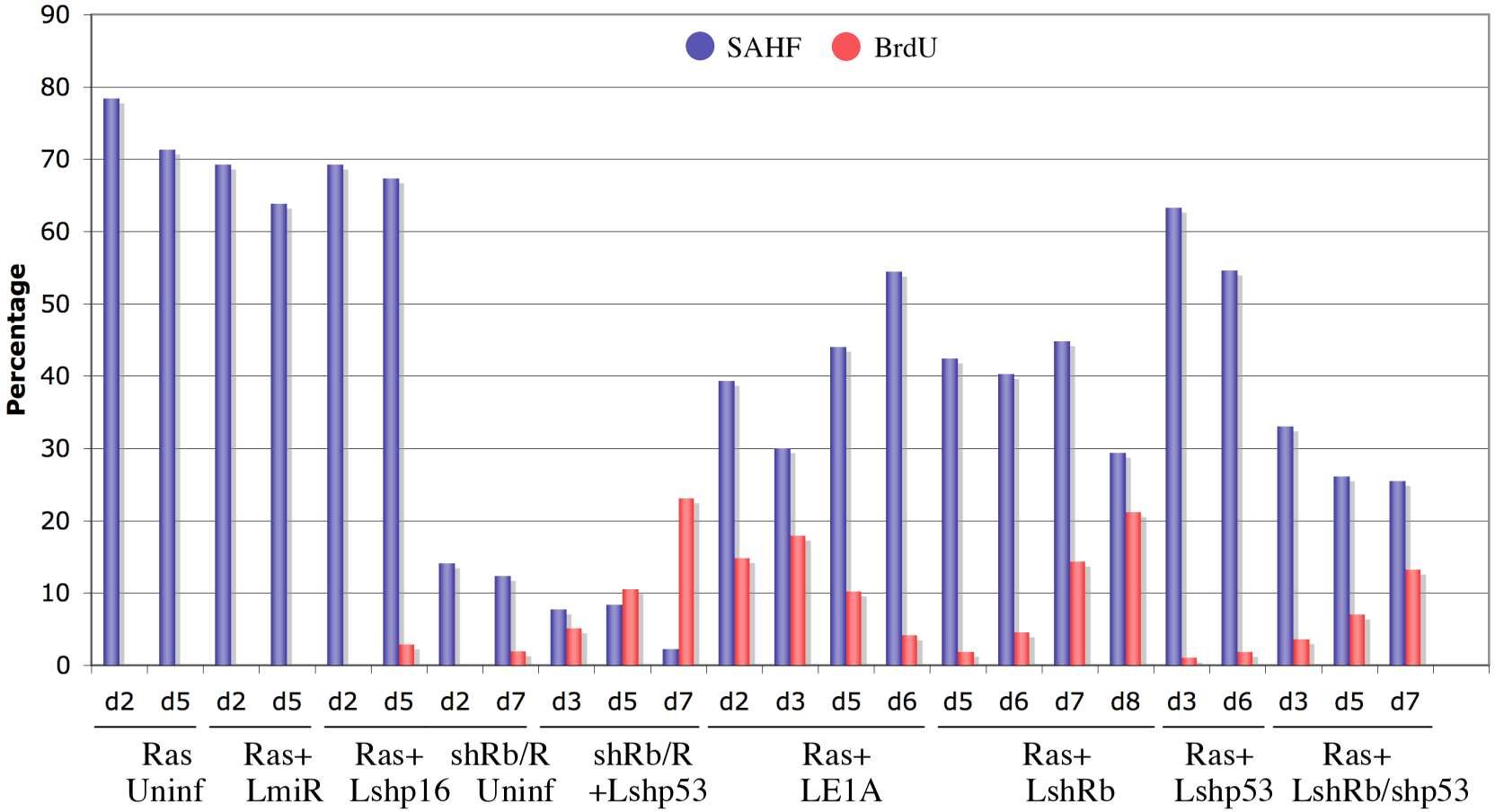

shp16+Ras
Ras
Unin
Lshp53
LmiR
Lshp16
LshRb
LshRb88
Lshp53
p53
p16
Rb
Actin

Figure 3.10

Rb but not p16 or p53 knockdown results in loss of SAHF and BrdU expression in Ras-senescent fibroblasts.

Quantitation of percentage of SAHF (blue bars) and BrdU incorporation (red bars) in senescent IMR90 cells at different time-point following lentiviral infection.

Treatments were as follow:
H-RasV12 senescent cells (Ras): uninfected (Uninf), infected with lentiviral E1A, infected with a lentivirus containing an empty short hairpin RNA cassete (LmiR), or lentiviruses expressing short hairpin RNAs targetting p16 (Lshp16), Rb (LshRb), p53 (Lshp53) or a conbination of LshRb and Lshp53.
H-RasV12 plus shpRb senescent cells (shRb/Ras):uninfected (Unif) or infected with Lshp53.


SAHF
BrdU
Percentage
90
80
70
60
50
40
30
20
10
0
d2 d5 d2 d5 d2 d5 d2 d7 d3 d5 d7 d2 d3 d5 d6 d5 d6 d7 d8 d3 d6 d3 d5 d7
Ras Uninf
Ras+ LmiR
Ras+ Lshp16
shRb/R Uninf
shRb/R +Lshp53
Ras+ LE1A
Ras+ LshRb
Ras+ Lshp53
Ras+ LshRb/shp53

senescent cells resulted in a slight reduction in SAHF levels but no significant change in BrdU incorporation. This same vector induced BrdU incorporation in SAHF-negative senescent BJ fibroblasts, as described in Chapter 2. As previously shown, lentiviral E1A resulted in rapid loss of SAHF and BrdU incorporation in Ras-senescent IMR90. In these cells the apparent increase in SAHF observed between days 3 and 5 post-infection reflects an increase in the ratio of uninfected to infected cells due to the preferential death of infected cells. Similar to the result observed with lentiviral E1A, infection of senescent cells with LshRb resulted in a reduction in levels of SAHF as well as BrdU incorporation (Figure 3.10). While the peak of the E1A effect is seen 2-3 days post infection, the LshRb effect peaks 7-9 days post-infection. This lag probably reflects the time necessary for the RNAi machinery to process short hairpin RNAs, as well as the rate of Rb protein turnover in senescent cells. This result identifies Rb as a cellular target of E1A in the context of SAHF-dissolution and indicates Rb is required for the maintenance of SAHF.

Since p53 inactivation can reverse senescence of SAHF-negative senescent cells (see chapter 2) and Rb is required for SAHF-formation, we speculated that the combined knockdown of Rb and p53 in senescent cells might allow them to resume growth. Concomitant downregulation of p53 and Rb resulted in a slightly higher reduction in SAHF levels than that observed by Rb knockdown alone. The levels of BrdU incorporation mirrored those seen in cells infected with LshRb alone, suggesting that there is no synergy between Rb and p53 downregulation in regards to the maintenance of SAHF and the cell cycle arrest. Senescent cells in which both Rb and p53 were knocked down also died, indicating that p53 downregulation is also unable to suppress the cell death.

Infection of senescent cells with E1A correlates with their loss of SAHF and death.

Following infection of senescent cells with E1A or LshRb, 98 to 100 percent of BrdU incorporating cells did not have SAHF (the numbers varied between experiments, the average value for all experiments is 99.2%). A representative image is shown in Figure 3.11A. This observation suggests that SAHF dissolution precedes S-phase re-entry, which is consistent with our model in which SAHF "lock in" the cell cycle arrest.

In general, dead cells detach from the plate or coverslip and are excluded from immunofluorescence studies. A fraction of the cells that died as a result of lentiviral E1A infection remained attached to the coverslip. Those cells always exhibit positive staining in immunofluorescence studies with antibodies directed against E1A (Figure 3.11B), thus correlating E1A expression with cell death.

Live microscopy visualization of SAHF dissolution and cell death in senescent IMR90 infected with lentiviral E1A.

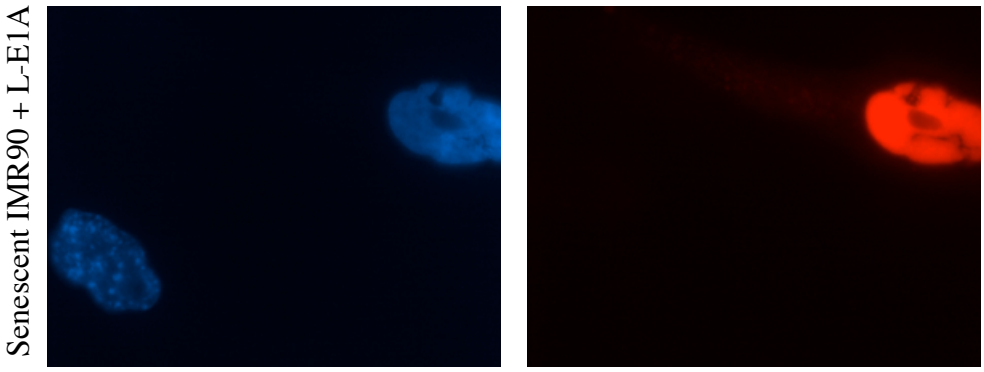
One unexplained feature of senescent fibroblasts is that only 80 to 90 percent of the population forms microscopically visible SAHF. It is formally possible that the cells that never form SAHF are the ones that incorporate BrdU and re-enter the cell cycle following infection with lentiviral E1A or shRb. Our data argues against that possibility, since the number of cells that incorporate BrdU is larger than the number of SAHF-negative cells (see for example Figure 3.8), which implies that, in the absence of cell

Figure 3.11

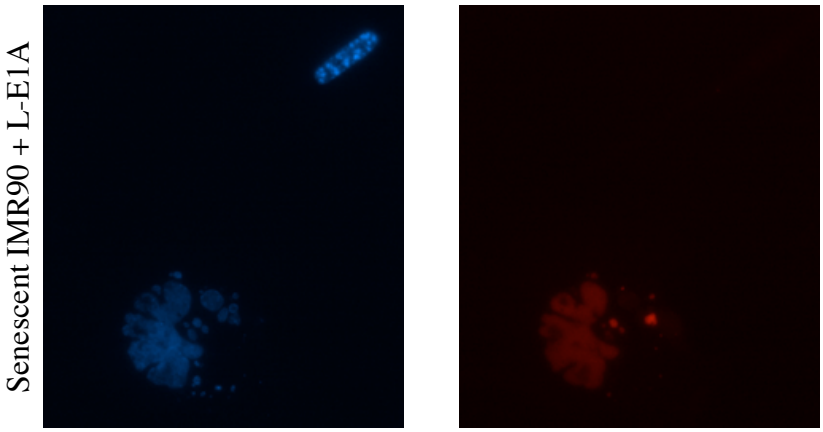
Infection of senescent cells with E1A correlates with their loss of SAHF and death

IMR90 replicative senescent cells were infected with lentiviral E1A and E1A expression was assayed by immunofluorescence. DNA was counterstained with DAPI. (A) The cells that lose SAHF exhibit E1A expression. A representative image is shown. (**B)** The apoptotic cells that remained attached to the coverslip and were analyzed by immunofluorescence as in (A) exhibit E1A expression. A representative image is shown.

A


Senescent IMR90 + L-E1A
DAPI
E1A

B


Senescent IMR90 + L-E1A
DAPI
E1A

division, SAHF-positive cells must lose SAHF and re-enter the cell cycle. Additionally, SAHF dissolution precedes BrdU incorporation (Figure 3.5). We therefore decided to utilize live microscopy to monitor the effect of E1A expression on SAHF at the single-cell level. The ultimate goal was to show that E1A does result in SAHF dissolution.

A histone H2B-YFP fusion protein was expressed along with H-RasV12 in IMR90 fibroblasts. The resulting senescent fibroblasts exhibited SAHF that could be visualized by YFP fluorescence. Initially, cells were imaged on plates for 24 hour periods. In cells imaged 48 to 72 hours post-infection with lentiviral E1A it was possible to see unraveling of SAHF (Figure 3.12). Imaging on an FSC2 perfusion camber allowed us to extend the imaging time to 48 hours. It was then possible to follow the complete unraveling of SAHF, followed by the death of the imaged cells (figure 3.13). Cells that were not infected with E1A did not lose SAHF during the timeframe of the experiment (Figure 3.14), indicating that loss of SAHF is not the result of photo-bleaching. No significant death was observed in uninfected cells (not shown), indicated that the death seen in cells that lost SAHF is not a product of the imaging procedure.

Ras-senescent IMR90 fibroblasts infected with lentiviral E1A and shRb have increased nuclease sensitivity compared to senescent cells infected with control lentiviruses.

Our results suggest that Rb maintains a form of facultative heterochromatin that is involved in the stable suppression of E2F-target genes in senescent fibroblasts.

Figure 3.12

Live microscopy visualization of SAHF unraveling in IMR90 cells infected with lentiviral E1A.

Ras-senescent IMR90 cells expressing an H2B-YFP fusion protein were infected with lentiviral E1A and imaged by live microscopy for a 24 hour period, starting 48 hours after the infection. Images were taken every 5 minutes. This particular cell moved out of the imaging field after 18 hours but displayed some unraveling of SAHF at the last time-point that it was imaged.

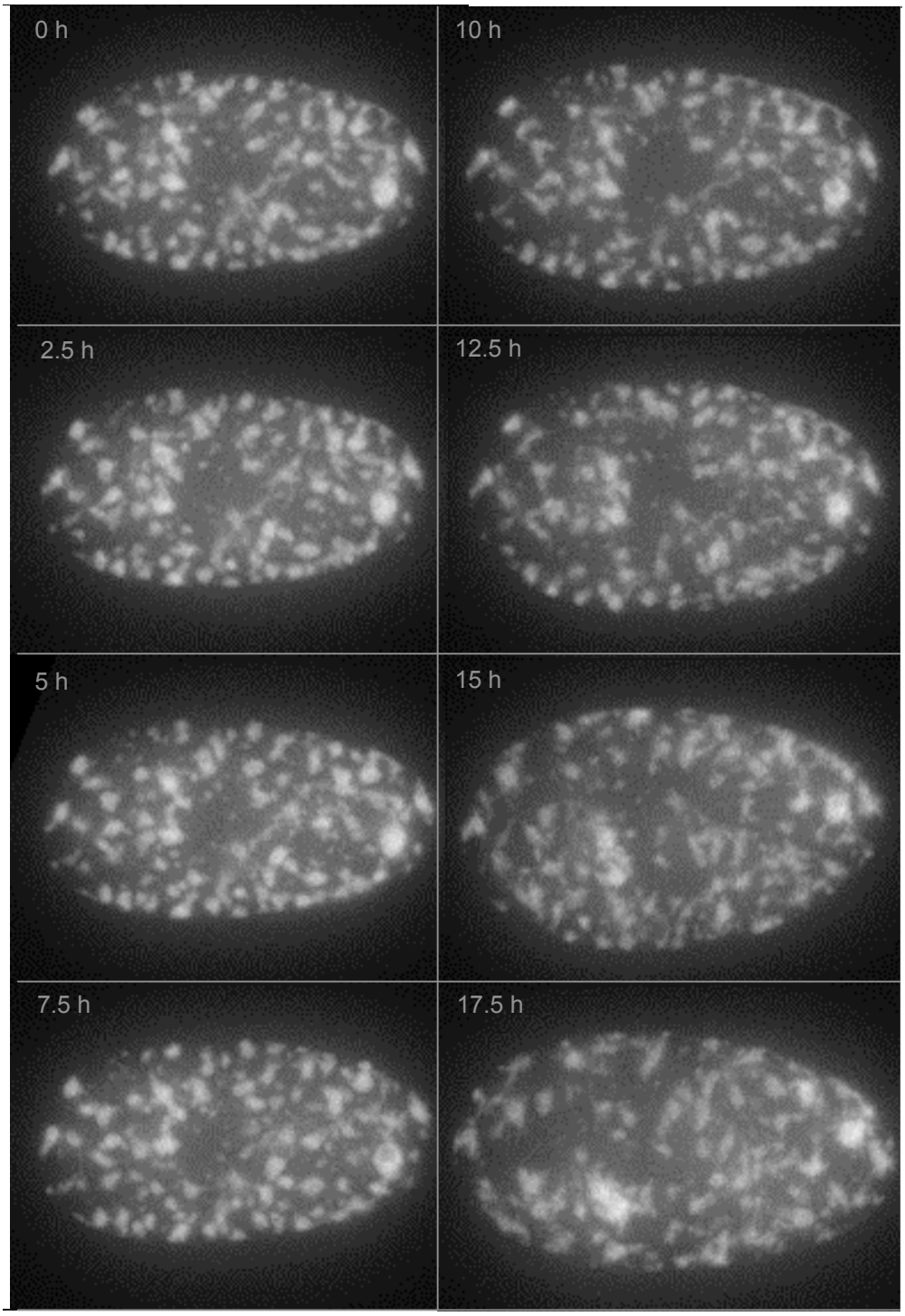

0 h
10 h
2.5 h
12.5 h
5 h
15 h
7.5 h
17.5 h

Figure 3.13

Live microscopy visualization of SAHF dissolution and cell death in senescent IMR90 infected with lentiviral E1A.

Ras-senescent IMR90 cells expressing an H2B-YFP fusion protein were infected with lentiviral E1A and imaged by live microscopy for a 48 hour period, starting 18 hours after the infection. Images were taken every 5 minutes and media perfusions were carried out every 4-6 hours. This particular cell showed significant SAHF dissolution 48 hours after the infection (30 hours into the imaging) and dies shortly after that time-point. The arrowhead points to what we believe is the inactive X chromosome, which appears to decondense.

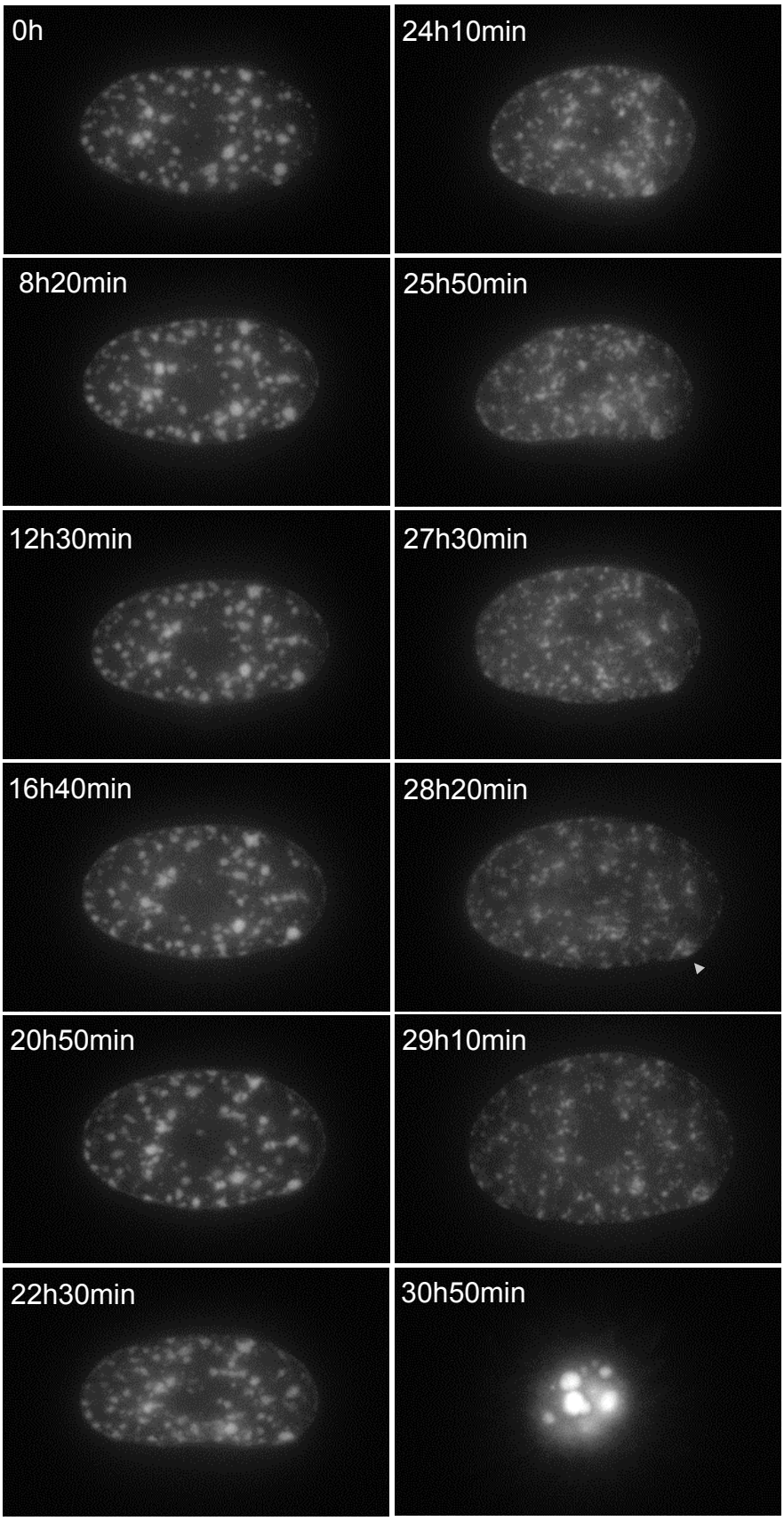
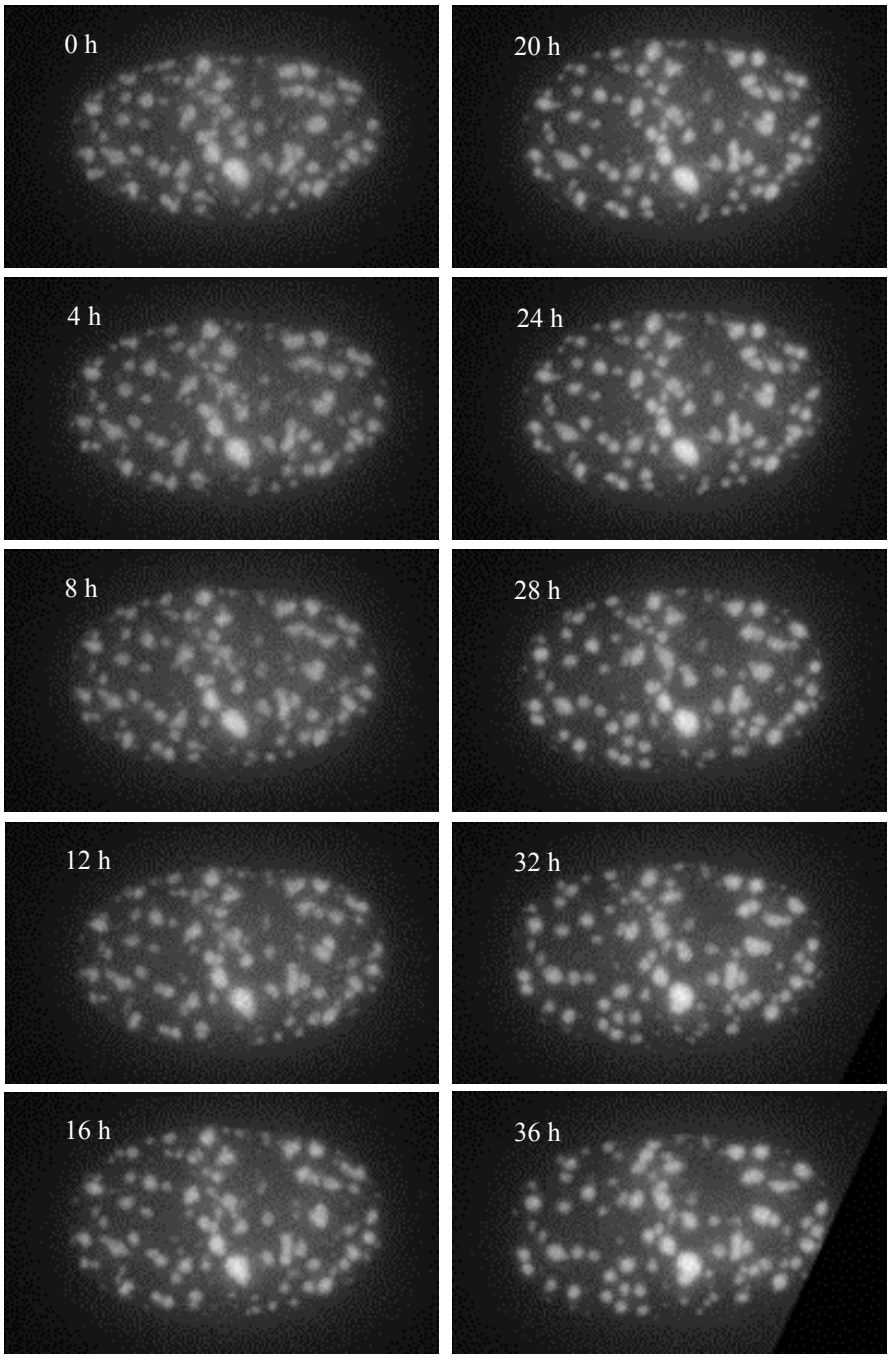

0h
24h10min
8h20min
25h50min
12h30min
27h30min
16h40min
28h20min
20h50min
29h10min
22h30min
30h50min

Figure 3.14

Uninfected senescent cells do not loose SAHF during the timeframe of the live imaging experiments.

Ras-senescent IMR90 cells expressing an H2B-YFP fusion protein were imaged by live microscopy for a 48 hour period. Images were taken every 5 minutes. No cells in this experiment exhibited signs of SAHF unraveling or dissolution. Images captured for a representative cell are shown.

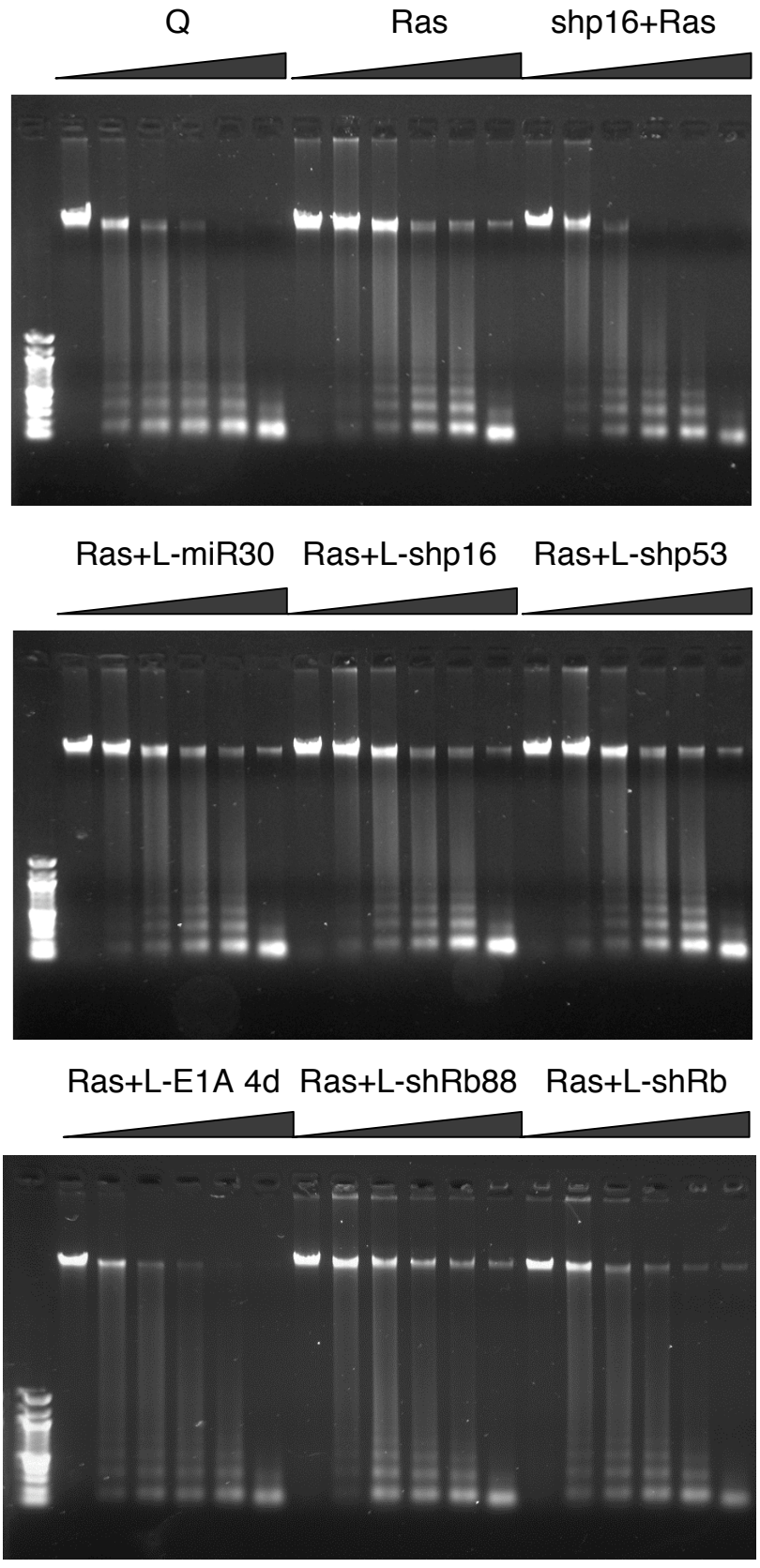

0 h
20 h
4 h
24 h
8 h
28 h
12 h
32 h
16 h
36 h

We previously showed that the chromatin of Ras-senescent IMR90 cells expressing shRb or shp16^{INK4a} displays greater nuclease sensitivity than that of cells made to senesce by Ras infection alone. This result supports our model in which senescence-specific chromatin changes directed by the p16/Rb pathway are responsible for the increased nuclease resistance observed in senescent cells. A prediction would therefore be that SAHF reversal by expression of L-E1A or LshRb would, like inhibition of SAHF formation, increase nuclease sensitivity as compared to the chromatin of Ras-senescent cells. Accordingly, the chromatin of Ras-senescent cells infected with lentiviral E1A or LshRb but not Lshp16, Lshp53 or LmiR, displays higher nuclease sensitivity than that of uninfected Ras-senescent cells (Figure 3.15). The effect observed due to Rb knockdown is more moderate than that that seen with E1A, but was reproduced in 4 independent experiments. Perhaps the reason for this difference is that E1A acutely inhibits Rb, resulting in a more concerted loss of SAHF. This allows for a window of time in which cell death can be prevented and a high proportion of the population has lost SAHF. Rb knockdown by means of a hairpin takes a longer time, since it is dependent on not only the RNAi machinery but also the turnover of Rb protein. Additionally, the level of knockdown is variable between cells, which might result in a less concerted dissolution of SAHF and subsequent death.

Figure 3.15

Ras-senescent IMR90 fibroblasts infected with lentiviral E1A and shRb have increased nuclease sensitivity compared to senescent cells infected with control lentiviruses

In vivo micrococcal nuclease digestion of detergent-permeabilized quiescent (Q), Ras-senescent (Ras), Ras-senescent cells expressing a short hairpin RNA targeting p16 (shp16+Ras), and Ras-senescent cells infected with lentiviral E1A (L-E1A) or the following lentiviral short hairpin RNAs: vector control (L-miR30), shp16, shp53, shRb88 and shRb. The assay was performed 8 days after lentiviral infection, except for cells infected with L-E1A, which were assayed 4 days post-infection. DNA was isolated from cells after digestion for the indicated time (min) and subjected to agarose gel electrophoresis.


Q
Ras
shp16+Ras
Ras+L-miR30
Ras+L-shp16
Ras+L-shp53
Ras+L-E1A 4d
Ras+L-shRb88
Ras+L-shRb

Upregulation of E2F-target genes in Ras-senescent cells infected with lentiviral E1A and shRb.

In the previous chapter we demonstrated a correlation between the presence of SAHF and the stable silencing of E2F-target genes. Senescent cells infected with lentiviral E1A and LshRb lose SAHF and exhibit BrdU incorporation, suggesting they have re-entered the cell cycle. It is therefore predicted that these cells will re-express E2F-target genes. We evaluated the levels of some E2F-target genes in Ras-senescent cells infected with lentiviral E1A or two different short hairpin RNAs targeting Rb. Five days following infection we observed increased levels of PCNA and mcm3 protein in these cells (Figure 3.16A). A similar upregulation was apparent in cells that senesced in the presence of shRb (Figure 3.16A, lanes 3 and 11) but not in uninfected cells or cells infected with an empty lentiviral vector, two different Lshp16 and L-shp53 (Figure 3.16A, lanes 1, 2, 4/9 and 8 respectively). Cyclin A, PCNA and Mcm4 mRNA levels also increased in senescent cells infected with LE1A or two different LshRb constructs but not in cells infected with the lentiviral control (Figure 3.16B).

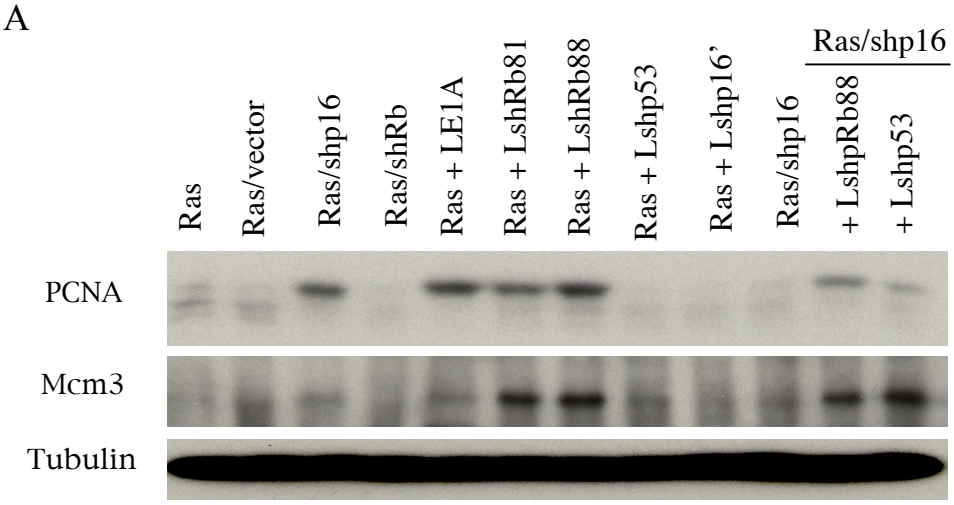
We then evaluated total protein levels 2 days after infection of Ras-senescent cells with E1A or 9 days after infection with the different lentiviral short hairpin RNAs. Z-vad was used to delay cell death. Senescent cells infected with E1A or three different short hairpins against Rb expressed high levels of PCNA and cyclin E protein (Figure 3.16C, lanes 9-12)). This upregulation was not seen in uninfected cells (lane 4), cells infected with the empty hairpin backbone (lane 7), Lshp16 (lane 8) or Lshp53 (lane 13).

Figure 3.16

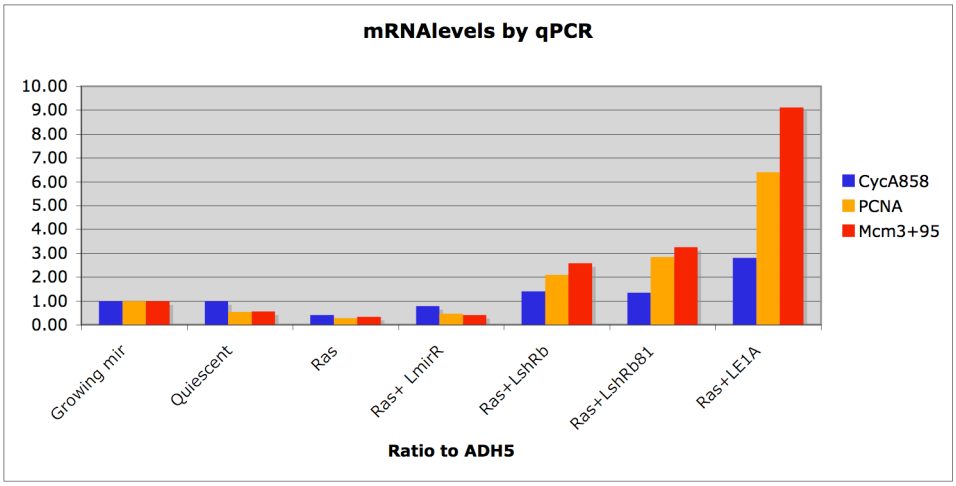
Upregulation of E2F-trget genes in ras-senescent cells infected with lentiviral E1A and shRb

A) Western blot analysis with antibodies against PCNA, MCM3 and tubulin The assay was done 5 days after lentiviral infection. (B) Quantitative PCR analysis of mRNA expression for cyclin A, PCNA and Mcm3 in growing, quiescent and Ras-senescent cells as well as Ras-senescent cells infected with LmiR, LshRb, LshRb81 or L-E1A. (C) Western blot analysis with antibodies against Rb, p53, p16, E1A, PCNA, cyclin E1 and actin.

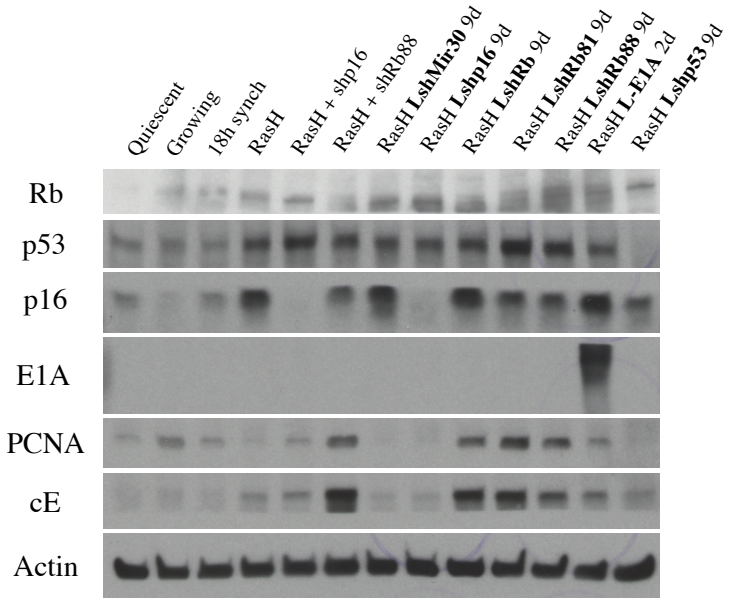
A


Ras
Ras/vector
Ras/shp16
Ras/shRb
Ras + LE1A
Ras + LshRb81
Ras + LshRb88
Ras + Lshp53
Ras + Lshp16'
Ras/shp16
Ras/shp16
+ LshpRb88
+ Lshp53
PCNA
Mcm3
Tubulin

B


mRNAlevels by qPCR
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
CycA858
PCNA
Mcm3+95
Growing mir
Quiescent
Ras
Ras+ LmirR
Ras+LshRb
Ras+LshRb81
Ras+LE1A
Ratio to ADH5

C


Quiescent
Growing
18h synch
RasH
RasH + shp16
RasH + shRb88
RasH LshMir30 9d
RasH Lshp16 9d
RasH LshRb 9d
RasH LshRb81 9d
RasH LshRb88 9d
RasH L-E1A 2d
RasH Lshp53 9d
Rb
p53
p16
E1A
PCNA
cE
Actin

We conclude that some E2F-targets are re-expressed in senescent cells as a result of infection with lentiviral E1A or LshRb. Rb is therefore required to maintain the suppression of E2F-target genes in senescent cells.

The chromatin-bound protein profile of senescent cells infected with lentiviral E1A and shRb reflect their loss of SAHF and cell cycle re-entry.

We have previously determined a signature profile of chromatin-bound protein that distinguishes growing and senescent IMR90 cells (Figure 2.12). Additionally, we have shown that the chromatin bound protein profile differs between SAHF-positive and SAHF-negative senescent cells (Figure 2.21). We therefore decided to assess the effect L-E1A and LshRb infection had on the chromatin-bound protein profile of senescent cells. Chromatin fractions were prepared from growing, quiescent, synchronized growing, shp16- and shRb-expressing Ras-senescent (which lack SAHF), and Ras-senescent cells either uninfected or infected with 6 different lentiviruses. Growing, quiescent and synchronized cells collected 18 hours after re-entering the cell cycle all showed high levels of chromatin-bound AcK9H3 and low levels of chromatin-bound HP1 γ (Figure 3.17A, lanes 1,2 and 3). In comparison, Ras-senescent cells exhibited lower levels of AcK9H3 and higher levels of bound HP1 γ (Figure 3.17A, compare lanes 2 and 4 and Figure 3.17B, compare lanes 1 and 4). Note that the baseline association of AcK9H3 to chromatin varies between experiments (compare AcK9H3 levels from figure 3.17A to 3.17B).

As shown in the previous chapter, cells that senesce in the presence of a short hairpin RNA targeting p16 have less HP1γ and more AcK9H3 associated with their chromatin than their Ras-senescent counterparts (Figure 3.17A, compare lanes 4 and 5). We were unable to see this change in the association of AcK9H3 and HP1γ in cells that senesced in the presence of a short hairpin RNA targeting Rb (Figure 3.17A, compare lanes 4 and 6 and figure 3.17B, compare lanes 3 and 4). However in these cells a number of E2F targets are in the chromatin-bound fraction (Figure 3.17B lane 3). Cyclin E cannot be detected on the chromatin of growing cells but accumulates on the chromatin of shRb expressing Ras-senescent cells (Figure 3.17A compare lanes 2 and 6; Figure 3.17B, compare lanes 1 and 3). In addition, we have recently performed microarray analysis and identified cyclin E as a gene whose downregulation during senescence depends on Rb (A.C and S.W.L, unpublished result). Consistent with this observation cyclin E increases its association with chromatin in all cells were Rb is knocked down, both before and after the establishment of senescence (Figure 3.17A lanes 6, 9, 10 and 11; figure 3.17B, lanes 3, 7, 8 and 9). E2F1 and Mcm3 are also present in the chromatin-bound fraction in shRb-expressing Ras-senescent cells (Figure 3.17B, lane 3).

As mentioned earlier, Rb increases its association with chromatin in senescent cells as compared to growing or quiescent cells (Figure 3.17B, compare lanes 1 and 2 to lane 4). As expected, Rb knockdown during the establishment of Ras-senescence abolishes this association (Figure 3.17B, lane 3). p53 is found in the chromatin fraction of senescent but not growing or quiescent cells (Figure 3.17B, compare lanes 1 and 2 to lane 4). Interestingly, the levels of p53 associated with chromatin increase in cells that senesce

in the presence of shRb (Figure 3.17B, compare lanes 3 and 4), which might reflect p53's role in the maintenance of senescence in those cells.

We observed a decreased association of Rb with the chromatin fraction in Ras-senescent cells infected with three different short hairpin RNAs targeting Rb (Figure 3.17B, lanes 7-9). These cells exhibit an increase association of cyclin E, E2F1 and mcm3 to the chromatin fraction (Figure 3.17A, lanes 8-10 and Figure 3.17B lanes 7-9), consistent with the upregulation of those E2F-target genes by both total mRNA and protein level (Figure 3.16). The level of chromatin-associated p53 also increases in these cells (Figure 3.17B, compare lanes 4 to lanes 7-9). As predicted, the levels of AcK9H3 increase in Ras-senescent cells infected with three different short hairpin RNAs targeting Rb, as compared to control Ras-senescent cells (Figure 3.17A, compare lanes 4 and 7 to lanes 9-11; Figure 3.17B, compare lane 4 to lanes 7-9). We also observed a decrease in the levels of HP1 γ in the chromatin fraction of senescent cells infected with one of the shRb lentiviral vectors (Figure 3.17A, lane 9; Figure 3.17B, lane 7) but not the other 2. The hairpin that results in a decrease in chromatin-bound HP1γ is the one that most efficiently knocks down Rb expression. Additional hairpins will need to be made to validate the result obtained with this one shRb. Additionally, the experiment is being repeated with chromatin fractions prepared at a later time-point following LshRb infection.

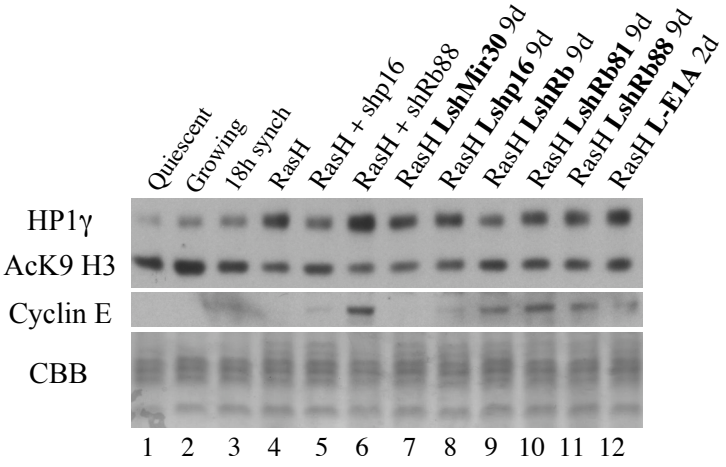
In Ras-senescent cells infected with lentiviral E1A, E1A can be found in the chromatin-bound fraction (Figure 3.17B, lane 6). Consistent with E1A's ability to bind and inactivate Rb, the association of Rb with chromatin is reduced in these cells as compared to Ras-senescent cells (Figure 3.17B, compare lanes 4 and 6). When assayed

Figure 3.17

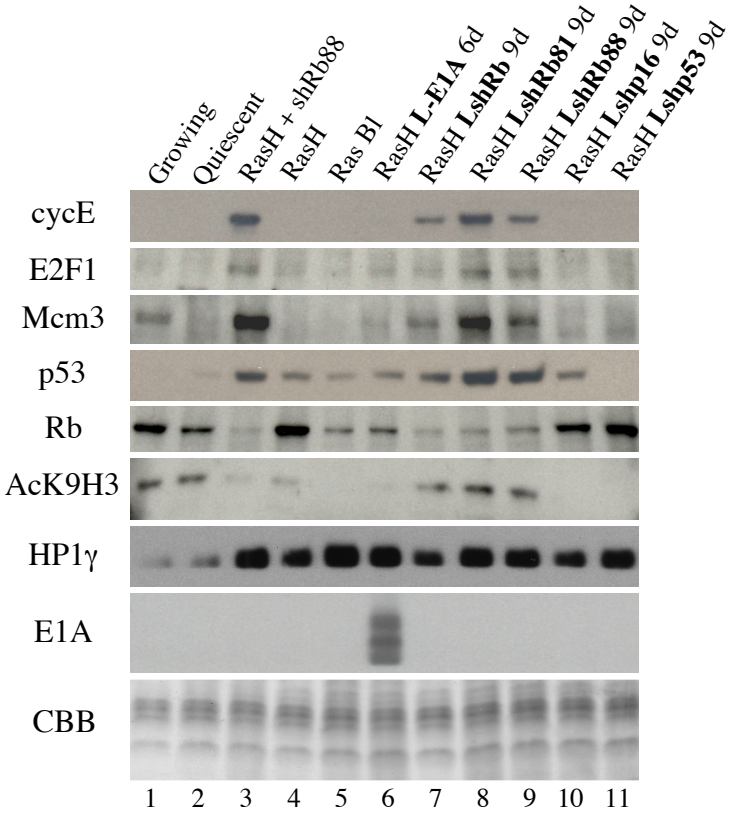
The chromatin bound protein profile of senescent cells infected with lentiviral E1A and shRb reflect the cells loss of SAHF and cell cycle re-entry

(A) The chromatin fraction from normal growing, quiescent, synchronized growing (18h synch), Ras-senescent, Ras-senescent plus shp16 or shRb and Ras-senescent cells infected with the indicated lentiviruses was resolved by SDS-PAGE followed by Coomassie brillian blue staining (CBB) to ensure equal loading of core histones (bottom panel). Proteins were transferred to an Immobilon-P membrane and immunoblotted with the indicated antibodies. (B) The chromatin fraction from normal growing, quiescent, Ras-senescent plus shRb88, Ras-senescent (RasH denotes the vector usually used while RasBl represents a vector that results in very low levels of Ras expression and has no relevance to the results presented) and Ras-senescent cells infected with the indicated lentiviruses was resolved by SDS-PAGE followed by Coomassie brillian blue staining (CBB) to ensure equal loading of core histones (bottom panel). Proteins were transferred to an Immobilon-P membrane and immunoblotted with the indicated antibodies.

A


Quiescent
Growing
18h synch
RasH
RasH + shp16
RasH + shRb88
RasH LshMir30 9d
RasH Lshp16 9d
RasH LshRb 9d
RasH LshRb81 9d
RasH LshRb88 9d
RasH L-E1A 2d
HP1γ
AcK9 H3
Cyclin E
CBB
1 2 3 4 5 6 7 8 9 10 11 12

B


Growing
Quiescent
RasH + shRb88
RasH
Ras Bl
RasH L-E1A 6d
RasH LshRb 9d
RasH LshRb81 9d
RasH LshRb88 9d
RasH Lshp16 9d
RasH Lshp53 9d
cycE
E2F1
Mcm3
p53
Rb
AcK9H3
HP1γ
E1A
CBB
1 2 3 4 5 6 7 8 9 10 11

shortly after lentiviral E1A infection, these cells exhibited a modest increase in AcK9H3 (Figure 3.17A, compare lanes 4 and 12). We were unable to detect a significant decrease in the association of HP1 γ with chromatin in these cells. These cells did however show a slight increase in chromatin bound E2F1 and Mcm3 compared to Ras-senescent cells (Figure 3.17B, compare lanes 4 and 6). Again, the assay is being repeated with chromatin fractions prepared at different time-points.

Ras-senescent cells infected with short hairpin RNAs targeting $p16^{INK4a}$ or p53 did not show any of the described changes in the profile of chromatin-bound proteins except for a slight increase in the levels of chromatin-bound p53 in cells infected with lentiviral shp16 (Figure 3.16B lanes 10 and 11). This may reflect an increase in the total protein level in these cells (Figure 3.16C).

Reduced tri-methylK9 H3 on the promoter of PCNA in senescent cells infected with lentiviral E1A

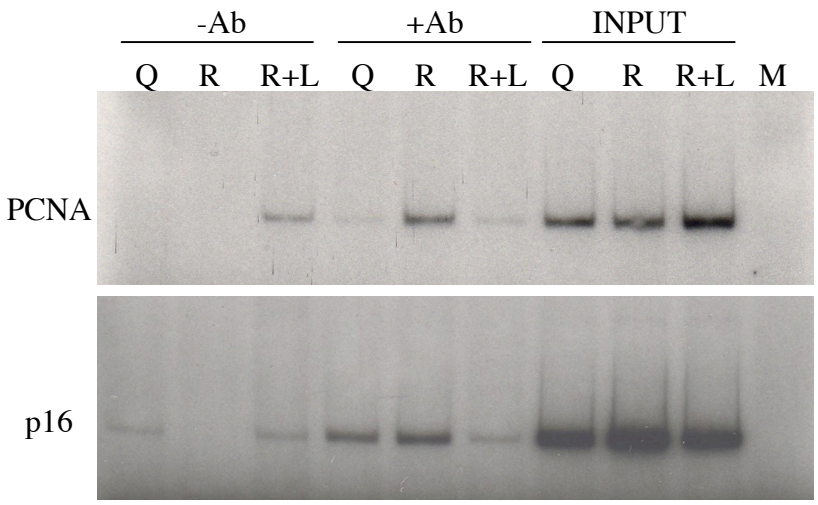
We have shown that TMK9H3 accumulates on the promoters of some E2F-targets in senescent but not growing or quiescent cells. Based on this result we would predict that such an association might be reduced or lost in senescent cells infected with lentiviral E1A. To test this we used a TMK9H3 antibody to perform chromatin immunoprecipitation analysis on quiescent, Ras-senescent and Ras-senescent cells infected with lentiviral E1A for 44 hours. Consistent with our previous results, histone 3 trimethylated on lysine 9 is found on the PCNA promoter in Ras-senescent but not

quiescent cells (Figure 3.18). Following infection of senescent cells with E1A much of the heterochromatic mark is lost from the PCNA promoter, suggesting that the removal of the TMK9 mark occurs prior to the re-expression of the E2F-target gene.

Figure 3.18

Reduced tri-methylK9 H3 on the promoter of PCNA in senescent cells infetcted with lentiviral E1A

Chromatin immunoprecipitation assay (ChIP) using an antibody against tri-methyl K9 histone H3, no antibody (- Ab) or nuclear extract (input). DNA fragments were amplified by PCR from the promoter regions of PCNA and p16INK4a. Buffer without nuclear extract served as Mock (M) control. Quiescent IMR90 cells (Q), Ras-senescent cells (R), and Ras-senescent cells 44 hours after infection with lentiviral E1A (R+L) were used.


-Ab
+Ab
INPUT
Q R R+L Q R R+L Q R R+L M
PCNA
p16

3.4 Discussion

Studies using viral oncoproteins E7 and Large T to inactivate Rb indicate that loss of Rb activity is not sufficient to reverse senescence in normal human fibroblasts (see chapter 1). The results presented in this chapter show that E1A is likewise insufficient to reverse the senescent arrest: while cells expressing E1A activate E2F-target genes and exhibit incorporation of BrdU, they do not appear to progress through the cell cycle and die before reaching M-phase. Quiescent cells successfully resume growth following E1A infection, suggesting that the death observed in senescent cells represents an additional barrier to reversal of senescence.

Despite the failure to induce cell cycle re-entry in senescent cells, E1A expression results in the loss of senescence-associated heterochromatic foci (SAHF). SAHF dissolution precedes the s-phase entry, consistent with the role of these structures in the stability of the arrest (see chapter 2).

As discussed in the introduction, E1A associates with a number of cellular proteins. It was therefore reasonable to assume that the loss of SAHF observed following infection with E1A was the result of the functional inhibition of one or more of the known E1A-interacting proteins. An interesting candidate was the retinoblastoma gene product (Rb), since our data indicates Rb is required for SAHF formation.

Through the use of a lentiviral short hairpin RNA targeting Rb (shRb) we show that Rb is a cellular function targeted by E1A, as downregulation of Rb is sufficient to reverse SAHF and allow cell cycle re-entry. This indicates that Rb is not only essential for the establishment of SAHF, but plays an equally important role in the maintenance of

these heterochromatic structures. Although this observation appeared surprising at first, emerging evidence has strengthened the correlation between Rb loss and global heterochromatic defects (see section 1.3.5). In our system Rb loss might affect other heterochromatic structures besides SAHF, and this possibility remains to be explored. Interestingly, we have observed some decondensation of the inactive X in cells monitored by live microscopy (Figure 3.13).

We previously proposed that during the initial phases of senescence Rb might control the nucleation of heterochromatin at specific sites throughout the genome, which then spreads by the action of histone methyltransferases and recruitment of HP1 proteins (see Chapter 2). Based on the results presented in this chapter we propose that, as proteins are turned over in senescent cells, Rb continues to recruit chromatin remodeling complexes to specific sites throughout the genome, thus ensuring the maintenance of SAHF and the cell cycle arrest.

The death of senescent cells following infection with lentiviral E1A or LshRb is delayed but not prevented by Bcl2, E1B19K and pan-caspase inhibitors. This suggests it is not a purely apoptotic form of cell death. The results from Philip Hinds' lab presented in the introduction to this chapter (Alexander et al., 2003; Tiemann and Hinds, 1998) suggest that the death that ensues following Rb inactivation in senescent Saos-2 cells can be completely blocked by Bcl2 or EIB19K. They also attribute a role for p73 in the induction of cell death in this setting. We have been unable to detect p73 upregulation following infection of Ras-senescent IMR90 cells with lentiviral E1A or LshRb. It is possible that such an upregulation is the result of a compensation mechanism resulting from the lack of p53 in Saos-2 cells. In our system we do see upregulation of p53, but

concomitant downregulation of p53 and Rb by short hairpin RNAs is not sufficient to block cell death. The differences between our results and those obtained by Hinds' group most likely reside in the choice of experimental system. While we use a primary cell line (IMR90 fibroblasts) Hinds and coworkers used the Saos-2 cell line. Saos-2 cells are a non-tumorigenic osteosarcoma cell line, which present a modal number of 56 chromosomes per cell, with over two-thirds of the chromosomes presenting complex rearrangements. The cells are both Rb and p53 null. It is therefore unclear whether the program executed in this cell line as a result of Rb re-expression is indeed senescence. Our results suggest that in primary human cells there is an additional barrier to the reversal of senescence that we are unable to overcome.

The fact that senescent cells die soon after being infected with lentiviral E1A or LshRb makes studying SAHF reversal by "bulk assays" like nuclease sensitivity of chromatin-bound protein analysis very difficult. The data from Alexander and co-workers suggests that progression through S-phase triggers the death of Saos2 cells following Rb inactivation (Alexander et al., 2003). The use of roscovitine (a kinase inhibitor that arrests cells in G1) or hydroxyurea (HU, that arrests cells in early S-phase) substantially delayed cell death in their system. Our data indicates that SAHF dissolution precedes BrdU incorporation in senescent cells infected with L-E1A or LshRb. Blocking cell cycle progression might prevent cell death but allow for the reversal of SAHF, thus facilitating "bulk assays".

Although most known E2F-regulated genes are characterized as functioning in cell cycle control and/or DNA replication, E2F also controls genes that are directly involved in cell-fate decisions such as apoptosis, differentiation, and early development

(Muller et al., 2001). Notably, cytochrome-C-binding protein APAF1, and the effector caspases 3 and 7 are direct targets of the E2F transcription factors (Muller et al., 2001). If E1A expression or Rb knockdown concomitantly de-repress all E2F targets in senescent cell, induction of apoptosis would be expected to accompany unscheduled S-phase entry. Indeed E2F1 can drive a variety of quiescent cells into S-phase, but overexpression of E2F-1 in normal cells induces both p53-dependent and p53-independent cell death (Dyson, 1998; Helin, 1998).

It is also possible that discordant senescent and proliferative signals are responsible for the observed death. While the execution of senescence happens in a carefully concerted manner, the reversal of the arrest results from the abrupt inhibition of a single cellular function, which in turn, disrupts the maintenance of the process in an unconcerted manner. Additionally, DNA damage foci are present in both replicative and premature senescent cells (see section 1.2.3); suggesting that this persistent DNA damage signals might trigger cell death upon cell cycle re-entry.

The observation that acute Rb loss can fully reverse senescence in replicative and H-RasV12 senescent MEFs (Sage et al., 2003) pinpoints an important difference between mouse and human fibroblasts. Cultured MEFs appear to senesce due to the accumulation of damaged DNA that results from culturing in 20% oxygen (Parrinello et al., 2003), and the DNA damage is sensed by a signaling pathway involving p53 (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007). It has been shown that in MEFs the p53-mediated senescent arrest requires a certain level of pocket proteins bound to E2F (Rowland et al., 2002). Acute loss of Rb might relieve a p53-imposed block in senescent MEFs, enabling them to continue proliferating with damaged DNA. We have shown that

p53 plays a dominant role in the maintenance of senescence established in the absence of p16 or Rb and, as discussed in chapter 2, p16INK4a is dispensable for senescence in MEFs (Lowe and Sherr, 2003). Consistent with this observation, SAHF formation is less pronounced in these cells, and p53 inactivation is sufficient to reverse the senescent arrest (Dirac and Bernards, 2003). Placing acute inactivation of Rb within the p53 pathway appears to reconcile all the available data, explaining why inactivation of Rb and p53 are both capable of reversing senescence in MEFs.

A recent study has indicated that the ability of E1A to rescue Ras-induced senescence relies on the inactivation of both p300/CBP and Rb family proteins (Deng et al., 2005). Interference with the p16(INK4A)/Rb pathway or inactivation of p300/CBP complemented the Rb-binding defective and the p300/CBP-binding defective mutants of E1A, respectively, to rescue premature senescence. The authors show that both p300 and CBP are required for the induction of p53 following Ras infection, suggesting that p300 and CBP might be transcriptional coactivators for p53 in the senescence pathway, and that inactivation of p300/CBP by either E1A or siRNA might contribute to senescence bypass by preventing p53 activation. Like Rb, CBP and p300 are critical for normal embryonic development, as mice completely lacking either CBP or p300 protein die at an early embryonic stage (Tanaka et al., 2000; Yao et al., 1998).

p300/CBP function primarily as transcription co-factors for a number of nuclear proteins. p300 and CBP function as histone acetyltransferases (HATs) and appear to couple transcription factor recognition to chromatin remodelling [reviewed by (Iyer et al., 2004)]. p300 and CBP are also capable of acetylating a number of non-histone proteins, including p53, p73, Rb, E2F, myb, myoD, HMG(I)Y, GATA1 and alpha-importin.

Acetylation of sequence-specific DNA-binding nuclear proteins such as p53, p73, Rb and E2F occurs on lysine residues adjacent to DNA-binding domains and augments promoter binding, resulting in activation of transcriptional activity. p300 and CBP have been implicated in embryonic development and cancer and are considered putative tumor suppressor genes. It would be interesting to evaluate the roles of p300 and CBP in the maintenance of the senescent arrest.

Other cellular functions certainly cooperate with Rb to maintain SAHF in senescent cells, and it is possible that combined inactivation of such functions together with Rb might allow the cells to resume growth. An RNAi based screen for genes that cooperate with Rb to reverse cellular senescence would most certainly be interesting.

Our findings identify Rb as being required for both the formation and the maintenance of SAHF in senescent human fibroblasts. Our data also suggests that Rb not only maintains SAHF but also maintains the heterochromatic marks associated with senescence. This is quite a departure for Rb's canonical role as a cell cycle regulator, and it suggests how Rb might function as a tumor suppressor. The data in this chapter provide the first mechanistic insights into the maintenance of senescence, which is essential for the tumor suppressor function of this cellular program.

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Chapter 4:

Concluding remarks and future directions

The body of work presented in this dissertation represents significant progress in our understanding of cellular senescence. We identified a distinct chromatin structure enriched with heterochromatin proteins that accumulates in senescent IMR90 human fibroblasts. We showed that heterochromatin-associated proteins and the Rb tumor suppressor can accumulate on the E2F-responsive promoters in senescent but not quiescent cells, and that these changes are associated with more stable repression of E2F responsive genes. Notably, Rb is required for SAHF formation and E2F target gene silencing. This observation led us to suggest that Rb-directed changes in heterochromatin organization contribute to senescence-associated changes in gene expression and the permanence of the senescent state. Lending further support to this model is our observation that p53 inactivation is sufficient to reverse senescence in cells lacking the p16/Rb-dependent chromatin changes.

We then extended our studies to the mechanism of maintenance of cellular senescence and identified Rb as being required for the maintenance of the senescent-specific chromatin changes. While Rb was generally considered a canonical cell cycle regulator our results show that it directs the formation of facultative heterochromatin in senescent cells and also maintain it. Since the heterochromatic changes regulated by Rb correlate with the stability of the senescent arrest, these remarkable findings broaden our understanding of how Rb might function as a tumor suppressor.

Whether SAHFs always occur in response to senescence and what the detailed mechanisms are that enable their formation are questions that remain to be answered. Despite this, SAHFs constitute a morphological feature of the nuclei of senescent cells that has been successfully exploited to identify the occurrence of oncogene-induced senescence (OIS) in vivo (Braig et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005). Significantly, as demonstrated in Chapter 2, SAHF represent a marker of senescence that can be predictive of the stability of the arrest.

The molecular pathways underlying SAHF formation remain to be elucidated. We have identified the p16INK4a/Rb pathway as being essential for SAHF formation (Narita et al., 2003). The non-histone chromatin proteins HMGA1 and 2 appear to act in a mutually reinforcing manner with p16 to promote SAHF formation. HMG proteins are structural components of SAHFs and contribute to the stable repression of proliferation-associated genes (Narita et al., 2006). The chromatin regulator ASF1 is also required for SAHF formation (Zhang et al., 2005). The ability of ASF1 to induce SAHF depends on its interaction with histone H3 and the histone chaperone HIRA (Zhang et al., 2007). We have established a correlation between the appearance of heterochromatic marks on E2F-targets and the appearance of SAHF, and have provided substantial evidence that E2F-targets are incorporated into SAHF in senescent IMR90 cells. It is unclear what other genomic regions might be incorporated into SAHF during the establishment of senescence.

Our characterization of SAHF is currently limited by our incomplete understanding of mechanisms of gene regulation. The nucleus is a dynamic entity and chromatin components (as well as individual DNA elements) are constantly interacting

and resolving (reviewed by (Fisher and Merkenschlager, 2002). We are still unable to integrate the various epigenetic correlates of gene activity—such as DNA methylation, replication timing and histone modifications —into a mechanistic explanation for the establishment and propagation of transcriptional states. If causal links between these epigenetic phenomena can be established, it might become possible to define changes in genome activity that ultimately determine cell fate. Our understanding of how chromatin changes in senescence are established and maintained, as well as their correlation to cellular process essential for the execution of this tumor suppressor pathway, will probably evolve as the chromatin field evolves. Interestingly, the heterochromatic changes associated with senescence might provide a good system for researchers in the chromatin field to study formation and maintenance of facultative heterochromatin.

In our limited evaluation of the histone modifications present in SAHF we have noted that histone H3 trimethylated at lysine 9 and histone H3 trimethylated at lysine 27 occupy distinct regions. While H3 trimethylated at lysine 9 is found at the core of the foci, histone H3 trimethylated at lysine 27 appears to occupy the outer layer (MN, unpublished observations). As predicted, HP1 appears to associate with the region occupied by H3 trimethylated at lysine 9. Interestingly it has been reported that the human inactive X chromosome (Xi) is packaged into at least two non-overlapping heterochromatin types, each characterized by specific Xi features: one is defined by the presence of Xi-specific RNA transcript, the histone variant macroH2A, and histone H3 trimethylated at lysine 27; the other defined by H3 trimethylated at lysine 9, heterochromatin protein 1, and histone H4 trimethylated at lysine 20 (Chadwick and Willard, 2004). Furthermore, regions of the Xi packaged in different heterochromatin

types are characterized by different patterns of replication in late S-phase. The authors suggest that this arrangement of facultative heterochromatin into spatially and temporally distinct domains might have implications for both the establishment and maintenance of the Xi. This previously unsuspected degree of epigenetic complexity might hold true for SAHF as well, where all these modifications have been reported. Indeed this study points out that elsewhere throughout the genome, other regions of H3TrimK27 immunostaining appear distinct from that of H3TrimK9. The two types of Xi heterochromatin are temporally and spatially distinct, with only H3TrimK9-modified heterochromatin showing the classic feature of replication in very late S-phase. H3TrimK9 and H3TrimK27 also occupy distinct territories at interphase, indicating that packaging of the Xi into the Barr body follows a prescribed pattern of folding that maintains the spatial distinction of the two types of heterochromatin apparent at metaphase. Further studies on the distribution of histone variants and chromatin-binding proteins within SAHF will be needed. Such a parallel between SAHF and the inactive X would be even more interesting if indeed Rb knockdown affects both regions of facultative heterochromatin (see discussion in Chapter 3).

Since we do not know what regions of the genome are contained within SAHF we cannot evaluate whether all the genomic regions that are incorporated into SAHF contribute to senescence or if their incorporation is just a "side effect" of the nucleation mechanism employed by the cells. There is one report in the literature that indicates that genes upregulated during senescence exhibit significant clustering (Zhang et al., 2003). Perhaps genes that need to be expressed during senescence localize to discrete regions of the genome that are somehow protected, and heterochromatin extends throughout the rest

of the genome. Two different groups have reported that each focus within SAHF represents a single chromosome (Funayama et al., 2006; Zhang et al., 2007). This idea is contradicted by 3D renderings of images obtained by confocal microscopy, which indicate that the number of foci in senescent cells is quite variable (S.N. andM.N., unpublished observation). This would suggest a somewhat random condensation of genomic regions into a variable number of heterochromatic foci. It is possible that large regions of the genome, close to the size of a complete chromosome, are incorporated into a single SAHF. Our data suggests that limited nuclease digestion of quiescent and senescent cells followed by microarray or sequencing analysis might allow for the identification of the genomic regions contained within SAHF.

Ultimately, all these *in vitro* studies on cellular senescence aim to characterize a process that inhibits tumor progression and modulates therapeutic response *in vivo*. As mentioned in the introductory chapter, senescence has been detected *in vivo*. The ultimate fate of these senescent cells is still unclear: while some senescent cells are swiftly eliminated from the organism (Ventura et al., 2007; Xue et al., 2007), others appear to persist (e.g. senescent melanocytes within nevi). For the later class of cells, it is important to understand whether senescence could be reversed, leading to tumor progression.

Mouse models could prove to be invaluable for *in vivo* studies of senescence maintenance. However, the differences between senescence in mouse and human fibroblast are discouraging. As mentioned previously, p16 plays an important role in SAHF formation and senescence in human fibroblasts but appears dispensable for senescence in MEF. Fortunately, the failure to upregulate p16 during senescence is not a general feature of mouse cells. On the contrary, most mouse cell types appear able to

induce p16 during senescence (see for example (Schmitt et al., 2002), (Tremain et al., 2000), (Lazzerini Denchi et al., 2005)). Moreover, p16 upregulation has been detected in vivo, correlating with the presence of SAHF (Collado et al., 2005; Lazzerini Denchi et al., 2005). A first step would involve the validation in mouse cells of the data obtained in human fibroblasts.

Work from the lab of Judith Campisi indicates that the senescence of MEFs cultured in 20% oxygen is the result of oxidative DNA damage (Parrinello et al., 2003). In their study MEFs did not senesce when cultured in physiological (3%) oxygen levels, consistent with their expression of telomerase. Studies done in MEFs expressing a LacZ mutation reporter confirmed this result and documented the impact of oxidative stress on the genomic integrity of murine cells during senescence and immortalization (Busuttil et al., 2003). These observations indicate that senescence studies performed in MEFs cultured under physiological oxygen conditions might yield similar results to those in human fibroblasts.

Understanding the mechanism underlying senescence requires that we understand the correlation between the initiating signals, the specific changes that that they direct on chromatin and the appearance of SAHF as macroscopic structures. At present we only hold a few pieces to this puzzle. The answers provided by our work raise a lot of questions. As scientists, we have to believe that is a good thing.

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Chapter 5:

Materials and methods

Cells and culture conditions

Human diploid IMR90 fibroblasts were purchased from ATCC (catalog #CCL-186) at population doubling 24. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Gibco cat#11995) supplemented with 10% FBS and 1% Penicillin/ Streptomycin sulfate with a subcultivation ratio of 1:4 every 3-4 days. The cells have been reported to be capable of attaining around 60 population doublings before the onset of senescence, which is equivalent to 30 passages under the culturing conditions used in these studies. In our hands the cells ceased proliferation at around passage 33. For all experiments involving retroviral mediated gene transfer the cells were used until passage 19 to ensure optimal growth and therefore optimal infection efficiency.

The BJ human diploid foreskin fibroblasts were purchased from ATCC (catalog # CCL-2522). Cells were provided at population doubling 19 and senesced around passage 42. The BJ cell line has a long lifespan in comparison with other normal human fibroblast cell lines. Although they are considered normal, these cells have a reported normal diploid karyotype at population doubling 61 but an abnormal karyotype at population doubling 82 (see discussion in text). BJ fibroblast were maintained in Minimum Essential Medium with Earle's salts (MEM) supplemented with non-essential amino acids (NEAA), 10% fetal bovine serum (FBS) and 1% Penicillin/ Streptomycin sulfate at a subcultivation ratio of 1:4 every 3 days.

The Phoenix amphotrophic cells used for packaging retroviruses were obtained from the lab of Dr. G. Nolan (Stanford University, CA). The 293T cells used for lentiviral production were a gift from Dr. G. Hannon. Both cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM, Gibco cat#11995) supplemented with 10% FBS and 1% Penicillin/ Streptomycin sulfate with a subcultivation ratio of 1:6 every 3-4 days.

All cells were cultured at 37°C in the presence of 5% CO_2.

Retroviral Vectors

The following retroviral vectors were used in this study: pWZL Hygro (H-*Ras*V12, $p16^{INK4a,}$ *HMGA2*) (Narita et al., 2006; Serrano et al., 1997), pLPC-Puro (EGFP-tagged human *HP1β* cDNA and *E1A*) (Narita et al., 2003; Samuelson and Lowe, 1997), pWZL-Blasticidin (H-*Ras*V12), pBabe-Puro (*hBmi1, MuthBmi1,* $p16^{INK4a}$) (Jacobs et al., 1999), pSin-Puro (Rb and *p16INK4a* shRNA) (Narita et al., 2003), pSin-Puro-miR30 (*INK4a and Rb* shRNAs) (Narita et al., 2006) and pCLNC (H2B-EYFP) (Kanda et al., 1998).

The target sequences in *INK4a*, *Rb* shRNAs are:

shRb: TGCAGCAGTTGACCTAGATGAGATGTC
shp16: TGCCCAACGCACCGAATAGTTACGGTC

Retroviral infection

Phoenix ampho cells ($4x10^6$) were seeded in a 10 cm dish 14-16 hrs before transfection. Transfection of 15 μg of a retroviral plasmid was done by calcium-phosphate precipitation using 2X BES transfection buffer (50 mM BES, 280 mM NaCl, 1.5 mM Na_2hPO_4, pH 6.95). 5 μg

were used in the case of EGFP-HP1 and H2B-YFP fusion constructs. The media of transfected cells was supplemented with 25 μM Cloroquine (acts to inhibit lysosomal DNases by neutralizing vesicle pH since DNA delivered by Ca_2PO_4 trasnsfection is thought to transit through lysosomes). 12-14 hours after transfection fresh media was added to the plates (10 ml of media per 10 cm plate), and 36 hr after transfection the virus-containing medium was collected, filtered (0.45 μm filter, Millipore) and supplemented with 4 μg/ml polybrene (believed to up-regulate receptor expression). Fresh media was added to each phoenix plate to allow for a second collection of viral supernatants. Target fibroblasts were plated at 5×10^6 cells per 10 cm dish 12-16 hrs before the first infection and incubated overnight at 37°C. For infections, the culture medium of the target cells was replaced by 2.5 ml of viral supernatant plus 2.5 ml of media with polybrene , and incubated at 37°C for 6 hr. If cells were being infected with 2 different viruses 2.5 ml of each viral supernatant was used for a total of 5 ml per 10 cm plate. 6 hr later the infection process was repeated. Media was changed on the recipient cells 6-8 hrs after infection and 24 later the cells were trypsinized and replated on selection media (100 μg/ml HygromycinB for 3 days (Roche Cat# 10843555001), 2 μg/ml Puromycin for 2 days (Sigma P-7255, Blasticidin 10μg/ml for 6 days (Invitrogen cat#46-1120)).

Adenoviral infections

Adenoviruses were introduced into quiescent and senescent IMR90 cells at a multiplicity of infection (MOI) of 30 or 60 plaque-forming units (PFU) $cell^{-1}$. Infection was allowed to proceed for 2 hours in 2.5 ml of media per 10 cm plate with regular rocking to prevent the cells

from drying out. 7.5 ml of media were subsequently added to each plate. Media was changed 24 hours later.

Lentiviral vectors and lentiviral infections

We used a third-generation lentiviral system from the Naldini lab (Dull et al., 1998). The Lentiviral GFP, LgtK1 and GSE22 has been described elsewhere (Beausejour et al., 2003; Dull et al., 1998). The lentiviral E1A was generated by subcloning Ad5 E1A 12s from the LPC-E1A retrovirus in place of GFP, using the BamHI and SalI sites. The following shRNAs were cloned into pRRL.Sin-18 lentiviral vector: *p16INK4a, p16INK4a –ex1, Rb, Rb-81, Rb-88 and p53* shRNA and the empty miR30 cassette. The p16 and Rb shRNAs correspond to the old hairpin design and are driven by the U6 promoter. They were cloned into the lentiviral vector in place of CMV-GFP. All other hairpins are expressed in the context of the endogenous miR30 micro RNA and were cloned downstream of the CMV promoter (which drives their expression). GFP was not removed from these vectors, and as a result, infected cells exhibit low GFP expression. The target sequences of the shRNAs are:

Rb: TGCAGCAGTTGACCTAGATGAGATGTC
p16INK4a: TGCCCAACGCACCGAATAGTTACGGTC
p16INK4a-ex1:CACGCACCGAATAGTTACGGTC
p53:CGGAGGATTTCATCTCTTGTAT
Rb81 CGGAAAGGACATGTGAACTTAT
Rb88 CGCAGTTCGATATCTACTGAAA

$0.5x10^6$ 293T cells were transfected with 10.0 μg of the indicated lentiviral DNA along with 6.5 μg of pMDLg/p RRE, 3.5 μg of pMD.G and 2.5 μg of pRSV.Rev. Calcium

phosphate transfection was done as indicated for retroviral infections. 12-14 hours after transfection fresh media was added to the plates and virus was collected 24 and 48 hours later. The 2 collections were pooled, filtered (0.45 μm filter, Millipore) and snap frozen in small aliquots. In order to infect cells lentivirus was diluted with culture media (200 μl /ml of lentivirus) and supplemented with 4 μg/ml polybrene. 4 ml of the diluted virus were used to infect $3.5x10^5$ post-selection 7 Ras-senescent cells. Infection was allowed to proceed for 14-16 hours before media change. In the experiments were Z-Vad was used to delay cell death Z-Vad was added to the media (20 μM). Media was changed every 3 days, until the collection of cells at the indicated time-points.

Micrococcal Nuclease Assay

Cells were washed with permeabilization buffer 1 (150mM sucrose, 80mM KCl, 35mM HEPES pH 7.4, 5mM K_2HPO_4, 5mM Mg_2Cl, 0.5mM $CaCl_2$) and permeabilized with 0.01% L-α-Lysophosphatidylcholine (Sigma L-4129) for 90 seconds. The L-α-Lysophosphatidylcholine solution was aspirated and cells were treated with 0.8 ml of 2U/ml micrococcal nuclease (Sigma N-5386) in permeabilization solution 2 (20mM sucrose, 50mM Tris pH 7.5, 50mM NaCl, 2mM $CaCl_2$) for the indicated time. Nuclease digestion was stopped by addition of 1 ml stop solution (20mM Tris pH 8.0, 20mM NaCl, 20mM EDTA, 1.5% SDS, 0.6 mg/ml proteinase K, 10 μg/ml RNase A) and samples were incubated at 37˚C overnight. DNA was recovered by phenol/chlorophorm extraction and resuspended in 10mM Tris, 0.1mM EDTA. The samples were electrophoresed in 0.8% agarose gel and photographed under UV light.

Southern Blot on MNase assays

DNA was transferred into a Hybond-N nylon membranes (Amersham pharmacia biotech) Probes specific for PCNA or stromelysin-1 promoter regions were generated by PCR. 30-40 ng of each oligos DNA was ^{32}P-labeled using the Magaprime DNA labeling system (Amersham pharmacia biotech) and labeled probes were cleaned with Quick Spin columns (Roche). Membranes were incubated at 65˚C for 3 hours in 10 ml Robbins hybridization buffer (250mM Na2HPO4 pH 7.4, 1mM EDTA, 7% SDS) before addition of 5,000,000 cpm of boiled probe. Hybridization was carried out overnight at 65˚C. Membranes were washed twice in 1X SSC, 0.1% SDS and twice in 0.25X SSC, 0.1% SDS. Each wash was done for 20 minutes at 65˚C. Membranes were exposed to Fuji BAS1000 phospho screens for 20 hours and read with Fujifilm image reader V 1.4E. Quantitation was done with Image Gauge V 3.0.

DNaseI sensitivity assay

Cells were washed with permeabilization buffer 1 (150mM sucrose, 80mM KCl, 35mM HEPES pH 7.4, 5mM K_2HPO_4, 5mM Mg_2Cl, 0.5mM $CaCl_2$) and permeabilized with 0.01% L-α-Lysophosphatidylcholine (Sigma L-4129) for 90 seconds. The L-α-Lysophosphatidylcholine solution was aspirated and cells were treated with 0.8 ml of 100U/ml DNAseI in permeabilization solution 2 (20mM sucrose, 50mM Tris pH 7.5, 50mM NaCl, 2mM $CaCl_2$) for the indicated time. Nuclease digestion was stopped by addition of 1 ml stop solution (20mM Tris pH 8.0, 20mM NaCl, 20mM EDTA, 1.5% SDS, 0.6 mg/ml proteinase K, 10 ug/ml RNase A) and samples were incubated at 37˚C overnight. DNA was recovered by phenol/chlorophorm

extraction and resuspended in 10mM Tris, 0.1mM EDTA. The samples were analyzed by quantitative PCR

Cell proliferation assay

IMR90 cells were plated on gelatin coated coverslips or 6-well plates and subsequently labeled with 5-Bromo-2'-deoxyuridine (BrdU, 100 mg/ml, Sigma) and 5-fluor-2'-deoxyuridine (FdU, 10 mg/ml, Sigma) for 6 hrs. Nuclei incorporating BrdU were visualized by immunolabeling. Cells were fixed in 4% formaldehyde made fresh from paraformaldehyde followed by permeabilization in 0.2% Triton X-100/ 0.5% normal goat serum/ PBS. DNA was denatured by incubation in 2N HCl/ 0.5% goat serum for 5 min at room temperature. The HCl was removed and the cells were incubated in 0.1M Sodium Borate pH 8.5 for 5 min. After 2 washes with PBS the cells were incubated with anti-BrdU antibody (Pharmingen, 1:400 in PBS/ 0.1% Tween20/ 0.5% goat serum) for 1 hr at room temperature. The cells were washed 3 times in PBS/ 0.1%Tween20 and incubated with 1:1000 dilution of Alexa Fluor Conjugates (Molecular Probes) in PBS/ 0.1% Tween20/ 0.5% goat serum containing 1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI). Following three washes with PBS/ 0.1%Tween20, the coverslips were mounted on slides using Vectashield mounting media (Vector Labs Cat# H-1000) and images were obtained using a Zeiss fluorescent microscope.

SA-β-gal assays

SA-β-gal activity was detected as previously described (Dimri et al., 1995) with slight modifications. Cells were washed once with PBS (pH 7.2), fixed with 0.5% glutaraldehyde (PBS [pH 7.2]), and washed in PBS (pH 7.2) supplemented with 1 mM MgCl2. Cells were stained in X-gal solution (1 mg/ml X-gal [Boehringer], 0.12 mM K3Fe[CN]6, 0.12 mM K4Fe[CN]6, 1 mM MgCl2 in PBS at pH 6.0) 4-8 at 37°C for 4-8 hours. In some cases DNA was visualized by DAPI (1 ug/ml) after 5 min permeabilization with 0.2% Triton X-100/PBS.

Cell viability

Adherent and non-adherent cells were collected and pooled. Cells were spun for 5 min at 1500 rpm, 4°C. The supernatant was aspirated leaving about 100 μl behind to resuspend the cell pellet. Samples were set on ice. 10 μl of cell suspension were mixed with 10 μl of trypan blue and the ratio of blue (dead) to total cells was determined by counting of at least 200 cells in a hemocytometer.

Immunolabeling

Cells were plated on gelatin coated coverslips and fixed 12-16 hours later by a 15 minute incubation in 4% formaldehyde made fresh from paraformaldehye. Cells were then permeabilized in 0.2% Triton X-100/0.5% normal goat serum/PBS and subsequently incubated for 1 hr at room temperature with the indicated primary antibody. Alexa Fluor Conjugates

(Molecular Probes) were used as the secondary antibodies (1:1000), and DNA was visualized by using 4',6-diamidino-2-phenylindole (DAPI) (1 μg/ml).

The following primary antibodies were used in immunofluorescence studies: anti-HP1a antibody (Chemicon), anti-HP1b antibody (Chemicon), anti-HP1g antibody (Chemicon), anti-K9,14Ac-H3 antibody (Upstate), anti-K9M-H3 antibody (provided by C.D. Allis), anti-TMK9 (Upstate), anti-K4M-H3 antibody (Upstate), anti-Rb antibody (G3-245, Pharmingen), anti-p107 antibody (Santa Cruz), anti-p130 antibody (Santa Cruz) anti-tubulin (Sigma) and anti-E1A (Santa Cruz). Where indicates, cells were pre-extracted in the CSK buffer with 0.3mg/ml digitonin (Sigma) before fixation for HP1γ staining.

Chromatin Immunoprecipitation assay

Chromatin immunoprecipitations were performed as described previously (Nahle et al., 2002) using anti-K9/14Ac-H3 (Upstate), K9M-H3 (ab7312, Abcam, Upstate, or provided by C.D. Allis), anti-TMK9 antibody (from our antibody facility, unpublished reagent), anti-TMK4 antibody (Upstate), HP1g (Chemicon), Rb (C-15, Santa Cruz), p107 (C-18, Santa Cruz), p130 (C-20, Santa Cruz) antibodies. DNA released from precipitated complexes was amplified using sequence specific primers by semi-quantitative PCR or Q-PCR. For semi-quantitative PCR products were labeled by [a-^{32}P]dCTP (Amersham Pharmacia) and separated on a 4% nondenaturing polyacrylamide gel. The primer sets used were: promoter regions of *cyclin A*, *PCNA*, *p16INK4a*, *stromelysin-1*, and *b-actin*. For Q-PCR reactions were carried out in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) on an IQ5 machine (BioRad). Relative levels were calculated as percent of input signal.

Western blotting

Western blotting analysis was carried out as previously described (Serrano et al., 1997). Briefly, cells were washed, collected by trypsinization, spun down and lysed in laemmli buffer. Samples corresponding to 15 μg of protein (BIO–RAD protein assay) were separated on 10% or 15% SDS–PAGE gels or 4-15% gradient gels (BioRad) and transferred to Immobilon-P membranes (Millipore). Western blot analysis was accomplished according to standard procedures using ECL detection (Amersham). Horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse antibodies (Amersham) were used as secondary antibodies.

Blots were probed with the following primary antibodies: anti- p16INK4a antibody (Santa Cruz, H156)), anti-Ras antibody (Oncogene, OP23), anti-Rb antibody (G3-245, Pharmingen) together with XZ-55 hybridoma supernatant, anti-E2F1 antibody (KH95, Santa Cruz), anti-E2F2 antibody (Santa Cruz, C20), anti-E2F3 antibody (Santa Cruz, C18), anti-cyclin A antibody (Sigma C19), anti-cyclin E1 antibody (Abcam ab3927), anti-PCNA antibody (Sigma, P8825) anti-MCM3 antibody (provided by B. Stillman), anti-MCM4 antibody (provided by B. Stillman), anti-cdc6 antibody (provided by B. Stillman), anti-E1A antibody (M73 hybridoma supernatant or Santa Cruz sc430), anti-p53 (DO1, Oncogene), anti-HMGA2 (1:1000, provided by V. Giancotti), anti-HP1α (Chemicon), anti-HP1β (Chemicon), anti-HP1γ (Chemicon), anti-histone H3 acetylated on Lys9/14 (Upstate 06-599), anti-histone H3 tri-methylated on Lys9 antibody (Upstate 07-523 and CSHL monoclonal facility), anti-histone H3 di-methylated on Lys9 (Upstate), anti-histone H3 di-/tri-methylated on Lys27 (provided by D. Reinberg), anti-tubulin (B-5-1-2, Sigma), and anti-actin antibody (ac-15, Sigma).

Real time Q-PCR

Total RNA was isolated by RNeasy Mini Kit (QIAGEN Inc.) and was converted to cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems). Gene–specific TaqMan primer sets were designed using Primer Express 1.5. Real time PCR was carried out in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7700 Sequence Detector (Applied Biosystems) and b-actin serves as an endogenous normalization control. Sequence Detector software (version 1.7) was utilized for data analysis and relative fold induction was determined by the comparative threshold cycle method.

Chromatin Isolation

Chromatin was isolated as described previously with minor modifications(Mendez and Stillman, 2000). Cells were resuspended in 5 volumes of buffer A (10 mM HEPES, [pH 7.9], 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride and Complete EDTA-free protease inhibitor cocktail [Roche; 92833700]). Digitonin (Sigma, 0.3 mg/ml) was added, and the cells were incubated for 10 min on ice. Nuclei were collected by low-speed centrifugation (4 min, 1,300 × g, 4°C) and washed once in buffer A. Nuclei were lysed in 7 volumes of buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitors as described above). Insoluble chromatin was collected by centrifugation (4 min, 1,700×g, 4°C), washed several times in buffer B, and centrifuged again under the same conditions. The final chromatin pellet was resuspended in DNaseI solution

(20mM Tris pH7.5, 7mM $MgCl_2$, 1/7 volume of 10U/μl DNaseI) and incubated on ice for 1 hr. 5X Laemmli buffer was added to the samples prior to subjecting them to Western blotting or Coomassie blue staining after SDS-PAGE. The final concentration of the samples was about $2x10^4$ cells/μl.

Live imaging

IMR90 cells were infected with retroviral H-Ras-V12 and H2B-YFP. The cells were selected in Hygromycin B for 4 days and 3 days later were seeded onto CellTak (BD Biosciences) treated 4cm round coverslips. 24 h later cells were infected with lentiviral E1A (300 μl unconcentrated stock with 1.2 ml of media). The coverslip was mounted on a FCS2 live-cell chamber (Bioptechs), which was mounted onto the stage of an LSM 510 microscope (Carl Zeiss MicroImaging, Inc.) and maintained at 37°C. For time-lapse observations of SAHF, Z-stacks (2-μm optical sections) were acquired at 6X every 5 minutes for 48 h. The chamber was perfused with fresh media every 6 hours. It was necessary to manually re-centered and re-focused every 30 minutes due to the excessive movement of fibroblast.

A projection of the z-stacks was obtained from the original dv File using the Softworx software. The projection was saved either as individual tiff image files or as a movie.

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