

Science is built up of facts, as a house is built of stones; but an accumulation of facts is no more a science than a heap of stones is a house.

Henri Poincaré

UNIVERSITY OF CALGARY

Genetic and Epigenetic Events Regulating Cellular Senescence and Aging

by

Philip Berardi

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Abstract

In the early 1960's, Hayflick demonstrated that primary human cells in culture show a progressive and spontaneous decline in growth rate eventually terminating in a quiescent, but viable state termed replicative senescence. It was later reported that the number of divisions a cell can undergo before it senesces is inversely proportional to donor age. This suggested a linear relationship between the senescence of individual cells and the aging of organisms. The objective of this doctoral research was to investigate the molecular mechanisms underlying the growth arrest and differential gene expression seen during cellular senescence.

Initially we identified novel cis-acting transcription regulatory elements in genes known to be differentially expressed during cellular senescence. A fifteen base pair cyclin D1 inhibitory element (DIE) was defined and shown to be responsible for the increased expression of cyclin D1 in senescent cells. Elements with sequence identity to the DIE were found in the promoters of other genes known to be activated during cellular senescence. The approximate molecular mass of the DIE-binding protein was determined by gel electrophoresis to be between 20-45 kDa.

Differential gene expression during cell senescence was further examined by characterizing expression of the splicing isoforms of the inhibitor of growth-1. The shifted focus of this investigation was aimed at elucidating epigenetic modifications associated with cell senescence and the role that the ING1 gene played in the formation of senescence associated heterochromatic foci (SAHF). The differential splicing of ING1, resulting in changing ratios of the two predominant ING1 species during cellular senescence, was implicated in altering chromatin structure. Furthermore, we showed that ING1a overexpression induced

formation of SAHF-like foci, a G1-phase cell cycle arrest and a senescence-like phenotype, while overexpression of the ING1b isoform induced apoptosis.

Taken together, this data reinforces the idea that differential gene expression contributes to enforcing the senescent phenotype. It also indicates that epigenetic modification of the human genome by chromatin remodeling during cellular aging may be responsible for altered accessibility of transcription factors, thus leading to the differential expression of growth regulatory genes. This is believed to contribute to and culminate in permanent growth arrest and provides evidence for an additional mechanism by which human primary cells induce and reinforce a program of gene expression specific to senescent cells.

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LIST OF ABBREVIATIONS

Units of Measure

bp	base pair
Ci	Curie
cpm	counts per minute
Da	Dalton
g	gram
hr	hour
kb	kilobase
kbp	kilobase pair
kDa	kiloDalton
M	molar
mg	milligram
min	minutes
ml	milliliter
mM	millimolar
ng	nanogram
pg	picogram
rpm	revolutions per minute
sec	seconds
V	volts
x g	times gravitational force
μ Ci	microCurie
μ g	microgram
μ l	microliter
μ M	micromolar
$^{\circ}$ C	degrees Celsius

Nucleic Acids

ADP	adenosine diphosphate
ATP	adenosine triphosphate
cDNA	complimentary deoxyribonucleic acid
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
mRNA	messenger ribonucleic acid
oligo(dT)	oligo-deoxythymidillic acid
poly(dI-dC)	poly(deoxyinosinic-deoxycytidylic) acid
RNA	ribonucleic acid

tRNA transfer ribonucleic acid

Techniques

CAT assay Chloramphenicol acetyl transferase assay
EMSA electrophoretic mobility shift assay
FACS fluorescence activated cell sorting
IP immunoprecipitation
Luc assay Luciferase assay
PCR polymerase chain reaction
RT-PCR reverse transcriptase polymerase chain reaction
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
TUNEL Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
WB western blot

Chemical Compounds/Buffers/Solutions

BrdU bromodeoxyuridine
DAPI 4',6-diamidino-2-phenylindole
DMEM Dulbecco's Modified Eagle's Medium
DTT dithiothreitol
EDTA ethylenediamine-tetraacetic acid
EtBr ethidium bromide
FBS fetal bovine serum
PBS phosphate buffered saline
PI propidium iodide
RIPA buffer radioimmunoprecipitation assay buffer
SDS sodium dodecyl sulphate
TAE tris-acetate EDTA buffer
TBE tris-borate EDTA buffer
TE tris-EDTA buffer
Tris Tris(hydroxymethyl)aminomethane

Transcription Factors/DNA Elements

AP1 Activator Protein 1
CRE Cyclic AMP Response Element
DIE Cyclin D1 Inhibitory Element
DP1 Deleted in Polyposis 1
E2F1 Transcription Factor E2F1
E-box Insulin Responsive Region
Egr-1 Immediate-Early Growth Response-1
NF- κ B Nuclear Factor-Kappa B
Oct1 Octamer-like Transcription Factor
Sp1 Promoter Specific Transcription Factor

TRE TPA Response Element
WT1 Wilms' Tumour-1

Cell Division Cycle Related

Arf Alternate Reading Frame
Cdc Cell Division Cycle
Cdk Cyclin-Dependent Kinase
CIP1 Cdk-Interacting Protein-1
Cyc Cyclin
G0 Gap 0 (Quiescence)
G1 Gap 1
G2 Gap 2
INK4a Inhibitor of Kinase 4a
KIP1 Cyclin-dependent Kinase Inhibitor Protein
M Mitosis
M1 Mortality Stage 1
M2 Mortality Stage 2
PCNA Proliferating Cell Nuclear Antigen
R Restriction Point
Rb Retinoblastoma
S DNA Synthesis
Waf1 Wild-type p53 Activated Fragment 1

General

Amp^r Ampicillin Resistance
ATCC American Tissue Culture Collection
BSA Bovine Serum Albumin
E. coli Escherichia coli
ECM Extracellular Matrix
FITC Fluorescein Isothiocyanate
GAPDH Glyceraldehyde Phosphate Dehydrogenase
HAT Histone Acetyltransferase
HDAC Histone Deacetylase
HDF Human Diploid Fibroblast
HPV Human Papillomavirus
Ig Immunoglobulin
IR Ionizing Radiation
LB Luria-Bertani Bacterial medium
MDM2 Mouse Double Minute-2
MPD Mean Population Doubling
Mu Mutant
PAI-1 Plasminogen-Activator Inhibitor Type-1
PARP1 Poly (ADP-Ribose) Polymerase 1
PIKK Phosphatidylinositol 3-Kinase (PIK)-related protein Kinase

PVDF	Polyvinylidene Difluoride
SV40	Simian Virus 40
SV40-LT	Simian Virus 40 Large T-Antigen
Tet ^r	Tetracyclin Resistance
UV	Ultraviolet
WT	Wild Type
5'-UTR	5'-Untranslated Region

Chapter 1
INTRODUCTION

Chapter 1: Introduction

1.1 Organismal Aging

Aging is amongst the most universal of biological phenomena known to man. Not surprisingly, academics from a variety of disciplines have studied this process extensively. In humans, aging is defined as a process of gradual and spontaneous change, resulting in maturation through childhood, puberty, and young adulthood and then progressing to decline through middle and late age (Beers, 2004). From a broader perspective, aging can be viewed as the progressive and deleterious biological changes that occur over time in nearly all multi-cellular organisms (metazoans). Ultimately, aging is a normal part of the life-cycle and is the process of growing old as a consequence of cumulative changes in an organism leading to a decrease in functional capacity.

1.1.1 Definition of Aging and Senescence

In order to understand the biological aging process it is necessary to emphasize the importance of contemplating aging in the context of the entire organism. Whole organism *aging* is believed to be a spontaneous, universal and progressive process that occurs intrinsically and has deleterious effects ultimately resulting in death (Strehler, 1977). An important characteristic of the aging process that is at least partially delineated within this dissertation is that both *intrinsic* and *extrinsic* factors concomitantly influence the 'rate' of aging and the probability of death.

At the cellular level, *senescence* is the process by which the capacity for cell division, growth and function is lost or changed through *in vitro* serial cultivation, ultimately decreasing the fitness of cells within their respective environments (Beers, 2004). Therefore, from a hypothetical cellular perspective the process of senescence contributes to aging and age-associated disease onset by having a negative effect on tissue integrity and expediting organismal death.

1.1.2 Theories of Aging

Understanding the aging process from a biochemical point of view has received the attention of many basic scientists in recent years as outlined in the February 25, 2005 directed issue of the journal *Cell* (2005). Many theories of aging have been proposed, all of which maintain the following criteria: They must occur in all individuals within and between species of the population (universal), they must produce changes in structure and/or function and they must occur in a time-dependent manner (*i.e.* progressively occur with age). Within the realms of these criteria, two opposing categories of aging theories have come to light. One category is that aging is a genetically controlled ‘programmed’ event that occurs within an organism to ultimately induce an aged phenotype. From this perspective, aging is similar to the way genes program other life-stages such as cell differentiation during embryological development or sexual maturation at adolescence (Sawada et al., 1993; Kirkwood, 2003; Austad, 2004). The other category of aging theories is that aging is the result of the accumulation of deleterious degenerative events (*i.e.* wear-and-tear). In contrast, this perspective

dictates that aging is not the result of any specific controlling program, but is the effect of many kinds of environmental assaults. Such damage can result in aging by affecting genes, proteins, cell membranes, enzyme function, blood vessels, etc. (Kirkwood and Proctor, 2003; Kirkwood, 2005). This concept was first suggested in 1882 by August Weismann. His theory of a 'disposable soma' delineated an important and unequal cellular dichotomy between germ-line or sex cells and somatic or body cells. Briefly, this theory proposes that aging evolved because organisms that segregate germ and soma must invest additional resources to reproduce instead of maintaining the soma, and this renunciation of the soma results in aging.

Unfortunately, none of these theories can explain the entire organismal aging process since aging is a multi-factorial phenomenon that has both a predictable 'programmed' component and an unpredictable stochastic component. Furthermore, aging occurs at different rates for different species, and even within a species, aging occurs at different rates among individuals. Within the same individual, tissues/organs lose viability at different rates and therefore the predisposition for various age-associated pathologies differs between organs (pacemaker theory of aging) (Hermann and Berger, 1999). With this in mind, one interpretation is that aging is genetically controlled, with a strong influence from stochastic environmental factors. Therefore many complex variables including lifestyle, environmental exposures and genetics have differential effects on

individual organisms within a species and between species thereby altering the aging process.

At the cellular level, many biochemists view senescence as a collection of random degenerative processes related only by the fact that they occur over time (Kadenbach et al., 1995; Rubin, 2002). The ‘wear-and-tear’ category of aging theories relies on the random accumulation of detrimental events; including, but not limited to, damage caused by free radicals and increased incorporation of errors into protein sequences also referred to as the ‘error catastrophe’ theory (Harman, 1956; Orgel, 1963). Other cellular theories of aging address the issues of whether senescence is more programmed than random entropy (genetically programmed category of aging theories), thus offering some advantage for a species (Hayflick, 1968). For example, senescence may have evolved because without it a species would accumulate ill-adapted older members. These members would compete with potentially better adapted younger members, slowing the rate at which adaptive mutations are introduced. Another common theory of aging and senescence, ironically the least elucidated, is that a combination of these and other theories rather than only one can accurately explain how aging occurs at the cellular and organismal level. This seems the most plausible since it takes into account both the genetic program that serves to determine the maximum life-span of a species and the random accumulation of detrimental events that explain the pathophysiological conditions that accompany aging.

1.1.3 Models of Aging

Similar to trying to understand the causes of complex disease states, the study of human aging is largely dependent on the development of valid/accurate models. Understandably, the development of an adequate model for complex multi-cellular organism aging is far from trivial. One must always consider whether the model of choice is a cause of aging or merely an effect of aging. For example, from a societal perspective one might consider the graying of hair and the formation of 'liver spots' (also known as old-age spots) a tell-tale sign of human aging. But do these features cause us to age or are they a consequence of aging? This simple example is merely to illustrate a point, since we all know that although gray hair and age-spots are associated with aging they are definitely not the underlying cause of whole-organism aging. However, there are more complex examples of this cause-and-effect relationship. For instance, do our genomes become increasingly unstable because we are getting older as described by the programmed theories of aging (Hayflick, 1968); or are we getting older as a consequence of cumulative genomic integrity loss as described by wear-and-tear theories of aging (Bowles et al., 1981)? Considering these questions is critical to developing accurate models of aging and to shedding light on a complex biological phenomenon such as aging and senescence.

Consequently, both whole-organism and cellular models of aging have been developed. Short-lived metazoan models are used by gerontologists to study human aging since it is assumed that aging is an evolutionarily conserved

phenotype among complex multi-cellular organisms. Since the life cycles of *Drosophila* (fruit fly), *Caenorhabditis elegans* (nematode) and *Mus musculus* (mouse) are short and amenable to examination in a laboratory setting, these organisms have been studied extensively and much of what we know about aging today is based on research using these models (Jazwinski, 2000; Warner, 2003). However, one must be wary of post-mitotic organism models such as *Drosophila* and *C. elegans* since these organisms have a limited capacity for replacement and repair of tissues (hence post-mitotic) and do not develop many of the diseases associated with aging in higher organisms such as cancer and vascular diseases. Additionally, unicellular organisms such as *Saccharomyces cerevisiae* (budding yeast) have been used with tremendous success in recent years (Sinclair et al., 1998), but one can argue that the life-cycle/longevity of a primitive, single-celled organism is not comparable to human aging.

Another common approach to elucidate the biochemical circuitry of aging is to try to understand the molecular mechanisms of premature-aging syndromes as outlined in table 1 (Table 1). These genetic models provide clues on the molecular pathways involved in aging since disease states caused by genetic mutation induce an aging phenotype. Interestingly, these syndromes are caused by defects in DNA damage repair pathways, suggesting that aging may be a consequence of accumulated DNA damage or a loss of genomic integrity/stability through the passage of time (Vijg, 2004). Moreover, whether organisms afflicted with these premature-aging syndromes are actually aging in an accelerated

manner similar to normal aging because of decreased genomic integrity or whether they represent a model in which the manifestation of cumulative DNA damage simply resembles an aging phenotype remains to be seen. Interestingly, since genomic stability is largely dependent on a number of molecular pathways including DNA packaging in appropriate chromatin structure, the potential 'cause' of aging *per se* in groups of various disease states is unique. The role of chromatin structure in DNA metabolism and in DNA damage responses is becoming increasingly obvious (Allard et al., 2004) and is the focus of a large portion of this doctoral dissertation that will be discussed in greater detail in upcoming sections.

Since my research interests lie in the understanding of human aging in the context of certain age-associated disease states, my model of choice is limited to growth competent (mitotic) cells such as fibroblast and epithelial cells derived from human or mouse origins. These cells retain the ability to undergo proliferation and include many of the cells that populate the complex supportive stromal tissues and contribute to the functional parenchymal tissues of organs. There is little doubt that organismal degeneration during aging has a cellular basis that involves maintenance of signaling between these various cell types (Pereira-Smith, 1992; Campisi, 1998; Pendergrass et al., 1999; Campisi, 2000; Busuttill et al., 2004). Therefore understanding how these cells gradually lose proliferative capacity *in vitro* is a good representation of how cells *in vivo* gradually lose their ability to replicate. The advantages of the normal human fibroblast model are that

it can be reproduced *in vitro* in a relatively short time-frame and that these cells should be most relevant to understanding the cellular aspects of human aging. Furthermore, the predictability and reproducibility of the loss of proliferative capacity that occurs in a maintained *in vitro* environment seems to parallel the loss of cellular growth potential *in vivo* (Rohme, 1981; Derwentzi et al., 1996).

The major disadvantages or caveats to this model are that *in vitro* cellular senescence may not occur in a similar fashion as *in vivo* senescence since the *in vitro* culture conditions are quite different from those of physiological conditions (Sherr and DePinho, 2000). In other words, we cannot assume that culturing cells *in vitro* is a benign process (Loo et al., 1987). In fact, the non-physiological conditions inherent to this *in vitro* system, including disruption of cell-to-cell contacts, lack of heterotypic interactions between different cell types, the medium-to-cell ratio, persistent Ras activation by mitogens, absence of appropriate survival factors, hyperoxia and seeding cells on plasma coated polystyrene are likely to induce various aberrant cellular responses – sometimes referred to as culture shock (von Zglinicki et al., 1995; von Zglinicki and Saretzki, 1997; Sherr and DePinho, 2000). Consequently, cells grown under more physiological conditions may have a greater proliferative potential than what we typically observe using our widely accepted *in vitro* protocols (Loo et al., 1987; Parrinello et al., 2003). Additionally, although there is increasing evidence that fibroblast senescence occurs *in vivo*, there are few reports confirming the existence of this senescent cell phenotype in the tissues of a normal healthy

organism (Dimri et al., 1995; Mishima et al., 1999; Pendergrass et al., 1999). Finally, *in vitro* cell culture limits the ability to interpret cellular senescence and/or aging as it pertains to organisms since these are cells cultivated in the absence of a normal tissue environment as is found *in situ*.

Recognizing both the limitations and advantages to certain models of aging is pivotal to understanding the development of age-associated disease states. We must now transcend reductionism and allow the future of aging research to progress based on the foundation we have established using *in vitro* cellular senescence models. Despite the drawbacks in terms of the cellular model used in the course of this study, I believe that the changes observed using *in vitro* replicative senescence are a valid representation of the cell deterioration and/or stress responses that occur *in situ*. Ultimately, I believe that these initial studies will contribute to the foundation of future investigations of replicative senescence as it occurs in tissues.

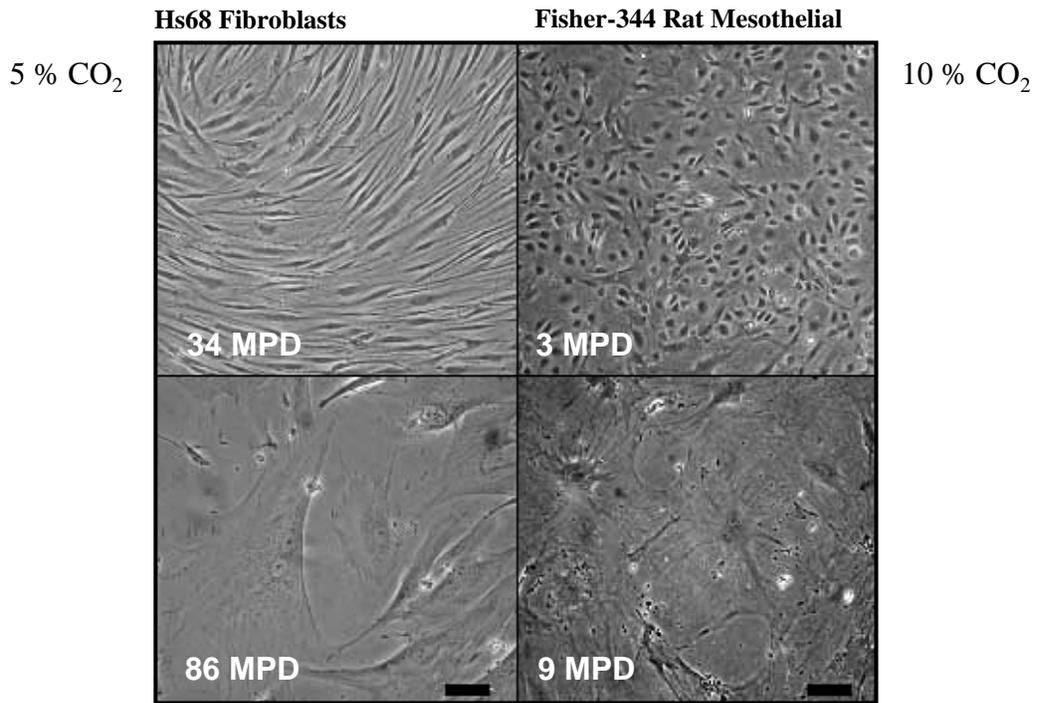
1.2 Impact of Cellular Senescence on Organismal Aging and Disease

The observation that cells from short-lived species generally undergo fewer divisions in culture than cells from long-lived species suggests that there is a cellular component to metazoan aging in which cellular replicative life-span correlates with organismal longevity (Strehler, 1977). To test this assertion, a simple experiment was performed using fibroblast and epithelial cells derived from human and rat origins. As predicted, the phenotypic markers of cellular replicative senescence such as a large, flattened morphology and accumulation of actin stress fibers, as well as biochemical markers such as senescence-associated β -galactosidase staining and activation of cyclin dependent kinase inhibitors p21^{Waf1} and p16^{INK4a} occurred in both species (Figure 1). However, the cells derived from the rat underwent many fewer divisions relative to human cells before they became senescent. This simple proof-of-principle experiment established that cellular senescence is universal insofar as human and rat cells exhibited common markers of aging and corroborated previous studies indicating that cellular replicative life-span is correlated with organismal longevity when using human and rat cell models.

On the other hand, the idea that there is an inverse relationship between the age of a donor organism and the replicative potential of fibroblast cells from that organism is not universally supported (Rohme, 1981; Harley et al., 1990; Cristofalo et al., 2004). This contention is of great importance in the justification of the senescent cell model of aging since it would offer definitive proof of a

Figure 1: Epithelial versus fibroblast senescence. Understanding the interplay between fibroblasts and epithelial cells was an important question not addressed by this project since it has been shown that cell microenvironments affect tissue homeostasis as maintained between these two cell types and that this plays a pivotal role in some age-associated disease states. **A.** Initial experiments defining morphological differences between these cell types proved to be very insightful. As shown here, young and senescent fibroblasts and epithelial cells grown in culture look very similar. They both adopt a large flattened morphology and show an accumulation of actin stress fibers during senescence. However, epithelial cells are more sensitive to oxidative stress and must be incubated in a higher percent CO₂ (5% versus 10%). Furthermore, epithelial cell medium must be augmented with hydrocortisone and hEGF to facilitate growth *in vitro*. Interestingly, the proliferative potential of cells derived from species of various life-spans show a linear relationship between the life-span of the species and the proliferative potential of primary cells derived from it. **B.** As shown previously, senescent fibroblasts have a unique biochemical signature that reinforces their arrested state and helps identify them. These biochemical changes include the up-regulation of key cyclin dependent kinase inhibitors, p21^{Waf1} and p16^{INK4a}. In order to test whether this also occurred in epithelial cells, western blots were performed on lysates from cells that were harvested in parallel with the cells shown in panel A. As shown here, p21 and p16 regulation during cellular senescence is similar in fibroblasts and epithelial cells from different species.

A



B

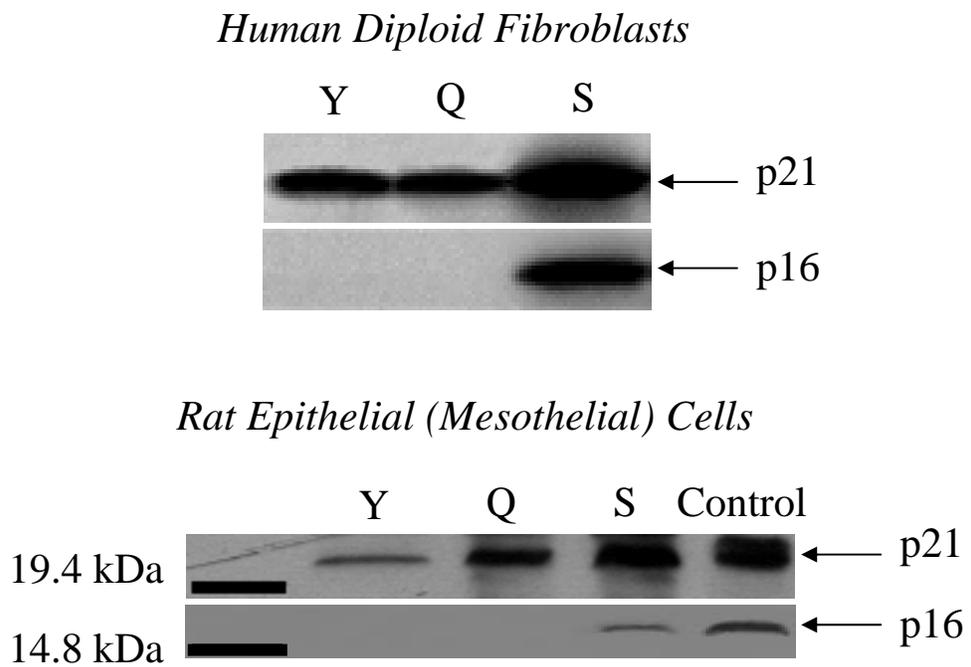


FIGURE 1

cellular and genetic component to age-associated organism degeneration. Unfortunately, I was unable to test this empirically as I did with the different species as noted above. However, I believe that the weak correlation between donor age and cellular replicative capacity is a consequence of the fact that multicellular organisms with renewable tissues are made up of a mosaic of heterogeneous cells and that adult progenitor cells are constantly repopulating tissues with 'younger' cells throughout life (Park et al., 2004). The shift in the secretory phenotype of senescent fibroblasts from an extracellular matrix (ECM) synthesizing to an ECM degrading role suggests that these cells do accumulate with age and contribute to the onset and progression of age-associated diseases since this is reflective of the tissue integrity loss that normally accompanies aging (Krtolica and Campisi, 2002; Parrinello et al., 2005). Further justification of the impact of this cellular model on organismal aging are the observations that DNA damage accumulation, oxidative stress, mitochondrial dysfunction and protein crosslinking occur *in vivo* over time (Rohme, 1981; Dimri et al., 1995; Smith and Pereira-Smith, 1996; Melk et al., 2003) and have an effect on cells *in vitro* (Rohme, 1981; Rubin, 2002; Serra and von Zglinicki, 2002). This suggests that the deteriorative processes that occur *in vivo* can be modeled *in vitro* and that these processes are likely to contribute to age-associated decline.

1.2.1 The Cancer and Aging Conundrum

Cellular senescence has been proposed to act as a tumour suppressive mechanism aimed at limiting the growth of cells (Sager, 1991). If this were true,

one might assume that the incidence of cancer would remain relatively constant throughout life even though exposure to carcinogens increases over the normal life course. However, after the age of approximately 35 years the incidence of cancer increases exponentially amongst North Americans (American Cancer Society, 2002). This cancer incidence trend is highlighted by the recent demographic shift occurring with the 65+ age group being one of the fastest growing cohorts (Yancik and Ries, 2000). Additionally, recent studies conducted by the American and Canadian Cancer Societies (American Cancer Society; Canadian Cancer Society) based on 2002 census data indicate that cancer is the leading cause of death in North America (American Cancer Society, 2002). For decades, heart disease has maintained the position of leading cause of death in North America; however due to the successful implementation of health promotion/disease prevention programs cancer is now the number one cause of death for North Americans 85 and younger (American Cancer Society, 2002). However, one often overlooked etiology of cancer is aging, and by extension cellular senescence, since research in this area has only recently been widely accepted. Since early research on the molecular mechanisms of aging has emphasized longevity rather than age-associated disease states *per se*, this field is just now emerging as a model in the study of disease onset and progression. Understanding the molecular mechanisms of cell senescence will likely shed light on the clear relationship between aging and cancer.

The seemingly sound hypothesis that cellular senescence offers a protective barrier against the effects of oncogenic transformation (Dykhuizen, 1974), has recently come into question with the continued functional characterization of senescent cells in the context of cell-to-cell interactions as is normally found in tissues (Campisi, 2005). These findings, discussed further in the next section, mainly show that senescent cells alter local cell microenvironments to promote oncogenic transformation of premalignant epithelial cells (Parrinello et al., 2005). This idea lends further weight to the idea that the accumulation of senescent cells in the elderly contributes to the increased incidence of cancer with age. Whether these cellular and biochemical changes result in multiple pleiotropic effects, in that genes that protect us from cancer early in life cause us to accumulate senescent cells and cause cancer later in life remains to be seen.

1.2.2 Senescence and the Effects on Tissue Microenvironments

The effects of senescent cells on tissue maintenance and viability have been reported for age-associated tissue changes such as those seen in cardiovascular disease and wound healing (Goldstein et al., 1994; Shelton et al., 1999; Minamino et al., 2004). These studies indicate that senescent stromal fibroblasts (mesenchymal derivatives) influence the adjacent epithelium (ectodermal derivative) in such a manner that tissue homeostasis is lost and diseases like cancer and heart disease are more likely to develop and progress. These findings bolster the hypothesis that cellular senescence plays an important

role in aging and longevity since the accumulation of damaged or senescent cells in these tissues ultimately results in the demise of the organism harbouring them (Krtolica and Campisi, 2002). Recent research has also shown that senescent fibroblast cells increase their secretion of inflammatory cytokines, proteases and epithelial growth factors that contribute to age-associated disease onset (Krtolica and Campisi, 2002; Parrinello et al., 2005). These observations were made by co-culturing pre-senescent or senescent fibroblasts with pre-neoplastic epithelial cells and observing the effect of senescent cells on inducing pre-neoplastic epithelial cell growth and pre-senescent cells being unable to transform pre-neoplastic epithelial cells. The understanding that cancer initiation is often driven by cellular microenvironmental changes coupled with the expansion of our current model of aging, in terms of the effects of cellular senescence on tissues in aging organisms, has helped us to understand how cellular senescence affects disease initiation and progression and is associated with the aging process itself.

Since tissue viability and the ability to maintain homeostasis is thought to decline with age, possibly due to an accumulation of senescent cells, the idea of *antagonistic pleiotropy* is reinforced. Antagonistic pleiotropy suggests that traits that are beneficial during reproductive years may have detrimental effects later in life during post-reproductive years (Williams and Day, 2003). Such traits could be passed on to progeny since they have an adaptation in reproductive years, and detrimental effects later could not be subject to natural selection. In other words, a single gene can have several distinct and seemingly unrelated (even antagonistic)

phenotypic effects at different stages in an organism's life. Supporting this point is the controversial finding that transgenic mice with a constitutively active p53 protein show enhanced resistance to spontaneous tumours compared to wild type littermates, but also exhibit early aging phenotypes (Garcia-Cao et al., 2002; Tyner et al., 2002). Therefore from one perspective, p53 helps to protect against cancer and increases overall fitness early in life by limiting growth/proliferation potential of potentially cancerous cells, but it also promotes aging, thereby limiting lifespan as discussed in section 1.2.1 (Atadja et al., 1995; Krtolica and Campisi, 2002; Feki et al., 2005).

1.3 Cell Growth, Replication and Senescence

The first reported observation of human cells having a finite replicative capacity was made by Leonard Hayflick in the early 1960's. Consequently, the phenomenon of finite replicative capacity is referred to today as the 'Hayflick limit' (Hayflick and Moorhead, 1961; Hayflick, 1965). It was then determined that the senescent cell phenotype, as measure by an inability to initiate DNA synthesis, is a dominant trait when performing somatic cell fusions between young and senescent cells and that this process is preserved in most multi-cellular organisms (Norwood et al., 1974; Wright and Hayflick, 1975; Stein and Yanishevsky, 1981). Together, these pioneering experiments demonstrated that normal somatic cells could not divide indefinitely as was once believed and that the inability to initiate DNA synthesis was at least in part responsible for the replicative arrest seen at senescence. These experiments also suggested that the replicative life-span of a cell should permit sufficient divisions for the proliferative requirements during the life-span of an animal, but it might act as a brake to excessive proliferation to protect against cancer. Oncogenic transformation and cellular senescence have therefore been speculated to be antagonistic events, the former results in rampant and uncontrolled cellular proliferation while the latter leaves cells proliferatively incapable (Campisi, 2000). Consequently, it has been postulated that cellular senescence acts in a tumour suppressive manner by which cells evade cancer formation simply by their inability to proliferate and possibly by limiting the growth of nearby cancerous

cells (Dykhuizen, 1974; Wynford-Thomas, 1999; Wynford-Thomas, 2000; Mathon and Lloyd, 2001).

Further supporting the notion that senescence acts to limit oncogenesis is that many events and stimuli, all of which put cells at risk for neoplastic transformation, can induce an apoptotic or a senescence response. These events include DNA damage and telomere erosion (Di Leonardo et al., 1994; Chen et al., 1995; Robles and Adami, 1998) as well as perturbations to DNA packaging and chromatin organization (Ogryzko et al., 1996; Jacobs et al., 1999; Bakkenist and Kastan, 2003; Itahana et al., 2003). Interestingly, the modulation of local chromatin domains at sites of DNA damage has now been recognized as playing a pivotal role in cellular responses to stress (Paull et al., 2000; Celeste et al., 2002; Celeste et al., 2003). Mounting evidence is also showing that the proliferative capacity of cells is regulated by post-translational modifications, including acetylation and methylation of conserved lysine residues, of the amino-terminal tail domains of histone proteins. The emerging model is that changes to the 'histone code' act to expose DNA damage sites, induce DNA damage repair or apoptosis cascades and limit the growth of normal cells by changing accessibility of cell cycle promoting transcription factors to target gene promoters (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Narita et al., 2003; Zhang et al., 2005). Therefore, the 'histone code' acts to extend the information potential of the 'genetic code' by imposing an additional level of DNA regulation based on the status of DNA packaging proteins (histones) (Jenuwein and Allis, 2001). This is

an attractive hypothesis since chromatin structure is known to be dynamic and dramatically affected through the normal life course (Narita et al., 2003; Allard et al., 2004; Jones and Divecha, 2004). This ultimately influences gene expression levels, DNA damage responses and cell growth and differentiation, all of which play a role in cellular senescence.

1.3.1 Molecular Hallmarks of Cellular Senescence

As Hayflick reported more than 40 years ago, senescence is the process by which the capacity for cell replication, growth, and function is lost with increasing cell divisions (Hayflick and Moorhead, 1961; Hayflick, 1965). It has since been postulated that senescence reflects a state of terminal differentiation (Bell et al., 1978); however, this is difficult to prove since molecular markers of fibroblast terminal differentiation are poorly defined and remain uncharacterized to date. In spite of this, the study of cellular senescence has become an experimental system of great value in understanding the fundamentals of organismal aging and cancer development.

Senescent cells show a large, flat morphology and exhibit a senescence-associated β -galactosidase activity (SA- β -gal) (Dimri et al., 1995; Severino et al., 2000). The cell-cycle arrest that is imposed on these cells is thought to be maintained by signals initiated by DNA damage sensors (d'Adda di Fagagna et al., 2003) that activate tumour suppressor genes such as p53, retinoblastoma (Rb), cyclin dependent kinase inhibitors (p21^{Waf1}, p16^{INK4a}) and acetyltransferase proteins such as the NAD-dependent deacetylase sirtuin 1 (SIRT1) and the

inhibitor of growth-1 (ING1) (Hara et al., 1996a; Lin et al., 1998; Stein et al., 1999; Vaziri et al., 2001; Feng et al., 2002; Nemoto et al., 2004). This group of functionally distinct proteins act to initiate and maintain DNA damage responses at every level of complexity in which complex genomes are organized thereby functionally linking DNA damage to transcription, DNA repair and cell suicide (Berardi et al., 2004). Abrogation of these tumour suppressor and DNA damage response pathways by viruses such as simian virus-40 (SV-40) and human papillomavirus (HPV) has been shown to enable cells to bypass the senescence-associated cell-cycle arrest by inhibiting key cell cycle checkpoint proteins such as p53 and Rb. These findings offered the basis for the previously discussed hypothesis that cellular senescence and oncogenic transformation are antagonistic events that operate through similar pathways (refer to section 1.2).

1.3.2 Telomeres, Telomerase and Senescence

The loss of repetitive DNA sequences (repetitive elements in ribosomal DNA) and genetic instability resulting in cellular replicative senescence and contributing to aging was hypothesized long before the association between telomere attrition and senescence was made (Johnson and Strehler, 1972; Strehler, 1986). More recently, replicative senescence in human primary cells has been linked to telomere attrition as a result of the DNA-end replication problem (Olovnikov, 1971; Watson, 1972; Olovnikov, 1973; Harley et al., 1990). However this is not a universal belief since primary cells derived from laboratory mice quickly undergo replicative senescence with telomeres approximately 5-fold

longer than human telomeres (i.e. ~ 12 kb versus ~ 60 kb) (Blasco et al., 1997). Therefore, one cannot assume that telomere attrition alone is responsible for the induction of normal cellular replicative senescence since mouse cells arrest with apparently long and functional telomeres. This indicates that the replicative capacity of cells is not based solely on telomere length; however these structures warrant study in the cellular senescence field since most evidence indicates that critically short telomeres can induce a senescent state in most cell systems.

Telomeres are repetitive sequences ((TTAGGG)_n in humans) at the ends of chromosomes that associate with various proteins to prevent chromosomal end-to-end fusions (Ducray et al., 1999). Telomere shortening as a consequence serial cultivation-induced cell division has been shown to result in activation of a telomere-initiated DNA damage response pathway (d'Adda di Fagagna et al., 2003). The two experiments delineating the difference between telomere ends of chromosomes and breakage-induced non-telomeric ends of chromosomes were done by Hermann Muller in 1938 and Barbara McClintock in 1941 using *Drosophila* and *Maize* respectively. Muller discovered that the ends of chromosomes were unique from radiation induced DNA double strand breaks in *Drosophila* (Muller, 1938) while McClintock further elucidated the stability of broken ends of chromosomes in *Maize* (McClintock, 1941). Sometime later Alexey Olovnikov and James Watson went on to propose an 'end-replication problem' that would result in telomere shortening with each round of replication (Olovnikov, 1971; Watson, 1972; Olovnikov, 1973). This was based on the now

clear observation that the mechanism of DNA replication in linear chromosomes is different for each of the two strands (called leading and lagging strands). Briefly, semi-conservative DNA replication requires a labile short RNA primer to begin DNA polymerization in the 5' to 3' direction. After DNA polymerization, the RNA primers are degraded and replaced by DNA synthesized from an upstream primer. Because there is no DNA beyond the end of the chromosome to serve as a template for an RNA priming event, the gap between the final lagging strand segment (Okazaki fragment) and the end of the chromosome cannot be filled in. Thus the 5'-end of the lagging strand will lose some nucleotides (approximately 50 to 100-bp loss *in vitro* per doubling) each time a cell replicates its DNA (Olovnikov, 1971; Watson, 1972; Olovnikov, 1973; Allsopp et al., 1992; Engelhardt et al., 1997). Interestingly, the rate of telomere sequence loss *in vivo* is not linear as is suggested by *in vitro* cell cultures (Frenck et al., 1998; Rufer et al., 1999). This is likely a consequence of differing cellular replicative activities *in vivo* over the normal life course and the enzymatic activity of a ribonucleoprotein that acts to maintain telomeres as discussed here.

The mechanism of telomere maintenance, solving the end-replication problem, was elucidated in the mid 1980's by Carol Greider and Elizabeth Blackburn. They discovered a telomere terminal transferase in the ciliate *Tetrahymena thermophila*, now referred to simply as telomerase, that contributed to the maintenance of these structures (Greider and Blackburn, 1985). In an elegant experiment, they found a novel activity in *Tetrahymena* cell free extracts

that adds tandem (TTGGGG)_n repeats (a slightly different sequence from mammalian TTAGGG telomere repeats) onto synthetic telomere primers and that repeat addition was independent of both endogenous *Tetrahymena* DNA and the endogenous alpha-type DNA polymerase (Greider and Blackburn, 1985). Telomerase was then further characterized to be a ribonucleoprotein enzyme composed of a single RNA and protein components that uses its internal RNA component, complementary to the telomeric single stranded overhang, as a template in order to synthesize telomeric DNA directly onto the ends of chromosomes (Blackburn et al., 2000). After adding six bases, the enzyme is thought to pause while it translocates the template RNA for the synthesis of the next six base pair repeat. This extension of the 3'-DNA template end permits additional replication of the 5'-end of the lagging strand, thus compensating for the end replication problem (Blackburn, 2005). In humans, telomerase activity has since been detected in various cell types including fetal tissues, normal adult male germ cells, inflammatory cells, in proliferative cells of renewable tissues, and in most cancer cells.

In a ground-breaking study, Calvin Harley and coworkers discovered that telomeres are specifically lost with age in normal dividing cells and that this process was correlated with cellular senescence (Harley et al., 1990). They also showed that cancer cells may escape mortality by activating the normally inactive enzyme telomerase (Allsopp et al., 1992) and as a result of this finding, telomerase was given the misleading title: 'the immortalizing enzyme' (Harley

and Villeponteau, 1995). The most recent addition to this evolving model of DNA replication and telomere attrition-initiated senescence came when it was discovered that critically short telomeres elicit a DNA damage response, thereby inducing a permanent cell cycle arrest by activating damage response kinases (d'Adda di Fagagna et al., 2003). This study was the first to provide evidence that telomere attrition-initiated senescence was linked to DNA damage response pathways.

Notably, although telomere length is believed to regulate replicative senescence and is often viewed as a type of mitotic 'clock' it is neither the only nor the ultimate timekeeper of cells, since cells with very long telomeres can still undergo replicative senescence (Blackburn et al., 2000). Additionally, when human cell lines and primary fibroblasts are telomerase-immortalized by overexpressing human telomerase reverse transcriptase (hTERT), several reports indicate that immortalized cells had shorter telomeres than growth-arrested controls when measuring telomeres by terminal restriction fragment (TRF) analysis (Bodnar et al., 1998; Ducray et al., 1999; Zhu et al., 1999). Surprisingly, immortalized cells featured less chromosome fusions than growth arrested controls in spite of having shortened telomeres (Hande et al., 1999). Similarly, it was noticed in yeast that certain telomerase-negative strains would senesce with longer telomeres than immortal telomerase-positive strains (Prescott and Blackburn, 1997; Roy et al., 1998). More recently, studies using primary fibroblast cells derived from various tissues of different individuals showed great

variability in telomere length at the time of senescence (Serra and von Zglinicki, 2002). This demonstrates that the maintenance of telomeres during human aging is not as linear and therefore predictable as was shown using cells in mass culture (Allsopp et al., 1992). Since telomere length does not always correlate directly with age and/or senescence, other factors must be involved in initiating and maintaining the permanent cell cycle arrest that typifies senescence and presumably contributes to aging.

1.3.3 The Effects of Stress on Cell Growth

Biological systems, by virtue of their environment, are subject to various types of stress. Interestingly, it has been shown that certain levels of stress can have beneficial effects on organisms and cells, a phenomenon known as *hormesis*, while others can result in system failure and deterioration. Consequently, cell responses to stressors are often evolutionarily conserved and follow a predictable signaling cascade made up of stress sensors, transducers, integrators and chromatin modulators that act to activate or repress transcription of target effector genes (Figure 2). Stressors can include environmental variables such as temperature, UV light, ionizing radiation, chemotherapeutic drugs, etc. They are also sometimes derived from internal sources such as free radicals (reactive oxygen species) or critically short telomeres.

According to telomere attrition theories, the senescent phenotype is believed to be induced by replicative exhaustion as a consequence of telomere attrition as described in the previous section (Allsopp et al., 1992). However, it

Figure 2: The DNA damage and cellular stress response cascade. The growing paradigm of how DNA damage and cellular stress responses are mediated first involves *sensors*, which recognize the cellular or genotoxic stress, *transducers*, which propagate the stress signal, *integrators*, which help to incorporate different facets of the stress response with other cell cycle regulatory machinery, and finally chromatin *modulators* which coordinately allow access to transcriptional *effectors*. This cascade always results in either repair mechanisms being initiated, programmed cell death (apoptosis) to eliminate irreparable cells, or a cell senescence response which contains the potentially dangerous cells ability to proliferate. Interestingly, the senescence mediated pathway of cell cycle arrest can be initiated by telomere-attrition initiated replicative exhaustion or by a stress-induced permanent cell cycle arrest. This suggests that replicative senescence and stress-induced premature senescence have common effector pathways resulting in similar cellular phenotypes.

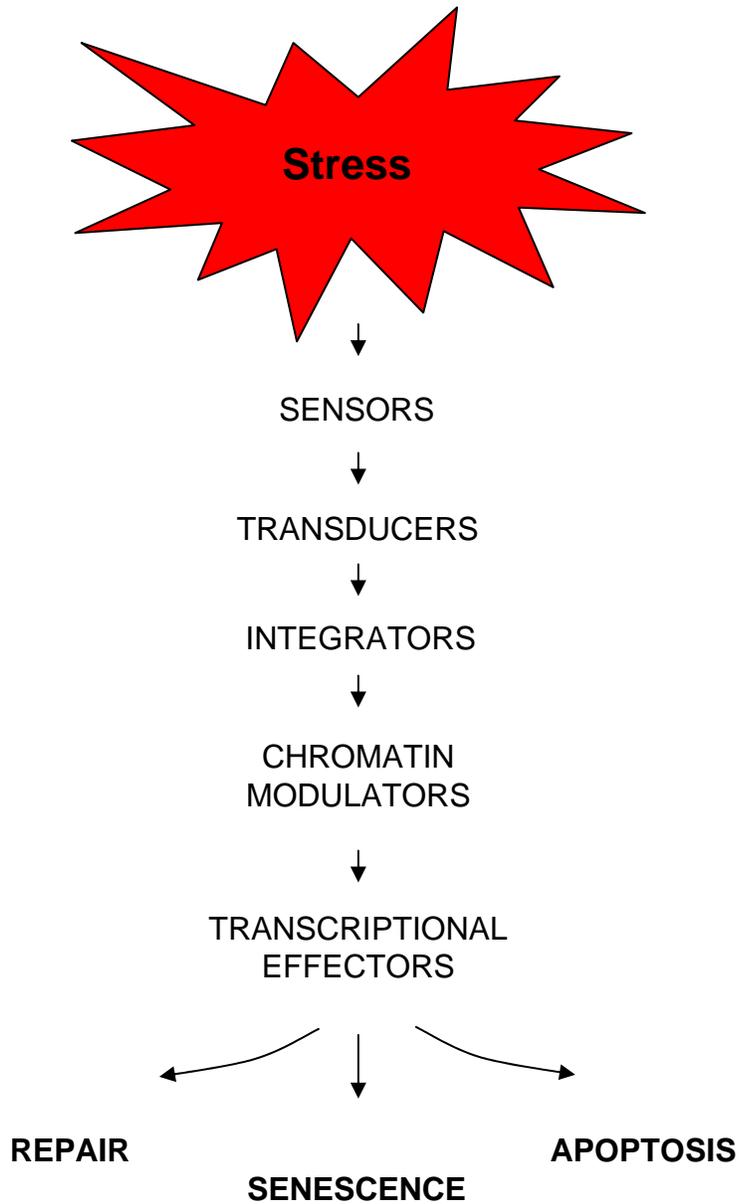
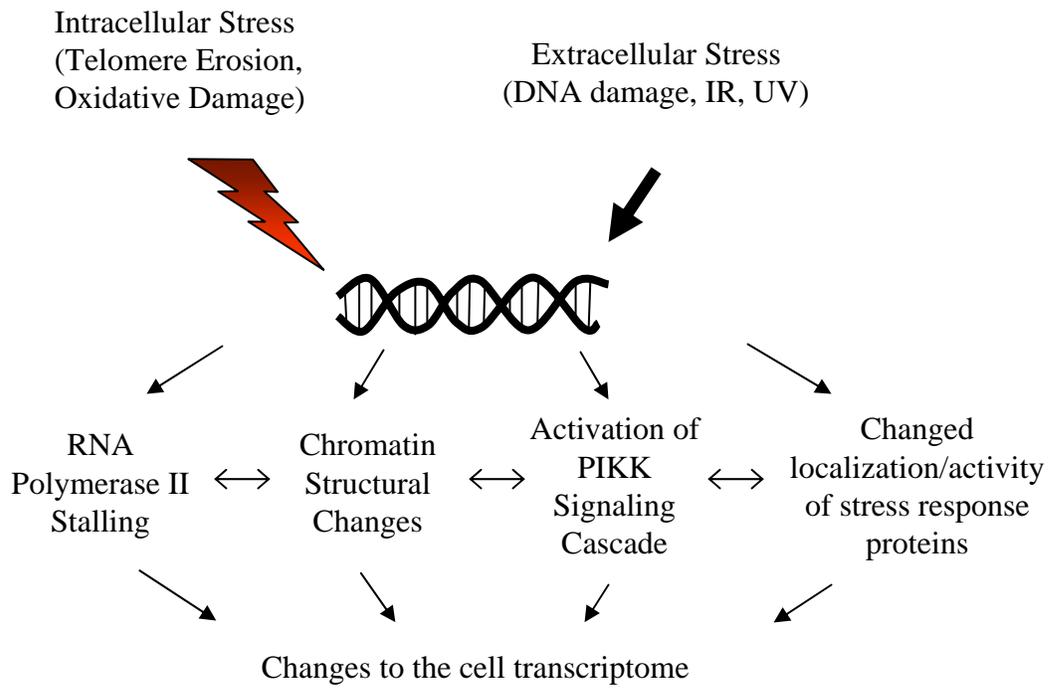


FIGURE 2

has also been shown that sublethal levels of cytotoxic stress on proliferatively competent young cells can induce a senescent-like phenotype in the absence of telomere shortening. This is referred to as stress-induced premature senescence (SIPS) (Toussaint et al., 2000). Interestingly, the telomere-attrition initiated cellular replicative senescence model shares a number of morphological and biochemical features with SIPS, however the role of telomere attrition in SIPS still remains unclear. Based on these data, it is tempting to speculate that telomere-initiated replicative senescence and SIPS are stress responses aimed at containing potentially dysfunctional cells, similar to the induction of apoptosis after irreparable DNA damage. The major difference may be that in the senescent states the stress is not sufficient to induce cell suicide, but rather a G₁-like cell cycle arrest (G_S) aimed at containing damaged cells (Cristofalo et al., 2004).

SIPS provides another useful model in understanding cellular aging and disease development. Since chronic exposure to sublethal levels of stress can induce SIPS, presumably in the absence of proliferation-induced telomere shortening, cellular senescence is likely to be a consequence of both replicative capacity as dictated by critical telomere length and environmental stress responses (*i.e.* a combination of these variables inevitably results in cell growth arrest). Furthermore, the downstream targets of these stress-initiated pathways act in a bidirectional fashion, possibly to reinforce the stress response pathway as outlined in figure 3 (Berardi et al., 2004).

Figure 3: Bidirectional model of transcription in response to stress. Several types of cellular stress can alter chromatin equilibrium, thereby activating similar downstream stress responses. Our model emphasizes how cellular stress responses impinge upon several common downstream targets that ultimately have effects on gene transcription. These targets also show a bi-directional activation in that one target such as chromatin structure influences another such as the PIKK signaling cascade and *vice versa*. Therefore the stress response pathways are reinforced by having effector proteins concomitantly activating one another. Key to the project undertaken are that cell stress responses, including DNA repair, apoptosis and senescence, are intimately linked to transcription factor driven gene activation and chromatin remodeling. Proteins such as ING1, Rb and p53 may act to integrate these stress responses through their interaction with histone acetyltransferase protein complexes and stress response molecules.



Berardi et al., 2004

FIGURE 3

Since organisms with premature aging syndromes often have abrogated genotoxic stress responses (Table 1), and increased stress resistance plays an important role in extended longevity (Longo, 1999), aging and stress resistance appear to be intimately related. Since replicative senescence, SIPS and to some degree organismal aging are all the result of genomic instability sharing common pathways resulting in a common phenotypic outcome, it reveals the interconnectedness of these three phenomena.

Table I: Genomic Vulnerability/Accelerated Aging Syndromes

Syndrome	Defective Gene(s) or Gene Product(s)	DNA Repair Defect
Ataxia-Telangiectasia	ATM	Unable to activate cell cycle checkpoints in response to DNA double strand breaks
Nijmegen Breakage Syndrome	NBS	Part of MRE11-RAD50-NBS1 (MRN) complex involved in initial processing of DNA double strand breaks
Ataxia-Telangiectasia-Like Disease	MRE11	
Werner's Syndrome	WRN helicase	Unclear role of WRN DNA helicase
Bloom's Syndrome	BLM helicase	Ablated homologous recombination repair and repair of damage at stalled replication forks
Xeroderma Pigmentosum	XPA, XPB, XPC, XPD, XPF	Ablated nucleotide excision repair (NER)
Trichothiodystrophy	XPB, XPD	Abrogated function of DNA helicases involved in transcription
Cockayne's Syndrome	XPB, XPD	Unable to repair lesions in the transcribed strand of active genes (TCR)
Fanconi's Anemia	Multiple 'FA Genes'	Abrogation of DNA damage response system
Rothmund-Thompson Syndrome	RECQL4	Unclear role of recQ-like DNA helicase

1.3.4 The Cell Division Cycle

The cell division cycle or simply the cell cycle is defined as the series of biochemical and structural events involving the growth, replication, and division of eukaryotic cells. It is controlled by numerous mechanisms that ensure correct cell division and propagation of stable genomes (Figure 4). The main processes which govern cell division are DNA replication during DNA synthesis (S-phase) and segregation of replicated chromosomes into two separate cells during mitosis (M-phase). Mitosis can be broken down further into prophase, metaphase, anaphase and telophase. Interphase is the general term to describe the interlude between two M-phases and can be sub-divided into gap-1 (G_1), DNA synthesis (S) and gap-2 (G_2) phases (Norbury and Nurse, 1992). The DNA synthesis phase of the cell cycle is referred to as the S-phase and we have called the permanent senescence arrest that occurs as a consequence of serial cultivation the G_S -phase.

The cell division cycle of normal primary cells is tightly controlled by the activity of cyclin and cyclin dependent kinase proteins acting in concert to allow cell cycle progression. The cell cycle must also involve the dynamic regulation of chromatin by histone and DNA modifying enzymes and transcription factors acting in concert to regulate gene expression (Krebs et al., 2000). When cell cycle regulatory mechanisms are dysfunctional (*i.e.* by inactivation using certain oncogenic viruses as shown in figure 5C) and the cell cycle checkpoints are inactivated, oncogenic transformation ensues.

Figure 4: Mortality pathways and the mammalian cell cycle. To understand the molecular mechanism of cell division and senescence we need to understand the life cycle of a cell. It consists of four main phases: G_1 , S, G_2 and M, where "G" stands for "Gap", "S" represents "Synthesis" and "M" means "Mitosis". After a cell divides in mitosis (M) it may either enter into another round of cell division or remain in the resting state for a long period referred to as G_0 . This is often referred to as a quiescent state. Upon mitogenic stimulation, cells in the quiescent G_0 phase may re-enter the G_1 phase. Cyclin protein complexes as well as tumour suppressor proteins such as p53, retinoblastoma (Rb) and ING1 play an important role in the successful progression of the cell cycle. Inactivation of the Rb pocket protein by cyclin/cyclin dependent kinase (cdk) complexes is necessary for cells to progress from G_1 to S phase. Late in the G_1 phase is a restriction point (R), often called the point of no return, where cells are committed to proceeding through a full round of the cell cycle. The permanent growth arrest resulting from serial cultivation is referred to as G_S for replicative senescence and typically occurs in mid to late G_1 . This state is defined by a unique biochemical signature as well as an accumulation of senescence associated heterochromatic foci (SAHF) that are induced by targeted histone deacetylase activity in part mediated by ING1a. The ING1b association with the proliferating cell nuclear antigen (PCNA) may also play an important role in the DNA synthesis phase progression.

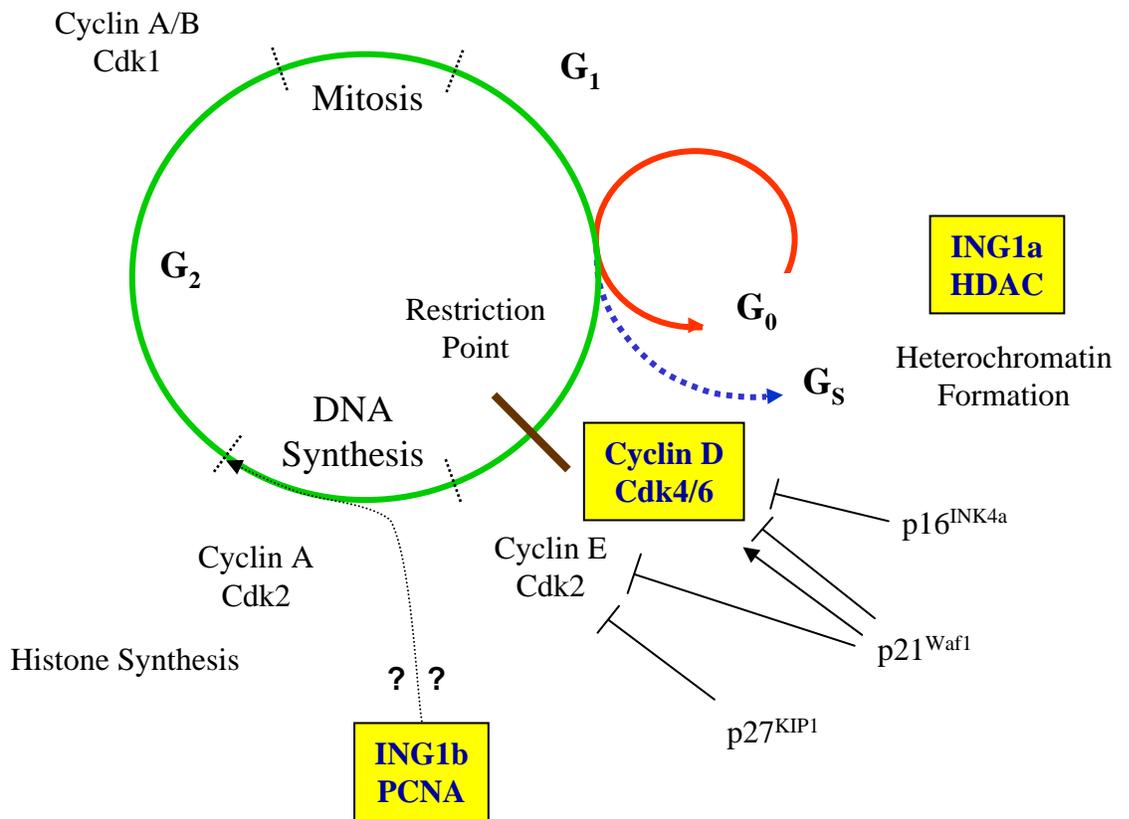


FIGURE 4

Much like the proposed role of senescence, cell cycle checkpoints exist as a quality control system maintained by cells to ensure that faulty genomes are not propagated (Evan et al., 1995; Sherr, 1996). Therefore, this system is in place to interrupt the cell cycle if something goes wrong somewhere along the complex series of events that allow cells to divide. The major cell cycle checkpoints are the DNA damage checkpoints that occur prior to the G₁/S transition (the G₁ checkpoint), the DNA synthesis checkpoint and the G₂ checkpoint that occurs after DNA synthesis (Kastan and Bartek, 2004). There are also mitotic spindle checkpoints that are in place to ensure that proper chromosome segregation takes place during every cell replication cycle (Dobles and Sorger, 2000). A defining feature of cellular transformation is that malignant tumour cells lose the ability to enforce checkpoints and therefore often do not enter the quiescent-like senescent (G_S) stage of the cell cycle. In contrast, normal cells maintain the ability to exit the cell cycle and enter this non-proliferative state.

G₀ (also referred to as quiescence) is a long-term non-proliferative stage that results from serum deprivation or contact inhibition *in vitro*; G₁ is a transient stage between the mitosis (M) and DNA synthesis (S) phases of growing cells; and G_S is the post-mitotic state which cells adopt when they reach the end of their replicative life-span (senescence). Senescent cells arrested in the G_S-phase of the cell cycle have a biochemical profile similar to the G₀ and G₁ phases of the cell cycle, yet the expression and activity of some cell cycle regulatory proteins during G₀, G₁ and G_S are distinct, implying that senescent cells exist in a unique, non-

proliferative state (Parr et al., 1998). Consequently, although cell cycle gap phases, G_0 , G_1 and G_S , are often viewed together, these stages are actually quite distinct (Schwarze et al., 2002; Zhang et al., 2003). Given that most cells in adult humans are in G_0 , and that defects in the ability to maintain the arrested G_S stage leads to cancer formation, it would be of great practical and clinical interest to elucidate mechanisms by which normal cells maintain this irreversible cell cycle arrest. The major physiological difference between cells in the quiescent G_0 -phase of the cell cycle and cells in the senescent G_S -phase is that quiescent cells can re-enter the cell cycle following mitogenic stimulation or changing *in vitro* culture conditions so that cells are no longer contact inhibited; in contrast senescent cells (G_S) are unable to re-enter the cell cycle regardless of mitogenic cues or changed culture conditions. In other words, senescent cells are permanently arrested in an irreversible quiescent-like state.

Pivotal to successful cell cycle progression is the phosphorylation and inactivation of the retinoblastoma (Rb) tumour suppressor by cyclin/cyclin dependent kinase (cdk) protein complexes near the restriction point of the cell cycle (Figure 5). Cyclin proteins were identified in yeast and named according to their ability to cycle or oscillate in overall protein levels through the cell division cycle (Nurse, 1975). These proteins are synthesized and assembled with cdk proteins in response to growth factor stimulation, thereby generating active holoenzymes that help inactivate the growth-suppressive function of the Rb protein through its phosphorylation.

Figure 5: The role of cyclin D1 in cell cycle progression and arrest. A. Cyclin D1 contains an N-terminal *LxCxE* motif that enables it to interact with the Rb tumour suppressive protein. It also contains an N-terminal cyclin box fold which is a protein binding domain functioning in cell-cycle and transcription control. This domain is present in cyclins, TFIIB and Rb. There is also a redundant C-terminal cyclin domain that functions to regulate cyclin dependent kinase activity. A C-terminal PEST sequence, a region rich in proline (P), glutamate (E), serine (S), and threonine (T) targets cyclin D1 for degradation by the proteasome. This is catalyzed by phosphorylation of threonine 286 (T286) by glycogen synthase kinase-3 β (GSK3- β) at the C-terminus of human cyclin D1. **B.** In order for cells to pass the restriction point, cyclin D/cdk4, cyclin D/cdk6 and cyclin E/cdk2 must sequentially phosphorylate the Rb pocket protein, resulting in Rb inactivation and allowing E2F transcription factors to transactivate cell cycle promoting genes. This ultimately culminates in cell proliferation. Cdk activities are tightly regulated by multiple mechanisms that include phosphorylation, reversible association with regulatory cyclin proteins and location within the cell. Cdks obtain full activity at binding with adenosine triphosphate (ATP) by phosphorylation of a threonine residue in the cdk. During cell cycle arrest/senescence Rb is hypophosphorylated, therefore it is in an activated state whereby it is bound to E2F transcription factors and inhibits cell cycle progression. The promoters of cyclin proteins are strongly influenced by extracellular growth factors including transforming growth factor- β (TGF- β), parathyroid hormone-related protein (PTHrP), insulin-like growth factors (IGFs) and other factors found in serum.

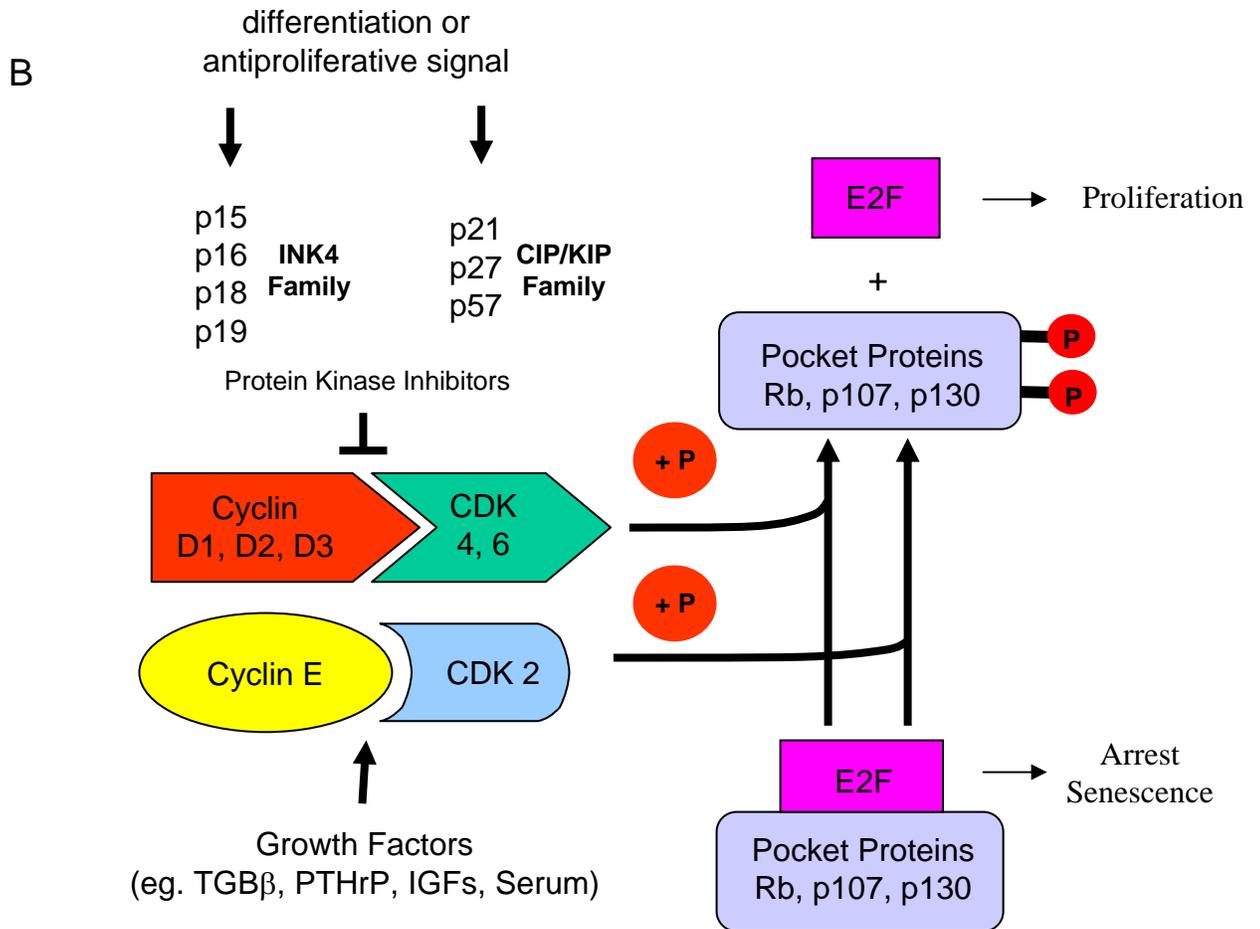
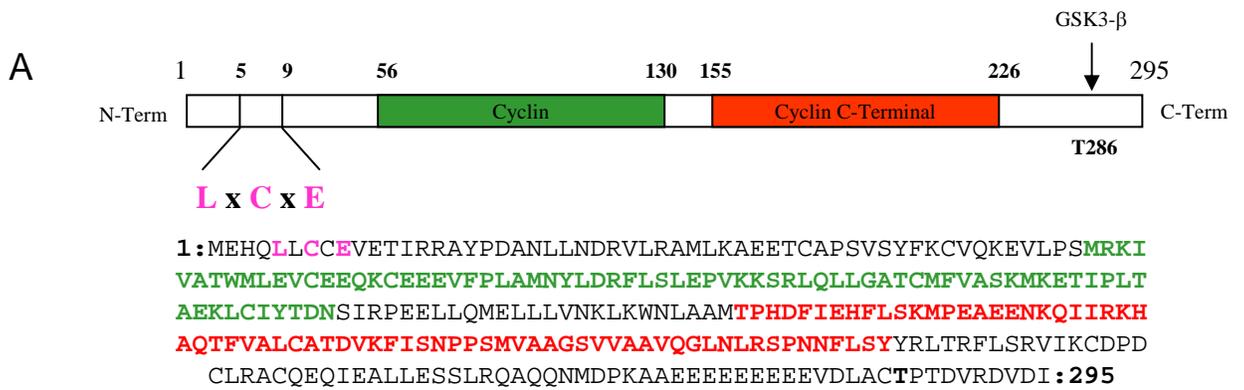


FIGURE 5

Figure 5 (continued): C. Inappropriate regulation of key cell cycle regulatory proteins is one of the distinguishing features in the development and progression of human cancers. Identifying mechanisms by which these genes are regulated can therefore provide useful targets for cancer therapy. Cyclin D1 impinges on both p53 and Rb tumour suppressive pathways. Rb also associates with other transcriptional repressor and activator proteins to coordinately regulate several downstream targets. Tumour viruses such as the human Papillomavirus (HPV) and simian virus-40 (SV40) are able to induce oncogenic transformation by their ability to inactivate Rb and p53 tumour suppressive pathways. MDM2 is an E3 ubiquitin ligase that targets p53 for degradation by the proteasome and can be sequestered to the nucleolus by p14^{ARF} resulting in p53 stabilization. Additionally, p53 is a potent transactivator of the cdk interacting protein p21^{CIP1} (originally identified as a wild type p53-activated fragment-1), which acts to inhibit cyclin dependent kinase activity and arrest the cell cycle. Other cyclin dependent kinase inhibitors such as p16^{INK4a}, which is encoded by the same gene locus as p14^{ARF}, act to inhibit phosphorylation of Rb resulting in cell cycle arrest. The polycomb complex protein Bmi-1 can inhibit expression of the INK4a locus (p16^{INK4a} and p14^{ARF}) resulting in cell cycle progression and stem cell self-renewal by impinging on two major cell cycle arrest pathways. Interestingly, Bmi-1 also induces telomerase activity and immortalizes human mammary epithelial cells possibly by a combination of these activities.

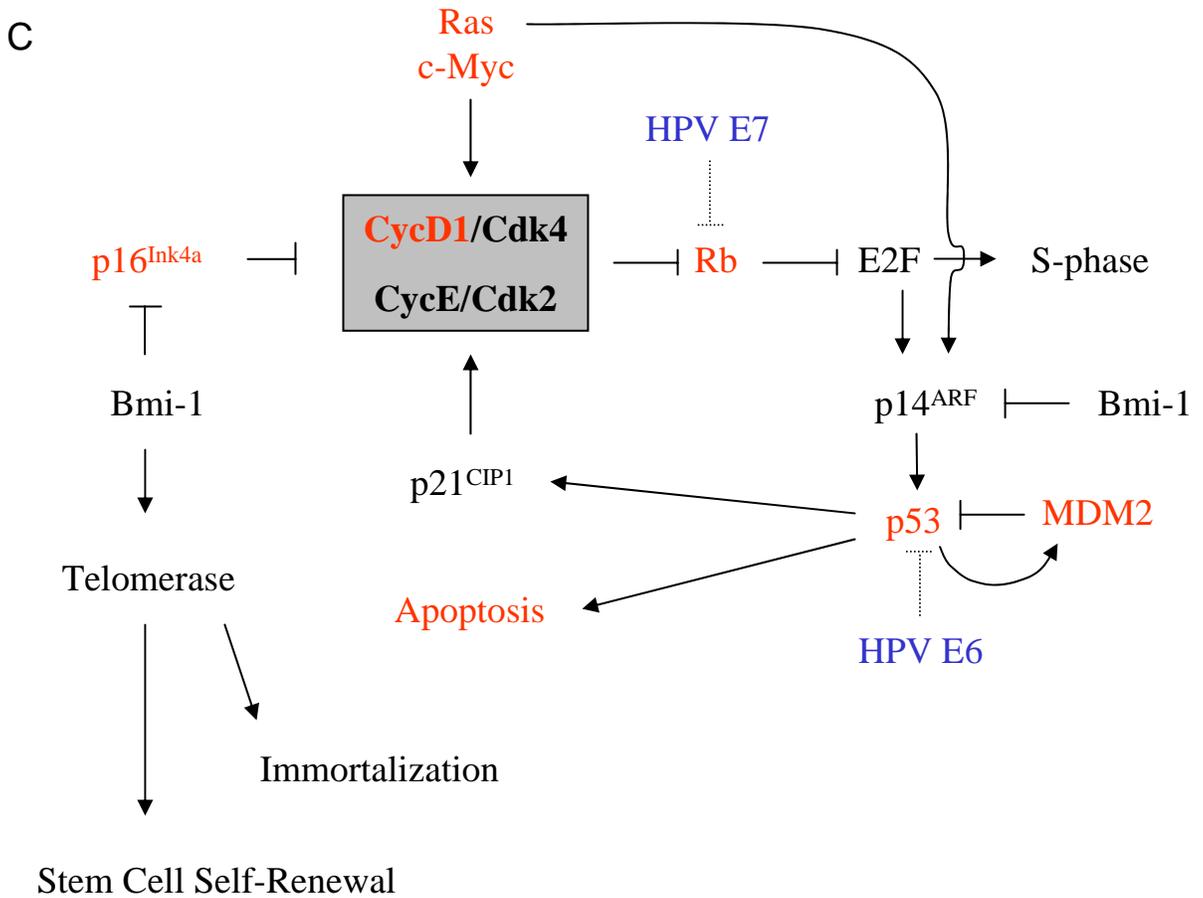


FIGURE 5

The restriction point, often referred to as the 'point of no return', is a cell cycle checkpoint in late G1 that dictates whether a cell can continue through DNA synthesis and mitosis. Therefore, before this point cells are able to leave the cell cycle and enter a quiescent, non-proliferative resting phase; otherwise, once cells pass this point they are committed to completing a full round of the cell cycle (Zetterberg and Larsson, 1985). The phosphorylation of Rb by cyclin/cdk complexes can be inhibited by two families of cyclin dependent kinase inhibitors. These are the inhibitor of kinase-4 (INK4) family which includes p16^{INK4a} and the cdk-interacting protein/kinase inhibitor protein (CIP/KIP) family which includes p21^{Waf1} and p27^{KIP1} (Figure 4 and 5). As shown in figure 5C, the cyclin D1/cdk and cyclin E/cdk complexes are central to this cell cycle progression and their differential activity plays a key role in cell proliferation arrest and senescence.

1.3.5 Cyclin D1 and Senescence-Induced Cell Cycle Arrest

The major cyclin proteins include D-type cyclins (D1, D2, D3), cyclin E, cyclin A and cyclin B (Gao and Zelenka, 1997). Each cyclin protein is activated at various points in the cell cycle by its association with one or more of the cyclin dependent kinase (cdk) proteins as shown in figures 4 and 5. Interestingly, cyclins display a high level of promiscuity in their ability to associate with different cdk proteins, possibly indicating alternative roles for these multiple complexes in regulating cell cycle progression (Gao and Zelenka, 1997). For example, cdk2 has about a 47% homology in amino acid sequence with cdk4, therefore it is not surprisingly that cdk2 can form complexes with cyclin D or cyclin E. Moreover,

this redundancy in cyclin binding has been proposed to play a role in the ability of cyclin D overexpression inhibiting cell cycle progression (Fukami-Kobayashi and Mitsui, 1999).

Cyclin D1 is a member of the D-type family of cyclins that preferentially associates with cyclin dependent kinases 4 or 6 (cdk4/6) (Matsushime et al., 1992; Meyerson and Harlow, 1994). Cyclin D1-cdk4/6 promotes the G1- to S- phase transition of the cell cycle by cooperating with cyclin E-cdk2 to sequentially phosphorylate the retinoblastoma tumour suppressor protein (Harbour et al., 1999). However, mice deleted for (null for) the cyclin D1 gene are rescued by cyclin E expression suggesting that cyclin D1 does not play a central role in regulating the cell cycle. This finding is further supported by the minimal phenotype of cyclin D1 knockout mice (Sicinski et al., 1995; Geng et al., 1999; Geng et al., 2001) and the normal proliferation of cyclin D-deficient (cyclin D1^{-/-}, cyclin D2^{-/-}, cyclin D3^{-/-}) fibroblasts (Kozar et al., 2004).

Mitogenic signaling events are capable of elevating cyclin D1 expression because the cyclin D1 gene promoter is sensitive to the growth factor induced signaling such as the Ras-GTPase signal transduction pathway (Shao et al., 2000). Therefore, cellular inhibition of cyclin D1/cdk4 or cyclin D1/cdk6 kinase activity is achieved through these external signaling cues (*i.e.* the Ras-Raf-Mek-Erk signaling pathway) and through association with inhibitory proteins such as p16, p21 and p27 (Figure 4 and 5) (Sherr and Roberts, 1999). Among these, p16 preferentially inhibits the kinase activity of cdk4 and cdk6 by re-assortment of

cyclin, cdk, and cdk-inhibitor complexes (McConnell et al., 1999). On the other hand, p21 and p27 are known to inhibit most cdks such as cdk1, cdk2 cdk4 and cdk6 (Russo et al., 1996a). Interestingly, p21 in normal fibroblasts is found in a quaternary complex throughout the cell cycle with cyclin, cdk and proliferating nuclear antigen (PCNA) and this complex has activity in proliferating cells but is inhibited by the addition of more p21 to the complex (Li et al., 1994). Therefore, p21 can both activate and inhibit cyclin/ckd kinase activity depending on its abundance as shown in figure 4.

Paradoxically, cyclin D1 which is typically associated with cell cycle progression is transcriptionally activated during replicative senescence (Lucibello et al., 1993; Fukami et al., 1995; Berardi et al., 2003). D-type cyclins are important in regulating the G₁/S transition by their ability to interact with and activate cdk4 and cdk6 and subsequently inactivate Rb by phosphorylation. Growth suppression by hypophosphorylated Rb (activated) is closely correlated with its ability to form complexes with a number of proteins through different protein binding domains of its amino acid sequence. This growth suppressive function is inhibited by phosphorylation of specific serine/threonine Rb residues by cyclin-dependent kinases. Rb contains at least 16 consensus sequences for cdk phosphorylation, but the significance of these sites is still unclear. Phosphorylation of various Rb C-terminal serine (S) or threonine (T) residues (S-807, S-811, T-821, T-826 and S-780) leads to disruption of binding of E2F (Knudsen and Wang, 1997). The E2F family of transcription factors is a key

regulator of cell growth control. To date, six different E2Fs have been identified, E2F-1 to E2F-6, each of which can heterodimerize with DP1 or DP2 (E2F dimerization partners) to form 12 different DNA binding transcriptional regulators (Farnham et al., 1993; Dyson, 1998). E2F transcription factors are normally inactivated by sequestration by one of three retinoblastoma family members, p105 (Rb), p107 or p130 (Dyson, 1998). The most well known of these tumour suppressors, Rb, is capable of binding E2F-1, -2, -3, -4 and plays an important role in the G₀, G₁ and S phases of the cell cycle (Dyson, 1998). Consequently, the E2F family of transcription regulators is able to transactivate cell cycle promoting genes (namely G₁/S transition genes) such as DNA polymerase II, cyclin E, PCNA, cyclin A and cdc2 (Wells et al., 2002; Vernell et al., 2003). During the course of this study it was also shown that the sequential phosphorylation of Rb by cyclin/cdk complexes progressively block Rb function as cells move through G₁ (see Figure 5B) (Harbour et al., 1999).

In summary, the protein regulators of the cell cycle clearly play a role in the normal implementation of cell cycle arrest. These proteins have also been shown to play a pivotal role in mediating the permanent cell cycle arrest seen during replicative senescence (Lucibello et al., 1993; Fukami-Kobayashi and Mitsui, 1998; Fukami-Kobayashi and Mitsui, 1999). Therefore the link between cell cycle machinery, with an emphasis on cyclin D1, and cellular senescence has been established.

1.4 Signaling Pathways Contributing to Senescence

Extracellular signaling pathways play an important role in the onset and maintenance of cellular senescence since these pathways influence fundamental processes such as cell growth, proliferation and apoptosis. In fact, research aimed at identifying pharmacological agents that induce the expression of senescence-inducing genes, such as cyclin dependent kinase inhibitors (discussed in section 1.3), may offer new treatment strategies for cancer. The hypothesis is that pharmacological inhibition of signaling pathways that are specifically down-regulated during replicative senescence may result in the reactivation of the senescence program in tumour cells (Lodygin et al., 2002). Interestingly, a recent study done by Gozani *et al.* indicates that a functional motif in the inhibitor of growth-1 (ING1) protein acts as a nuclear receptor for the phosphatidylinositol monophosphate signaling pathway (Gozani et al., 2003). Furthermore, this activity was shown to occur in a stress-inducible manner, therefore it supports our finding that ING1 acts to modulate chromatin structure after stress and during replicative senescence (Scott et al., 2001b; Vieyra et al., 2002b; Berardi et al., 2005). This may offer a new link between extracellular growth signaling pathways and chromatin modulation as will be discussed here.

1.4.1 Mitogenic and Stress Signal Transduction-Dependent Cell Proliferation

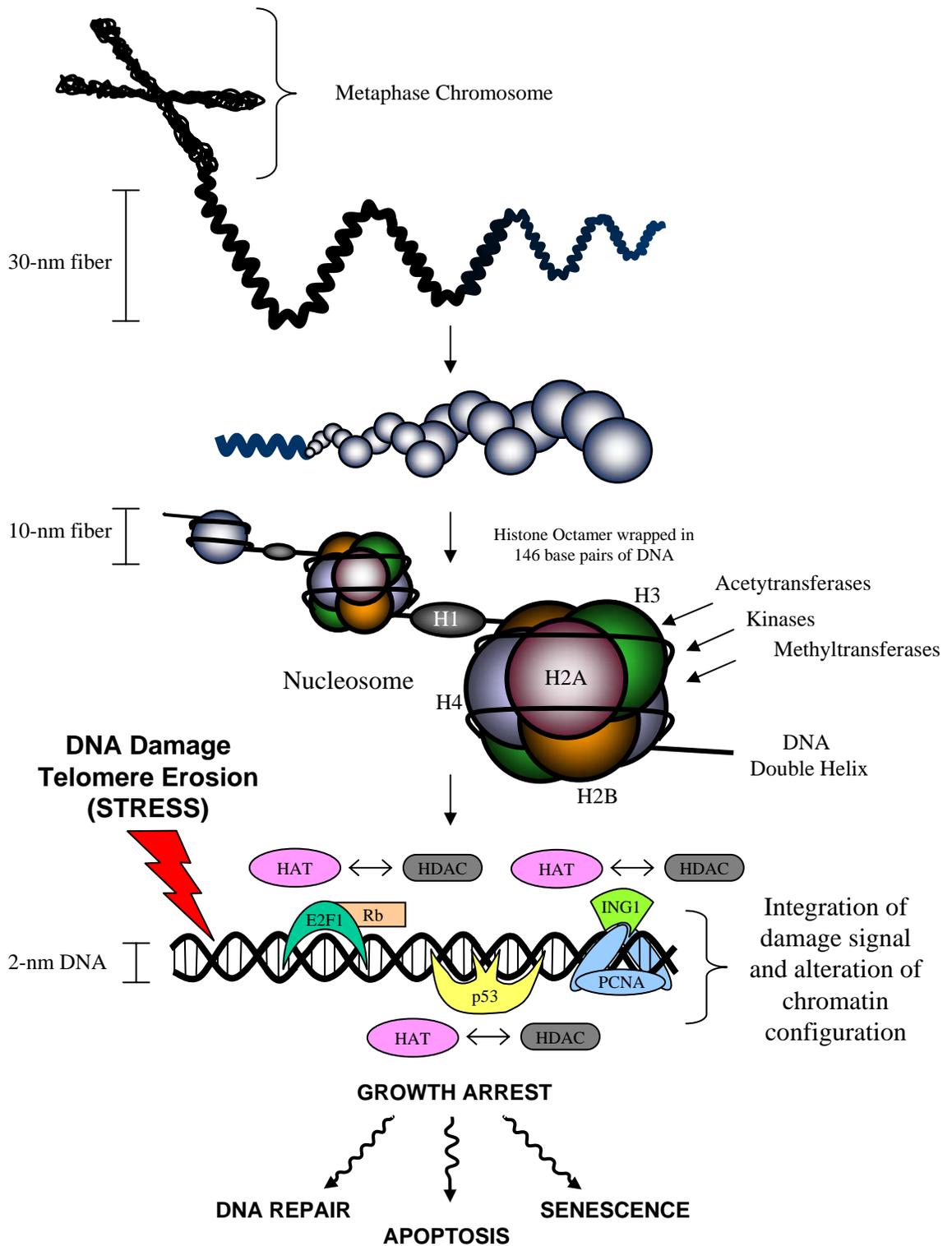
Senescence, like many cellular responses to environmental cues, is mediated by activation of complex signaling cascades that invariably induce

changes in gene expression (Riabowol, 1992). The impaired responsiveness of senescent fibroblasts to environmental factors, particularly the lack of response to mitogens, suggests cells are unable to maintain appropriate transmission of growth signals. This hypothesis is supported by the observation that infection of senescent fibroblasts with simian virus-40 (SV-40) results in one more round of DNA replication (Gorman and Cristofalo, 1985) indicating that replicative machinery of cells is intact. However this finding does not provide definitive evidence that a true receptor-block in a downstream signaling cascade functions in terms of maintaining cells in a non-proliferative state.

Studies have shown that senescent cell cultures elicit proper activation of mitogenic growth factor receptors, however nuclear events that are targets of these receptor initiated signaling pathways are sometimes impaired (Atadja et al., 1994; Meyyappan et al., 1996). These defects may involve processes that occur in response to receptor ligation such as calcium mobilization, multiprotein complex formation, protein kinase activation and phospholipid turnover; or may be a product of accessibility changes to gene promoters during senescence as a consequence of aberrant chromatin structure/homeostasis (Figure 6) (Berardi et al., 2004).

Figure 6: Functional links between transcription, DNA repair and apoptosis.

Human genes are packaged by an octamer of small basic proteins called histones (H2A, H2B, H3 and H4) that make up chromatin which is organized in coiling chromosome structures within the nuclear envelope. These structures resembling ‘beads-on-a-string’ are called nucleosomes and are compacted into 30nm chromatin fibers. Modulation of these structures can occur as a consequence of both intracellular and extracellular stressors ultimately inducing a common downstream signaling cascade. There are several proteins involved in maintaining this dynamic equilibrium of these highly ordered structures including histone modifying enzymes such as histone acetyltransferases (HATs) and deacetylases (HDACs). Histone acetylation is a process of charge neutralization of basic, positively charged lysine residues. This destabilizes the binding of histones to the negatively charged DNA so that other enzymes like RNA polymerases, with their associated machineries, are capable of unwinding the chromatin, access the DNA at selective sites and express the genes through transcription. Several recent studies have highlighted the importance of this ‘epigenetic’ control of the human genome. Here we propose that structural changes to chromatin such as those seen at sites of DNA damage as well as those induced by extracellular stressors can activate common key transducers of cellular stress. This signaling cascade impinges upon diverse integrators of the stress response including Rb, p53 and ING1 and results in changes to the transcriptome by post-translational protein modifications including acetylation, phosphorylation and methylation. Ultimately, the mammalian cell’s fate is dictated by how severe the stress is and culminates in survival when the stress is repaired or removed, apoptosis or senescence when the stress is too severe, or transformation when the damage remains unchecked and induces constitutive growth in the absence of mitogenic cues. This demonstrates the hierarchy of gene expression control from the decondensation of chromatin to the binding of transcription factors on specific gene promoters.



Berardi et al., 2004

FIGURE 6

Examples of this reported by our laboratory and others include the reduced expression of the immediate-to-early response transcription factors c-fos (cellular - FBJ osteosarcoma oncogene), erg-1 (early growth response-1), Id-1H and Id-2H (inhibitors of DNA binding) (Seshadri and Campisi, 1990; Hara et al., 1996b; Meyyappan et al., 1999) and the reduced binding activity of the transcription factors serum response factor (SRF), T-cell factor (TCF) and activator protein-1 (AP-1) (Riabowol et al., 1992; Atadja et al., 1994; Ding et al., 2001; Tresini et al., 2001). Based on these findings it has been hypothesized that during replicative senescence there are signaling defects that are downstream of growth factor receptor activation (Riabowol, 1992).

Other signaling pathways important for cell growth and stress responses are the lipid-mediated signal transduction pathways (Gozani et al., 2003; Jones and Divecha, 2004). It is speculated that these pathways play a pivotal role during cellular senescence and are involved in organismal aging and disease (Rouser et al., 1971; Krasilnikov, 2000). Phosphoinositides, which form a minor family of phospholipids, are intracellular second messengers that play a key role in propagating cell growth, proliferation and stress response signals (Payraastre et al., 2001). Lipid kinases and phosphatases respond to extracellular signals leading to remodeling of the phosphoinositide profile which in turn regulate downstream targets. These downstream targets control diverse intracellular processes such as cell proliferation and survival (Datta et al., 1999), gene transcription (Ellson et al., 2002) and recently it has been shown to play a role in cell stress responses

(Gozani et al., 2003). Therefore, the biochemical cascade involved in stress signaling, as outlined in figure 2, supports the role of phosphoinositides as *transducers* of the stress response (Berardi et al., 2004). Interestingly, it is the conserved inhibitor of growth (ING) family plant homeodomain (PHD) zinc finger (Figure 7) that acts as a nuclear receptor of phosphatidylinositol monophosphates (PtdIns(3)P, PtdIns(4)P and PtdIns(5)P) (Gozani et al., 2003). Furthermore, previous studies show that these phosphoinositide species localize to heterochromatin, nucleolar-associated heterochromatin and sites of pre-mRNA processing (Boronenkov et al., 1998). This localization is consistent with ING1 protein isoform localization as a consequence of stress induction or senescence (Scott et al., 2001a; Scott et al., 2001b; Berardi et al., 2005). The role of ING1 proteins in the onset and maintenance of cellular senescence is a major component of this doctoral dissertation and will be discussed in greater detail in the following sections.

Figure 7: The ING family plant homeodomain zinc-finger. The ING1 PHD Zn-finger is commonly found in chromatin-regulatory proteins and has been shown to bind phosphoinositide monophosphates (PtdIns(3)P, PtdIns(4)P, PtdIns(5)P). PtdInsPs are ephemeral molecules that are rarely found in the nucleus since they play critical roles in cytoplasmic signal transduction pathways. R₁ and R₂ are long-chain hydrocarbon tails of fatty acids found on these molecules. The enzymatic activity of phospholipases, lipid kinases and lipid phosphatases in response to extracellular signaling leads to remodeling of the phosphoinositide profile. Therefore the phosphoinositide pathway is a second messenger signaling system regulated in response to a variety of extracellular and intracellular stimuli. These include growth factor and differentiation signals as well as cell cycle and DNA damage signals. A recent study demonstrating the ING family PHD domain interacting with phosphoinositide monophosphates to directly regulate nuclear stress responses and previous studies showing that PtdInsPs localize to heterochromatin and nucleolar-associated heterochromatin corroborate the data presented linking ING1 binding to chromatin in a growth dependent manner and inducing the formation of heterochromatin during senescence. This suggests that changes in nuclear phosphoinositide profile may have a direct role in modulating chromatin structure. The ING1 PHD finger is a C₄-H-C₃ type zinc-finger made up of approximately 50 residues at the C-terminus of the ING family proteins. Since it is encoded by the common exon of ING1, this functional motif is conserved in all ING1 isoforms and is thought to act in concert with other ING1 functional motifs to target accessory proteins to chromatin. The residues speculated to maintain the zinc-mediated structures of the PHD finger are highlighted in red.

STRESS

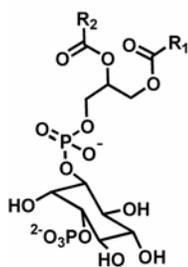


Phosphatidylinositol 3-phosphate (PtdIns(3)P)

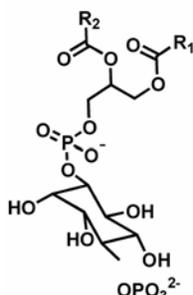
Phosphatidylinositol 4-phosphate (PtdIns(4)P)

Phosphatidylinositol 5-phosphate (PtdIns(5)P)

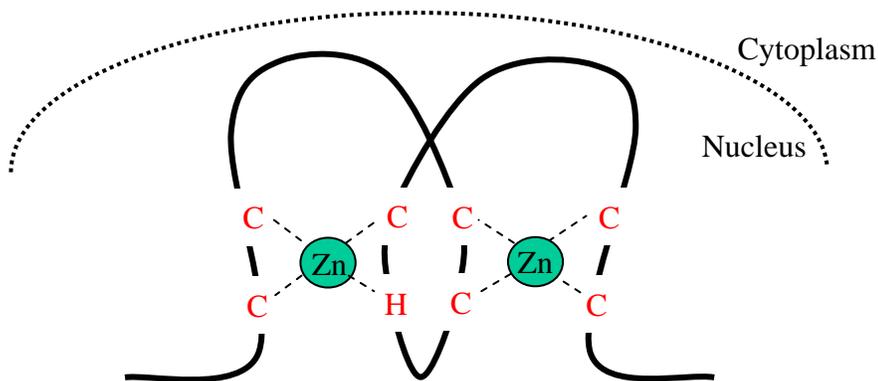
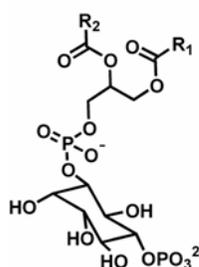
PtdIns(3)P



PtdIns(4)P



PtdIns(5)P



50 Residues Total

PTYCLCNQVSYGEMIGCDNDECPIEWFHFSCVGLN~~HK~~PKGK~~WY~~CPKCRGE

Pro Thr Tyr Cys Leu Cys Asn Gln Val Ser Tyr Gly Glu Met Ile Gly Cys
Asp Asn Asp Glu Cys Pro Ile Glu Trp Phe His Phe Ser Cys Val Gly Leu
Asn *His* Lys Pro Lys Gly Lys Trp Tyr Cys Pro Lys Cys Arg Gly Glu

FIGURE 7

1.4.2 Tumour Suppressor Gene Expression/Activity

Tumour suppressors that play a key role in cell cycle regulation and the maintenance of senescence-induced cell cycle arrest are p53 and Rb (Shay et al., 1991a; Shay et al., 1991b; Atadja et al., 1995; Mallette et al., 2004). Not surprisingly, the inhibition of these proteins using viruses, such as the human papillomavirus (HPV), results in the uncontrolled proliferation of cells as outlined in figure 5C (Bischof et al., 2005). The p53 protein was originally characterized as an antigen that was upregulated as a consequence of chemical or viral transformation of cells (DeLeo et al., 1979; Lane and Crawford, 1979; Benchimol et al., 1982; Rovinski and Benchimol, 1988). Paradoxically, the gene was originally believed to encode an oncogene since it was found associated with malignancies; however it was later found to be a mutated form of p53 (Lane and Benchimol, 1990; Hollstein et al., 1991). Thus, the wild type p53 is now defined as a *bona fide* class I tumour suppressor since a loss of function mutation can result in oncogenic transformation. Briefly, class I tumour suppressors (sometimes referred to as *gatekeepers*) are genes that acquire somatic cell mutations from the environment or hereditary mutations resulting in their loss of function. This is based on Alfred Knudson's 'two-hit hypothesis' which states that tumour suppressor genes (TSG) such as Rb must undergo inactivation at both alleles in order to induce cancer formation (Knudson, 1971). However, these observations were extended and class II tumour suppressors were described which contribute to oncogenic transformation because of decreased expression levels in

the absence of dual allele interruption (Lee et al., 1991). Members of this class of tumour suppressors are sometimes referred to as *caretakers* because their changed expression levels often predispose genomes to additional mutations thereby resulting in oncogenic transformation.

There are several emerging models involving diverse biochemical pathways describing how p53 functions as a negative regulator of growth (Sharpless and DePinho, 2002; Gu et al., 2004; Meek, 2004). One of these is by binding to DNA in a sequence-specific manner and affecting transcription of growth regulatory genes (Mietz et al., 1992). Genes encoding negative cell cycle regulators, which are transactivated by p53, include cyclin dependent kinase inhibitor p21 (originally named wild type p53 activated fragment, Waf1) and GADD45 (growth inhibition after DNA damage-45). Interestingly, p53 has also been shown to inhibit the expression of growth promoting genes such as c-fos and c-jun (Ginsberg et al., 1991; Santhanam et al., 1991; Chin et al., 1992). Consequently, it is tempting to speculate that p53 functions to maintain cellular senescence by activating expression of growth inhibitory genes and repressing the expression of growth stimulatory genes. However, upon closer examination it becomes evident that this is not the only role p53 plays in cell cycle progression control. Recent studies have also implicated p53 and p53-induced target genes such as GADD-45 in epigenetic regulation of chromatin structure (Smith et al., 2000).

Tumour suppressors are well known to be activated or transcriptionally upregulated during cellular senescence and are thought to play a pivotal role in the non-proliferative state maintained by these cells (Atadja et al., 1995; Garkavtsev et al., 1998b). In fact, the transcriptional activation of tumour suppressor genes during cellular senescence has been exploited to identify new tumour suppressors using subtractive hybridization approaches (Garkavtsev et al., 1996). Subtractive hybridization is an experimental strategy used to identify mRNA present in one sample but not in others, so by comparing cDNA libraries of genes preferentially expressed in a normal cell lines but not in cancer cell lines one can effectively identify candidate tumour suppressors (Garkavtsev et al., 1996). The ING1 gene was isolated using this strategy by comparing cDNA from normal and malignant human breast epithelial cells followed by screening of a senescent cell cDNA library to enrich for growth inhibitors and an *in vivo* biological screen (Garkavtsev et al., 1996; Garkavtsev and Riabowol, 1997). Continued functional characterization of ING1 has identified it as a protein associated with chromatin modulation as will be discussed (Aasland et al., 1995; Feng et al., 2002; Kuzmichev et al., 2002; Vieyra et al., 2002a).

Therefore, the developing model of cellular senescence with respect to tumour suppressor gene activation and carcinogenesis has suggested that replicative senescence might represent a mechanism of suppressing cancer development. However closer evaluation of cellular senescence in the context of tissue homeostasis and morphogenesis might indicate otherwise. In fact, recent

studies have shown that stromal-epithelial interactions in aging may actually promote cancer development (Parrinello et al., 2005). This finding further supports the previously mentioned theory of aging based on the *antagonistic pleiotropy* model in which natural selection has favoured genes conferring short-term benefits to the organism at the cost of deterioration in later life (Kirkwood and Rose, 1991; Campisi, 2001).

1.4.3 Senescence-Associated Epigenetic Gene Regulation

Epigenetics refers to the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in primary DNA sequence (Russo et al., 1996b). In other words, an epigenetic event is one that involves the alteration of the expression of genetic information at the transcriptional, translational or post-translational level. Induction of a cellular senescence-like phenotype by epigenetic modulation has been studied for some time. Early efforts focused on the effect of aging on histone acetylation (Oh and Conard, 1972). Then DNA promoter methylation was evaluated using an inhibitor of promoter methylation and cytidine analogue, 5-aza-cytidine, to induce a senescent phenotype (Adams and Burdon, 1982). Recently, interest in this field has resurfaced with reports addressing the mechanism by which epigenetic gene regulation affects the cellular senescence phenotype by changing the accessibility of growth regulatory gene promoters to key transcription factors. These include DNA promoter methylation (Ahuja and Issa, 2000), polycomb protein complexes in gene silencing (Gil et al., 2004) and histone modification by acetylation of

conserved lysine residues (Bandyopadhyay et al., 2002). As mentioned, ING1 plays an important role in chromatin modulation by affecting histone acetylation thereby altering chromatin structure (Aasland et al., 1995; Feng et al., 2002; Kuzmichev et al., 2002; Vieyra et al., 2002a).

The first of the growing number of functional motifs identified in the ING1 protein was the plant homeodomain (PHD) zinc finger (Feng et al., 2002). Zinc fingers are very abundant with an estimated 2%, or about 700 human genes, encoding zinc finger proteins (Klug, 1999). Zinc finger proteins can be subdivided into a growing number of classes; however the classical zinc finger (C₂H₂) is the most frequently used class of transcription factor due to its *bona fide* ability to bind DNA. Not surprisingly, zinc finger proteins have also been shown to play both a direct and indirect role in chromatin modulation (Kadam and Emerson, 2003; Nagashima et al., 2003). The zinc finger motif was discovered during biochemical studies on the transcription factor-III A (TFIIIA), which regulates the 5S ribosomal RNA genes of *Xenopus laevis* (Klug, 1999). The classical C₂H₂ motif consists of a sequence of about 30 amino acids containing two histidines (H), two cysteines (C) and three hydrophobic residues, which are all at conserved positions. It forms a small, independently folded domain stabilized by the transition element divalent cation Zn²⁺, which can be used repeatedly in a modular tandem fashion to achieve sequence-specific recognition of DNA. The domains all have the same structural framework, but achieve chemical distinctiveness through variations in key residues. The modular design

thus offers a large number of combinatorial possibilities for DNA recognition, so it is not surprising that zinc-finger domains are so widely found in nature (Klug, 1999).

The ING1 plant homeodomain zinc-finger (Figure 7) is a C₄-H-C₃ finger and has been shown to bind phosphatidylinositol monophosphates (PtdInsP) in a stress-inducible manner (Gozani et al., 2003). This is of particular importance for several reasons. First, ING1 is a strong inducer of apoptosis and cell cycle arrest under various cellular contexts (Berardi et al., 2005). This is in accordance with its role in a stress response as mediated by PtdInsP. Secondly, ING1 has been shown to have the capacity to bind DNA (Kataoka et al., 2003) and to chromatin in a stress inducible manner (Vieyra et al., 2002b; Berardi et al., 2005). Regulation of this binding activity by an ephemeral species of molecules such as PtdInsP seems plausible since these stress signals must be rapidly transduced and short-lived in order to have an appropriated physiological effect. A large portion of this doctoral dissertation focused on the physiological effects of ING1 on inducing and reinforcing the senescent state through its ability to alter chromatin structure and induce the formation of heterochromatin in chromatin domains associated with cell growth and proliferation.

1.4.4 ING1-Induced Gene Regulation and Growth Control

The ING family is encoded by five genes (Feng et al., 2002; He et al., 2005) that all contain well conserved plant homeodomains (PHDs). ING1 was originally identified as a gene preferentially expressed in normal, compared to

cancer-derived immortal epithelial cells, with significant levels seen in senescent fibroblasts (Garkavtsev et al., 1996). Ectopic expression of ING1 protein in human breast cancer cell lines and in normal human fibroblasts initially inhibits cell growth and arrests cells in the G₀/G₁ phase of the cell cycle (Garkavtsev et al., 1996; Garkavtsev and Riabowol, 1997; Garkavtsev et al., 1998a) and at later time points induces apoptosis (Helbing et al., 1997; Shinoura et al., 1999; Wagner et al., 2001a; Vieyra et al., 2002b). Conversely, ING1 antisense mRNA promotes cell growth in mouse mammary epithelial cells *in vivo* and focus and colony formation *in vitro* (Garkavtsev et al., 1996; Takahashi et al., 2002). ING1 expression is regulated through the cell cycle (Garkavtsev and Riabowol, 1997), its cellular localization is altered by UV-induced DNA damage (Scott et al., 2001a; Scott et al., 2001b; Gozani et al., 2003), and in some cell systems it appears that ING1 levels are increased by UV irradiation thereby enhancing the rate of DNA repair (Cheung et al., 2001). ING1 expression is down-regulated in a wide spectrum of human tumour types (Toyama et al., 1999; Feng et al., 2002; Nouman et al., 2003; Tallen et al., 2003), and mislocalization of ING protein has been demonstrated in varied tumour types (Nouman et al., 2002; Vieyra et al., 2003; Tallen et al., 2004).

The human ING1 gene locus encodes two predominant isoforms, p47^{ING1a} (ING1a) and p33^{ING1b} (ING1b), that appear to have opposing effects in different biological systems (Skowyra et al., 2001; Feng et al., 2002; Berardi et al., 2005). The ING1b isoform, but not the ING1a isoform, binds to the proliferating cell

nuclear antigen (PCNA) through a PCNA-interacting-protein (PIP) domain (Warbrick, 1998) in response to UV and this interaction is required for the efficient induction of UV- and ING1-induced apoptosis (Scott et al., 2001b). The ability to induce apoptosis is in part linked to transcription since the ING proteins affect the expression of a subset of genes, either independently, or in collaboration with the p53 tumour suppressor (Garkavtsev et al., 1998a; Nagashima et al., 2001; Nourani et al., 2001; Takahashi et al., 2002; Vieyra et al., 2002a; Nourani et al., 2003). ING1 may also affect gene expression by directly binding to AT-motif rich gene promoters such as p21^{Waf1} (Kataoka et al., 2003), or by interaction with hormone regulated signal transduction pathways including thyroid hormone (Wagner et al., 2001b) and the estrogen receptor (Toyama et al., 2003).

Localization studies of the ING1 PHD-binding phosphoinositides indicate their presence in heterochromatin, nucleolar-associated heterochromatin and sites of pre-mRNA processing (Boronenkov et al., 1998; Yokogawa et al., 2000; Osborne et al., 2001). ING1 has also been shown to bind chromatin with high affinity and to localize to the nucleolus in a UV-stress inducible manner (Scott et al., 2001a; Vieyra et al., 2002a). ING proteins interact with histone acetyltransferases (HATs), histone deacetylases (HDACs) and factor acetyltransferases (FATs) and affect histone and transcription factor lysine acetylation (Loewith et al., 2000; Choy et al., 2001; Nagashima et al., 2001; Nourani et al., 2001; Skowyra et al., 2001; Howe et al., 2002; Kuzmichev et al., 2002; Vieyra et al., 2002a; Shiseki et al., 2003; Toyama et al., 2003). HATs and

HDACs play an important role in controlling the transcription of genes by modifying chromatin structure, with higher levels of histone acetylation neutralizing the basic charge on histone lysine residues, thereby reducing nucleosome stability which is believed to 'open' chromatin structure and favour transcription. In contrast, increasing HDAC activity generally favours nucleosome compaction and transcriptional repression (Kuo and Allis, 1998; Narlikar et al., 2002). Furthermore, moderate levels of acetylation can destabilize nucleosomal arrays, and the acetylation of specific lysine residues can regulate the chromatin binding or enzymatic activity of other non-histone proteins (Nagashima et al., 2001; Narlikar et al., 2002; Shiseki et al., 2003). Both human and yeast ING orthologues have been shown to bind to and activate different HAT complexes (Loewith et al., 2000; Choy et al., 2001; Choy and Kron, 2002; Vieyra et al., 2002a) and to bind HDAC1 (Skowyra et al., 2001; Kuzmichev et al., 2002; Vieyra et al., 2002b).

As previously outlined, the strategy used to isolate ING1 was based upon the idea that cellular senescence limits the growth of normal human cells and is thought to play a pivotal role in hyperplasia and malignant transformation (Goldstein, 1990; Ferbeyre and Lowe, 2002). Cell senescence is believed to represent a state in which a telomere- or genotoxic stress-generated DNA damage signal causes a subsequent signal to be initiated, perhaps involving Rb, p53 and the PI3 kinase related kinase (PIKK) family members ATM and DNA-PK, that results in permanent cell cycle arrest (Atadja et al., 1995; Vaziri et al., 1997;

Baird et al., 2003; d'Adda di Fagagna et al., 2003). How this signal is generated and transduced remains poorly defined, but one mechanism is thought to be through the induction of differential gene expression with activation of growth inhibitory tumour suppressors and inactivation of growth promoting genes. Although many genes show altered expression levels in senescent cells (Wong and Riabowol, 1996; Shelton et al., 1999; Kyng et al., 2003; Zhang et al., 2003) the biochemical mechanisms by which senescence is induced and maintained remain cryptic. Accumulating evidence suggests that chromatin remodeling during cellular senescence plays a key role in the differential expression of genes during this anti-proliferative state in normal human somatic cells (Wagner et al., 2001a; Vieyra et al., 2002b; Narita et al., 2003). Inherent in this idea of altered chromatin structure during senescence is the fact that chromatin-remodeling proteins must be regulated in activity and/or localization in order to alter gene expression selectively. In this study we present data showing that one mechanism in the senescence signaling pathway is the differential expression of various ING1 gene isoform transcripts, paralleling the INK4a gene transcript (Zhu et al., 2002), which promotes histone deacetylation and contributes to the altered chromatin conformation typical of senescent cells.

1.5 Hypothesis and Objectives

1.5.1 Hypothesis

The hypothesis upon which this dissertation is based is that cell growth and senescence are dependent on both genetic and epigenetic maintenance and regulation of metazoan genomes. Furthermore, post-translational histone modification as a consequence of serial cultivation and genotoxic stress reflects the epigenetic reprogramming that occurs *in vivo* over the normal life course which affects chromatin homeostasis, and by extension gene transcription, as cells approach the end of their replicative life-span. This ultimately results in aberrant DNA metabolism and cell growth arrest that typifies the senescent state.

The specific objectives of this study include:

1.5.2 Objectives

- ✓ **Identification of novel gene promoter transcriptional regulatory elements that act to reinforce the senescent cell state.** Initial studies focused on further annotating the promoter of cyclin D1 and by extension the promoters of several genes transcriptionally activated during cellular senescence. We reasoned that cyclin D1 was a good model for senescence-associated differential gene expression since it is speculated to play a unique role in the establishment of the senescent state and is transcriptionally activated in a senescence specific manner as opposed to a cell growth specific manner. In order to establish sequence specific protein binding, a series of electrophoretic mobility shift assays were

performed using wild type and mutant oligonucleotide probes designed to represent the cyclin D1 promoter.

- ✓ **Elucidation of genetic mechanisms by which differentially expressed genes are regulated.** To further understand how this unique sequence, which was shown to differentially bind proteins in a senescence specific manner, contributed to differential gene activity, luciferase reporter constructs were designed with wild type and mutant sequences. Dual luciferase assays showed that the wild type sequence, but not the mutant sequence, was capable of differentially activating the luciferase reporter when transfected into young or senescent fibroblasts.
- ✓ **Establishing the differential expression patterns of ING1 during cellular replicative senescence.** Initial studies on ING1 indicated that this gene was activated in senescent cells, hence the name inhibitor of growth-1. However upon testing this assertion, it was found that the isoform initially reported to be differentially expressed, p33^{ING1b}, was in fact down-regulated during replicative senescence and that the other predominant ING1 splice variant, p47^{ING1a}, was activated. Further functional characterization of the ING1a splice variant showed that it played a unique and possibly antagonistic biochemical role compared to the better understood ING1b isoform.
- ✓ **Evaluation of how epigenetic modifications to chromatin structure influences gene regulation during cellular senescence.** ING1a was shown to preferentially associate with histone deacetylase complexes and activity using a functional assay

in which ING1 isoforms were immunoprecipitated and tested for HDAC activity *in vitro*. This was further explored by identifying genes that were transcriptionally activated or repressed by ING1 in an isoform specific manner. Interestingly, the proliferating cell nuclear antigen (PCNA) was found to be strongly affected by ING1 isoform overexpression suggesting that ING1 plays a role in targeting histone acetyltransferase and deacetylase activity to the PCNA promoter.

Chapter 2

MATERIALS AND METHODS

Chapter 2: Materials and Methods

2.1 Cell Culture

2.1.1 Cell types cultivated

Fibroblast cell strains used in these studies include WI-38 human lung fibroblasts (ATCC# CCL-75) and HS-68 human foreskin fibroblasts (ATCC# CRL-1635). Additionally, Werner's syndrome derived fibroblasts WS-2, WS-11 and WS-12 (a generous gift from Dr. Sam Goldstein & Ms. Elena Moerman; Department of Medicine, University of Arkansas for Medical Sciences and Geriatric Research, Little Rock, USA) and mouse embryonic fibroblasts (MEF) were serially cultivated and stocks were maintained from early to late passage stages. Cells were grown in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 100 U/ml of penicillin and 100 mg/ml of streptomycin (Gibco-BRL). Transformed cell lines used in these studies include HeLa (ATCC# CCL-2), MCF-7 (ATCC# HTB-22), SNB19 (glioblastoma cell line a generous gift from Dr. Peter Forsyth), HEK293T (ATCC# CRL-1573) and NIH3T3 (ATCC# CRL-1658). These cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 100 U/ml of penicillin and 100 mg/ml of streptomycin (Gibco-BRL). Maintenance of cells was carried out in a humidified atmosphere of 5% CO₂ and 95% air at 37⁰C. Plastic (polystyrene) tissue culture vessels were supplied by Becton Dickinson Labware (Franklin Lakes, NJ. USA) or Sarstedt Canada (St. Laurent, Canada). These are

non-pyrogenic, plasma coated vessels intended for culture of adherent cell types. Plasma treatments ensure that hydrophobic polystyrene is permanently rendered hydrophilic to support cell attachment and spreading (Ramsey et al., 1984).

2.1.2 Freezing and thawing cells

Cryopreservation of cells was done by harvesting using trypsin-EDTA treatment followed by centrifugation at 500 x g for 10 min. Pelleted cells were then resuspended in a medium containing 90% FBS and 10% sterile dimethylsulfoxide (DMSO, Sigma) to yield approximately $1 \times 10^6 - 2 \times 10^6$ cells/ml. One milliliter aliquots of cell suspension were transferred to cryovials (Nalgene, Rochester, New York) and vials were placed in styrofoam racks at -80°C for 12-18 hrs. Frozen cells were then placed upright in liquid nitrogen for long-term storage.

To thaw cells, a vial of frozen cells was removed from liquid nitrogen and placed in a 37°C water bath for 3 minutes. The thawed cell suspension was then transferred to a 15-cm plate containing 20 ml DMEM/10% FBS/1% penicillin/streptomycin and was incubated at 37°C in an atmosphere of 5% CO_2 .

2.1.3 Cultivation and splitting of cells

Cells nearing confluence were incubated in trypsin-EDTA solution (0.05% trypsin, 0.53 mM EDTA dissolved in Hanks Balanced Salt Solution) until they just began to lose anchorage from the tissue culture plates. An equal volume of DMEM/10% FBS was then added to the dish and cells were dislodged by titration

with the supernatant and seeded onto fresh tissue culture dishes. Uniform spreading of cells was achieved by gently shaking plates containing medium and cells in different directions.

2.1.4 Determining cell growth potential

In order to accurately and quantitatively assess the growth of cells, $\{^3\text{H}\}$ -thymidine or 5-bromo-2'-deoxy-uridine (BrdU) incorporation assays were performed. Cells were incubated in DMEM containing $1\mu\text{Ci/ml}$ of $\{^3\text{H}\}$ -thymidine for 24 hrs. Cells were then rinsed 3X with phosphate-buffered saline (PBS) and fixed by the addition of methanol at -20°C . For emulsion autoradiography the cells were rehydrated in PBS and coated with Kodak NTB2 nuclear track emulsion. After 24 hrs at 4°C , the emulsion was developed with one-half strength D-19 developer (Kodak). For microscopy, the cells were rehydrated with water, examined and photographed.

Alternatively, subconfluent cultures were pulsed for 4 hr with $1\mu\text{l/ml}$ of the thymidine analog 5-bromo-2-deoxyuridine (BrdU) (Amersham) at 37°C . Cells were then detached from culture plates with trypsin, fixed in 60% ethanol, and treated as follows with PBS washes between each step: 0.1 mg/ml of RNase A for 30 min at 37°C ; 2 M HCl, 0.5% Triton X 100 for 30 min at room temperature; FITC conjugated anti-BrdU (Becton Dickinson) diluted 1:5 for 1 hr at room temperature; 5 $\mu\text{g/ml}$ propidium iodide, 0.1 mg/ml RNase A, 0.1% NP-40, and 0.1% trisodium citrate for 10 min. at room temperature. Samples were analyzed

using a BD FACScan™ (BD Biosciences) at the University of Calgary Flow Cytometry Core Facility to detect both fluorescein and propidium iodide signals. For cytological analyses, cells were plated on coverslips, labelled with BrdU as above, and fixed in 5% acetic acid, 95% ice-cold ethanol for 15 min at -20°C . Positive nuclei were visualized using the Amersham cell proliferation kit, except that BrdU was detected with a FITC-conjugated secondary antibody (Calbiochem). Cells were counterstained with Hoechst (or DAPI depending on availability) to identify all nuclei, and the percentage of BrdU labelled cells (FITC/DAPI) was determined using a fluorescence microscope.

Based on these assays, HS68 fibroblasts were considered early passage cells when they had undergone up to 38 mean population doublings (MPDs). Over a 72 hr time period, these cells typically incorporated $\{^3\text{H}\}$ -thymidine or BrdU into 85-90% of cells in contrast to late passage cells which had undergone 84-86 MPDs and which incorporated $\{^3\text{H}\}$ -thymidine or BrdU into less than 10% of cells.

2.1.5 Synchronizing of cells

In order to adequately synchronize human fibroblast cells, cells were grown to confluence and split from one tissue culture dish into two in DMEM containing 10% FBS. Cells were then incubated at 37°C for 4-6 hrs to allow adherence. Following this short incubation, media containing FBS was aspirated and replaced with media lacking FBS and cells were incubated at 37°C for 4 to 5 days. The sub-confluent serum deprived cells were then stimulated by the addition

of media containing 20% FBS enabling a large portion of the cell population which had been arrested in the G₀ phase of the cell cycle to synchronously enter the G₁ phase. Alternatively, cells were split and treated with the fungal metabolite and antibiotic aphidicolin at 1 µg/mL. Since aphidicolin is a reversible inhibitor of eukaryotic nuclear DNA replication, it arrests cells prior to S-phase. Following aphidicolin-induced arrest at the G₁/S transition, cells were split and allowed to re-enter the cell cycle in normal growth media. This treatment enabled higher yield of a G₂ population of cells.

HeLa cells were grown in 10-cm plates to 25–30% confluence and synchronized using a double-thymidine-block protocol, with a first block for 16 hrs, a 10 hr release, and a second block for 15 hrs. The final concentration of thymidine used in the block medium was 2 mM.

2.1.6 In vitro Cell Transfections

Primary human fibroblast cells were transiently transfected using Lipofectamine-2000 transfection reagent (Invitrogen). Cells were grown to 95% confluence in complete medium, washed 3X in PBS and media was replaced with Opti-MEM (Invitrogen) serum-free medium. Plasmid DNA and Lipofectamine reagent were then mixed separately in Opti-MEM for 5 minutes at room temperature and the two solutions were mixed together for 20 minutes with agitation at room temperature. The ratio of µg of DNA to µl of Lipofectamine was typically 2:3. After 20 minutes of incubation, the DNA: Lipofectamine

complexes were added drop-wise to cells. Growth medium was then replaced after incubation for 4-6 hrs at 37⁰C. Cells were harvested and assessed for transgene expression 12 hrs after Lipofectamine treatment.

Electroporation was also used to transfect cells. Briefly, 5 x 10⁵ log phase young and senescent HS68 fibroblasts were seeded in 150 mm plates and harvested when ~80% confluent. Cells were trypsinized, suspended in 400 µl of serum free DMEM and transferred to 4 mm gap cuvettes (BTX Inc., San Diego). Cyclin D1 promoter-chloramphenicol acetyl transferase (CAT) reporter plasmid (30 µg) was added to 5 µg of a CMV driven β-galactosidase expression construct and 15 µg of salmon sperm DNA to make a total DNA content of 50 µg per cuvette. Electroporations were done using a Biorad gene pulser at 250 V and 960 µF and, following transfection, samples were transferred to 10 cm plates. Cells were harvested 48 h post-transfection and assayed for CAT activity.

2.2 Protein Purification

2.2.1 Preparation of cell lysates

Cell culture plates were washed 3X with cold (4⁰C) PBS and then scraped on ice in RIPA (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM KCl, 1 mM EDTA, 0.25% deoxycholate, 0.25% Nonidet P-40, 0.25% Tween-20) lysis buffer if lysate was used for immunoprecipitations. Cell suspensions were transferred into 1.5 ml eppendorf tubes, sonicated 3 times for 5 sec. each on ice, pelleted for 10 min. at 4⁰C at 13000 x g and the supernatants were used immediately or snap frozen in liquid nitrogen and stored at -80⁰C. Whole cell extracts used for western blotting experiments were harvested by rinsing cell culture plates with PBS and then scraping cells in 1 ml of sample buffer added directly to cells on the plate. Protein lysates in sample buffer were then used immediately or frozen indefinitely.

2.2.2 Nuclear isolation and high salt extraction

Cells grown to the desired density under appropriate conditions were rinsed with cold PBS, scraped and triturated in PBS containing 0.05% NP40. The released nuclei were pelleted by centrifugation at 500 x g for 30 sec. and extracted with a buffer containing 25 mM HEPES (pH 7.8), 500 mM KCl, 5 mM NaF, 0.5 mM MgSO₄, 1 mM DTT and the protease inhibitors aprotonin, pepstatin and leupeptin at a concentration of 0.1 mg/ml each.

2.2.3 Electro-transfer of proteins from gel to solid support

SDS-PAGE gels were run for 1000 volt hours when using the large gel apparatus and 150 volt hours when using the mini-gel apparatus. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose (PALL Corporation) or polyvinylidene fluoride (Millipore Corporation) was done using the Quick-E Blotter (Shadel Inc.) for large gels, or the Mini-Protean II vertical electrophoresis system (Biorad) for mini-gels. Proteins were isolated as described above, electrophoresed through polyacrylamide gels and transferred onto appropriate membrane support for 2.5 hours at 65 volts in a transfer buffer containing 120 mM Tris base (pH 8.0), 40 mM glycine and 20% methanol.

2.2.4 Placental tissue protein extraction

A freshly delivered placenta was cut into small pieces and washed in cold PBS to remove blood and mucous. The pieces were then homogenized for 5 min. in a buffer containing 150 mM Tris (pH 7.5), 60 mM KCl, 150 mM β -mercaptoethanol, 300 mM sucrose and the protease inhibitors aprotonin, pepstatin and leupeptin at 0.1 mg/ml each. The homogenate was strained through a piece of gauze to separate liberated cells from unbroken tissue. The cell suspension was briefly centrifuged at 500 x g and the pellet of cells that was recovered was resuspended in PBS containing 0.05% NP40. The resuspended pellet was then homogenized using a Dounce homogenizer to release nuclei. The nuclei were

briefly pelleted and nuclear proteins were extracted by incubating the nuclear pellet in three times its volume of a buffer containing 500 mM KCl, 25 mM HEPES (pH 7.8), 5 mM NaF, 0.5 mM MgSO₄, 5% glycerol, 1 mM DTT and 1 mg/ml each of aprotonin, pepstatin and leupeptin for 30 min. with mixing. The mixture was then centrifuged at 10 000 x g and the supernatant was aliquoted, snap frozen in nitrogen and stored at -80°C.

2.2.5 DNA affinity chromatography

The desired amount of Affigel-10 matrix (Biorad) was poured into a conical tube and washed in ice-cold coupling buffer by resuspending the beads in buffer and pelleting in a tabletop centrifuge 3-4 times. Coupling buffer is 50 mM Hepes (pH 7.6), 100 mM KCl or 100 mM Hepes (pH 7.6), 80 mM CaCl₂, 1 mM MgCl. Wild type or mutant DIE complementary sequence oligonucleotides were synthesized with a primary amino group on the 5'-terminus of sense strands. Complementary oligonucleotides were then annealed in oligonucleotide annealing buffer containing 10 mM Tris·HCl (pH 7.5 – 8.0), 50 mM NaCl and 1 mM EDTA and conjugated to the washed Affigel-10 matrix as described by the manufacturer. This allowed a stable amide bond to be formed between the matrix and the modified 5'-terminus of the sense strand of the double-stranded oligonucleotide. In parallel, aliquots of supernatant were sequentially removed to measure ligand (double-stranded oligonucleotide) to matrix (Affigel-10 support) coupling efficiency. The coupling reaction was stopped by adding 1 M Tris (pH 7.5) to a

final concentration of 50 mM. Placental nuclear extract containing 500 mg of total protein was further clarified by centrifugation at 45 000 x g for 60 min. followed by a heparin-agarose ion exchange column to enrich for DNA-binding proteins. The lysate was then passed through a 5 ml bed volume column of Affigel-10/ligand in a binding buffer containing 20 mM Tris (pH 7.6), 50 mM KCl, 1 mM EDTA, 0.2 mM ZnCl₂, 0.05% Nonidet, 1 mM DTT, 0.5 mM benzamidine and 1 mg/ml each of aprotonin, pepstatin and leupeptin. The column flow-through was pre-incubated with 5 mg/ml poly d(I)d(C) or sheared salmon sperm DNA for 30 min., loaded onto another 5 ml column and the column was washed with 100 ml of binding buffer containing 250 mM KCl. The column was then eluted with a step gradient of KCl in binding buffer. The fractions were collected and concentrated using Centricon 30 (Diamed Lab Supplies Inc.) concentrator tubes, dialysed against a buffer containing 50 mM Tris pH 7.4, 10 mM KCl and 1 mM DTT and proteins were analyzed by SDS-PAGE.

2.3 Protein Assays

2.3.1 *Bradford Assays*

The Bradford reagent was prepared by dissolving 100 mg of Coomassie brilliant blue G250 (Biorad) in 50 ml of 95% ethanol. 100 ml of concentrated phosphoric acid was added followed by distilled water to a final volume of 200 ml. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. Within the linear range of the assay, the more protein present, the more Coomassie binds (Bradford, 1976). Standard curves were routinely prepared using at least six dilutions of 100 mg/ml bovine serum albumin (New England Biolabs).

2.3.2 *Western Blotting*

For western blots, denatured whole cell lysates (~30 µg) in Laemmli sample buffer were electrophoresed (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Detection of ING proteins was done with a panel of monoclonal α -ING1 (CAb) antibodies or with ING1 isoform-specific polyclonal rabbit antibodies where indicated (Boland et al., 2000). Both rabbit and mouse α -ING1 antibodies recognize native and denatured forms of ING1b, ING1a and the truncated p24^{ING1c} isoform. Additional antibodies used for western blots in the course of this study include cyclin D1 (Ab-1: DCS-6,

Lab Vision: NeoMarkers) mouse monoclonal, p21 (F-5: SC-6246, Santa Cruz) mouse monoclonal, p16 (Ab-1: DCS-50.1, Lab Vision: NeoMarkers) mouse monoclonal and PCNA (PC10: SC-56, Santa Cruz) mouse monoclonal or PCNA (FL-261: SC-7907, Santa Cruz) rabbit polyclonal. Antibodies used as loading controls for western blots include α -Tubulin (Ab-1) mouse monoclonal (Oncogene Research Products) Actin (C-2: SC-8432, Santa Cruz) mouse monoclonal and QM (C-17: SC-798, Santa Cruz) rabbit polyclonal.

2.3.3 Immunofluorescence

Cells were cultured, transfected, fixed and mounted as described (Atadja et al., 1995). Fixed cells were incubated with CAbs1-4 (Boland et al., 2000), washed and incubated with phalloidin-conjugated-FITC to stain actin plus a goat anti-mouse IgG-Alexa 488 (Cedarlane) or -Cy5 (Chemicon) to visualize ING1 proteins. After rinsing, samples were mounted in 1 μ g/ml paraphenylenediamine in PBS/90% glycerol containing 4',6-diamidino-2-phenylindole (DAPI) at 1 μ g/ml. Imaging was performed using an Olympus FluoView confocal laser scanning system (model FV300), capturing images using the Olympus FluoView (FV300) software.

2.4 DNA Techniques

2.4.1 Plasmid Preparations

Small scale and large scale plasmid preparations were done using Qiagen plasmid preparation kits. These plasmid purification protocols are based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, dyes and low molecular weight impurities are removed by a medium salt wash. Plasmid DNA is then eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

2.4.2 Restriction Endonuclease Digestion

Optimal buffer and temperature conditions for each enzyme used were chosen according to manufacturer's protocol. A typical reaction included 0.2 – 20 µg of DNA, and one unit of restriction enzyme per microgram of DNA in a final volume of 10 – 50 µl. Analysis of restriction fragments was done using agarose gel electrophoresis as described below. Restriction enzymes were purchased from the following companies: Pharmacia (Baie d'Urfe, Quebec), Gibco BRL (Burlington, Ontario), Boehringer Mannheim (Laval, Quebec) and New England Biolabs (Pickering, Ontario).

2.4.3 Fractionation of DNA by Agarose gel electrophoresis

Fractionation of DNA according to size or conformation (linear, coiled or super-coiled) was done as outlined in the *Molecular Cloning* manual (Sambrook

et al., 1989). DNA samples were mixed in DNA gel loading buffer (120 mM EDTA, 60 mM Tris·HCl (pH 8.0), 18% Ficoll, 0.15% bromophenol blue, xylene cyanol) and loaded into wells in agarose gels containing 0.6 – 2.0% agarose (Invitrogen). Gels were run in TAE running buffer (40 mM Tris-acetate (pH 8.0) and 1 mM EDTA) in a horizontal system for submerged gel electrophoresis (Shadel Inc. or Biorad) at room temperature. DNA was stained with ethidium bromide using different methods depending on application. Diagnostic agarose gels were generally made with the addition of 0.5 mg/ml of ethidium bromide to the gel. DNA agarose purification gels were stained after the gels were run by soaking the gel in TAE solution containing 0.5 mg/ml of ethidium bromide at room temperature for 20 mins. DNA was visualized using ultraviolet illumination (Transilluminator Model VWR LM-20E, VWR Scientific Products) at 302 nm or 365 nm. Images were captured digitally using a KODAK DC-120 zoom digital camera and analyzed using KODAK 1D Image Analysis Software (Mandel Scientific Company Inc., Guelph, ON).

2.4.4 Ligation of DNA Molecules

DNA fragments used for ligations were purified by agarose gel electrophoresis. Reactions were performed as outlined in the *Molecular Cloning* manual (Sambrook et al., 1989). Ligations were done using various molar ratios of insert to plasmid DNA and typically included restriction digested plasmid DNA, insert DNA, ligation buffer, 1 mM ATP and 1 U of T4 DNA ligase

(Pharmacia) in a 20 μ l final volume. Incubations were generally done at 15⁰C for 12 – 16 hrs for cohesive-end ligations or room temperature overnight for blunt-end ligations. In some cases blunt-end ligations were done using higher concentrations of T4 DNA ligase for shorter incubation times. Digested plasmids were always ligated in the absence of insert as a control.

2.4.5 Nested Deletions of the Cyclin D1 Promoter

The cyclin D1 promoter analysis project was initiated by Dr. Muthupalaniappan Meyyappan prior to my arrival in the laboratory. Nested deletions of the cyclin D1 promoter were made by Dr. Meyyappan. The 1.3 kb human cyclin D1 promoter in pUC118 vector was a generous gift from Yue Xiong (University of North Carolina, Chapel Hill). The vector was digested with SacI/PvuII to release the 1,291 bp promoter containing 138 bp of the 5'-UTR. The ends of the fragment were filled in with Klenow polymerase (Gibco-BRL) and it was subcloned into the SmaI site of pBluescript II KS. Nested deletions were performed with a double stranded nested deletion kit (Pharmacia). For 5' nested deletions, the cyclin D1 promoter in pBluescript was digested with HindIII, filled in with thionucleotides and redigested with EcoRV at the 5' end. Deletions were made by Exonuclease III digestion for various time points followed by S1 nuclease treatment. The deleted constructs were analysed by agarose gel electrophoresis, ligated and transformed to obtain clones, which were screened and sequenced for the desired deletions. Deleted clones in pBluescript were

digested with Sall/BamH1 and subcloned into the Sall/BamH1 site of the pBLCAT3 vector. Clones were designated as -1,154 (full length), -858, -580, -459, -425, -198, -85, -23 and +26 relative to the transcriptional start site.

For 3' deletions of the 5'-UTR, the -23 clone in pBluescript was cut with SpeI, filled in with thionucleotides and redigested with BamH1 at the 3' end. After Exonuclease III/S1 nuclease treatment, the deleted clones were ligated, screened and sequenced. Clones with 3' deletions were digested with Sall/XbaI and subcloned into the Sall/XbaI sites of the pBLCLAT3 vector. Two clones with the 3' end deleted were designated as -23 to +74 and -23 to +24.

2.4.6 Site-directed Mutagenesis

The cyclin D1 promoter analysis project was initiated by Dr. Muthupalaniappan Meyyappan prior to my arrival in the laboratory. Site-directed mutagenesis of the cyclin D1 promoter was performed by Dr. Meyyappan. A total of 8 bp between +117 and +131 of the 5'-UTR were mutated using a Quik-change site directed mutagenesis kit (Stratagene). The Quik-change site-directed mutagenesis kit can be used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids. Briefly, a mutagenic primer (46mer) was synthesized and annealed to the double stranded pBLCAT3 construct containing the full-length cyclin D1 promoter. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Pfu DNA polymerase was used to synthesize the mutagenic promoters followed by digestion of the parental plasmid by DpnI. The DpnI endonuclease (target

sequence: 5'-Gm6ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to DpnI digestion. The nicked vector DNA containing the desired mutations is then transformed into XL1-Blue competent cells and the resulting plasmid was isolated and sequenced to confirm the mutation. The small amount of starting DNA template required for this method, the high fidelity of the Pfu DNA polymerase and the low numbers of thermal cycles all contribute to the high mutation efficiency and decreased potential for generating random mutations during the reaction.

2.4.7 DNA Sequencing

DNA sequencing was done by the University Core DNA (UCDNA) facility (Calgary) using high-throughput Applied Biosystems 3730S and 377 automated DNA sequencers and ABI BigDye® Terminator chemistry. This is an automated fluorescence-based dideoxy DNA sequencing method based on the Sanger sequencing method. Briefly, dideoxy DNA sequencing is an enzymatic reaction used to decipher the nucleotide sequence of previously cloned DNA. In its simplest form (manual sequencing), this method is based on using four different chain termination nucleotides (ddATP, ddGTP, ddCTP, ddTTP) in separate reactions to generate DNA products that are resolved by polyacrylamide gel electrophoresis. The 2',3'-dideoxynucleotide triphosphates (ddNTPs) used in this method differ from deoxynucleotides by the having a hydrogen atom attached

to the 3' carbon rather than a hydroxyl group resulting in molecules that terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide. Automated dideoxy DNA sequencing using fluorescently labeled ddNTPs is typically used today.

2.4.8 Preparation of Genomic DNA

To isolate genomic DNA from adherent cell cultures, cells were trypsinized and centrifuged for 5 min. at 500 x g and the supernatant was discarded. Pelleted cells were then washed in 1 ml of ice cold PBS and centrifuged. After washing cells were resuspended in approximately 1 volume of digestion buffer (100 mM NaCl, 10 mM Tris (pH 8.0), 25 mM EDTA, 0.5% SDS and 0.1 mg/ml proteinase K). Cells were then incubated with shaking at 50⁰C for 12 – 18 hrs. After overnight digestion, nucleic acids were extracted as described below.

2.4.9 Phenol/Chloroform Extraction and DNA Precipitation

An equal volume of tris-saturated phenol/chloroform/isoamyl alcohol was added to digested cells and vortexed. Samples were then centrifuged for 2 min. at 13 000 rpm at 4⁰C and the supernatant (aqueous phase) was transferred to a fresh tube. An equal volume of chloroform was then added to remove residual phenol and the samples were vortexed and centrifuged again. The supernatant was transferred to a fresh tube and 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol were added. The solution was then vortexed and incubated

overnight at -20°C . The following day the samples were centrifuged for 30 min. at 13 000 rpm at 4°C and the supernatant was discarded. A final 70% ethanol wash was then done to remove excess salt and the pelleted DNA was dissolved in 10 mM Tris (pH 7.6). The final DNA concentration was determined by absorbance (A_{260}) using a GeneQuant spectrophotometer (Pharmacia) and the A_{260}/A_{280} ratio was determined to assess DNA purity (pure DNA $A_{260}/A_{280} = 1.8$).

2.4.10 DNA Sequence Analysis

All sequence analyses were performed using the Wisconsin Package™ (Version 9.1) from the Genetics Computer Group (GCG) available through the Canadian Bioinformatics Resources (CBR) website (<http://cbr-rbc.nrc-cnrc.gc.ca/>). Promoter sequences in the GeneBank DNA sequence database were obtained from the National Center for Biotechnology Information (NCBI). Accession numbers identifying particular sequences are listed in the results. Sequences were searched for the 15 bp DIE using the FINDPATTERNS program, allowing for 33.3% (5/15) random mismatch. Further DIE-like element comparison was done using the PRETTY program to find those elements with highest sequence similarity to the DIE.

2.5 RNA Techniques

2.5.1 Isolation of RNA from Cells in Culture

Total RNA extraction was done by lysing cells directly on the culture dish by addition of 1.0 ml of Trizol reagent (Gibco BRL) and passing the cell lysate several times through a pipette. Homogenized samples were then incubated for 5 min. at room temperature and 0.2 ml of chloroform was added. After a short incubation (3 min.) samples were centrifuged at 12 000 x g for 15 min. at 4⁰C. The upper aqueous phase was then removed and RNA was precipitated using isopropyl alcohol. The RNA pellet was then washed in 75% ethanol and the pellet was allowed to air-dry. After a short time drying at room temperature, the RNA pellet was resuspended in diethyl pyrocarbonate (DEPC) water and used immediately or stored at -80⁰C. DEPC is a histidine-specific alkylating agent that acts to modify histidine residues within the RNase active site thereby rendering it inactive. The final RNA concentration was determined by absorbance (A_{260}) using a GeneQuant spectrophotometer (Pharmacia) and the A_{260}/A_{280} ratio was determined to assess RNA purity (pure RNA $A_{260}/A_{280} = 2.0$). To eliminate any DNA contamination, RNA samples were treated with DNaseI for 30 min at 37⁰C. DNaseI was then inactivated by treating sample for 5 minutes at 75⁰C.

2.5.2 Synthesis of cDNA from mRNA

Reverse transcription of mRNA was done using the method described in the Current Protocols manual (Ausubel et al., 1987). Briefly, first strand synthesis

was done in a solution containing 100 pmol of oligo(dT) or random hexanucleotide primers (Pharmacia), 10 mM of each dNTP (Pharmacia) and 2 µg of isolated RNA in DEPC water. These components were mixed and incubated for 5 min. at 70°C and immediately placed on ice for 5 min. to disrupt any secondary structures formed by RNA. 1X first strand synthesis buffer (Invitrogen), 0.1 M DTT and RNase Out (Invitrogen) were mixed during the 5 min. incubation of samples on ice and were added to the mix and placed in the PCR for 10 min. at 25°C for primer annealing followed by 2 min. at 42°C. At this point 1µl of Superscript II reverse transcriptase (Invitrogen) was added to the reaction and the reverse transcription proceeded as follows: 42°C for 50 min. for primer extension and 70°C for 10 min. to terminate the reaction. The final cDNA concentration was determined by absorbance (A_{260}) using a GeneQuant spectrophotometer (Pharmacia) and the A_{260}/A_{280} ratio was determined to assess cDNA purity (pure DNA $A_{260}/A_{280} = 1.8$). RNase Out (Invitrogen) and RNA Guard (Pharmacia) are both recombinant ribonuclease inhibitors and were substituted based on availability.

2.5.3 *Semi quantitative RT-PCR*

Multiplex PCRs in 50 µl reaction volumes contained 2 µl of reverse transcriptase (RT) reaction product as template DNA (corresponding to cDNA synthesized from 100 ng of total RNA), 1X PCR buffer, 80 µM of each deoxynucleotide and 20 pmol of each 5' and 3' primer. Two units of Taq DNA

polymerase (Gibco) were added to each tube during the first denaturation step and equal aliquots (20 pmol) of secondary primer sets were added at the appropriate cycle number by the “primer-dropping” method (Wong et al., 1994). Aliquots of PCR products chosen to give equivalent signals from the internal control (GAPDH) were electrophoresed (2% agarose gels; 100 V) and incubated in 0.01 µg/ml of ethidium bromide to visualize bands that were quantitated by digital densitometry using the *Discovery Series Quantity One 1-D Analysis Software* (Biorad). PCR primers had GC contents of 40-60% and were separated by introns. Oligonucleotide sequences of primers (5'-3') and the predicted PCR product sizes were:

Name	Primer Sequences (5'-3')	Product Size (bp)
ING1a	GAGAATTCGGGTGGAGGGTGGAC TTGCGAATTCTGTTTCCACGGCCA	800
ING1b	GAAGATCCAGATCGTGAGCC GAGACCTGGTTGCACAGACA	300
p21	AGTGGACAGCGAGCAGCTGA TAGAAATCTGTCATGCTGGTCTG	380
p16	AGCATGGAGCCTTCGGCTGACT CTGTAGGACCTTCGGTGACTGAT	514
Cyclin D1	AGCCATGGAACACCAGCTCCTGTG GATGGAGCCGTCGGTGTAGATGCA	399
Cyclin A (A2)	TCCAAGAGGACCAGGAGAATATCA TCCTCATGGTAGTCTGGTACTTCA	466
PCNA	TCCATCCTCAAGAAGGTGTTGGAG CAGACATACTGAGTGTCCACCGTTG	659
GAPDH	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC	150

2.6 Functional Assays

2.6.1 *Beta-Galactosidase Assay*

In vitro senescence-associated beta-galactosidase (SA- β -gal) activity as a measure of senescence was detected as described (Dimri et al., 1995). Cells were washed with phosphate-buffered saline (PBS pH 7.2), fixed with 0.5% glutaraldehyde in PBS for 3-5 min at 22⁰C, washed with PBS (pH 6.0), and stained for 16-24 h at 37⁰C with 1 mg/ml of 5-bromo-4-chloro-3-indolyl- β -galactoside (X-gal) in PBS (pH 6.0) containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mM MgCl₂.

2.6.2 *Electrophoretic Mobility Shift Assay*

Nuclear extracts from young and old HS68 HDFs were prepared as described previously (Riabowol et al., 1992; Atadja et al., 1994). A double stranded 64 bp oligonucleotide spanning nucleotides +75 to + 138 and the overlapping oligonucleotides {both wild-type (Wt) and mutant (Mu)} were synthesized with 5'-overhangs, end labelled with Klenow enzyme (0.5U, GIBCO-BRL) and 50 μ Ci { α ³²P}-dCTP (3000 Ci/mmol, Amersham), and gel purified. 20,000 cpm of labelled probe (0.1 to 0.5 ng) was incubated with 5 μ g of nuclear extracts from young or old cells in binding reactions that contained 20 mM Hepes (pH 8.0), 25 mM KCl, 5 mM MgCl₂, 5% glycerol, 2 mM DTT, 0.1 mM EDTA and 2 μ g poly (dI-dC) as described previously (Ince and Scotto, 1995). The reactions were incubated for 30 min at room temperature, electrophoresed through

5% non-denaturing polyacrylamide gels at 150 V at room temperature, dried and visualized by autoradiography.

Oligonucleotides corresponding to the cyclic AMP response element (CRE) and binding conditions for this element have been described previously (Atadja et al., 1994). Briefly, 20,000 cpm (0.1 to 0.5 ng) of gel purified $\{\alpha^{32}\text{P}\}$ -dCTP labelled probe was incubated with 5 μg of nuclear protein extract in a buffer that contained 20 mM Tris (pH 7.6), 4% Ficoll, 50 mM KCl, 1 mM EDTA, 0.2 mM DTT, 10 μM ZnCl_2 , 1 μg partially denatured salmon-sperm DNA and 30 μg bovine serum albumin in a final volume of 20 μl for 30 min at room temperature. Unlabelled wild type (Wt) and mutant (Mu) competitor DNA was added at a 100-fold excess and incubated for 10 min at room temperature before the addition of hot probe.

2.6.3 UV cross-linking of DNA-protein complexes

The cyclin D1 promoter analysis project was initiated by Dr. Muthupalaniappan Meyyappan prior to my arrival in the laboratory. UV cross-linking of DNA-protein complexes were performed by Dr. Meyyappan. Photochemical crosslinking is a powerful method for studying protein-nucleic acid interactions. UV-light is a zero-length crosslinking agent that predominantly or exclusively cross-links proteins to nucleic acids at their contact points. It can therefore provide strong evidence for close protein-nucleic acid interactions. However, to achieve an acceptable degree of crosslinking with conventional UV-

light sources, exposure times ranging from minutes to several hours are necessary. Such prolonged irradiation allows for the artifactual redistribution of proteins and precludes kinetic studies. The use of UV lasers overcomes these difficulties since the number of photons required for the crosslinking may be delivered in time intervals on the order of nano- or picoseconds.

To estimate the relative molecular weight of the DNA binding proteins, binding reactions using various oligonucleotides as described for the EMSA assays were performed. Twice the amount of labelled probe (40,000 cpm) and nuclear extract (10 µg) were used. After 30 min. incubation at room temperature, the binding reaction was subjected to UV light and unprotected DNA was digested using DNase I. Samples were irradiated by a 305 nm inverted UV transilluminator at 7 mW/cm² for 5 min. The crosslinked reactions were electrophoresed through 15% sodium dodecyl sulphate (SDS) polyacrylamide gels, dried and visualized by autoradiography.

2.6.4 Chloramphenicol Acetyltransferase (CAT) Reporter Assay

The cyclin D1 promoter analysis project was initiated by Dr. Muthupalaniappan Meyyappan prior to my arrival in the laboratory. *In vitro* CAT assays of the cyclin D1 promoter were done by Dr. Meyyappan. Briefly, electroporated cells were washed twice with ice-cold PBS, harvested and centrifuged. The cell pellet was washed with PBS, resuspended in 100 µl of lysis buffer (0.25 M Tris-Cl, pH 7.8) and subjected to three freeze-thaw cycles in liquid

nitrogen with rigorous vortexing between each step. The cell lysates were centrifuged at 10,000 rpm for 5 min and the supernatants containing the cell extract were used for β -galactosidase assays as previously described (Swisshelm et al., 1994). Briefly, 10 μ l of cell extract was added to a tube containing 90 μ l of lysis buffer, 350 μ l of LacZ buffer (60 mM Na_2HPO_4 -40 mM NaH_2PO_4 (pH 7), 10 mM KCl, 1 mM MgSO_4 , 50 mM β -mercaptoethanol) and 50 μ l of chlorophenolred- β -D-galactopyranoside (CPRG) (30 $\mu\text{g}/\mu\text{l}$) (Boehringer Mannheim). The mixture was incubated for 1 hr at 37°C and the absorbance at 574 nm was determined. Activity resulting from the co-transfected CMV- β -galactosidase plasmid was determined by subtraction of endogenous basal activity values obtained from mock-transfected young and old fibroblasts. Extracts corresponding to equal units of β -galactosidase activity were used for CAT assays as described previously (Gorman et al., 1982). The proportion of acetylated product was determined by liquid scintillation counting of excised thin-layer chromatography spots.

2.6.5 Luciferase Reporter Assay

Constructs containing Wt and Mu DIE elements were made using pGL3 Control reporter plasmids. The 15 bp HPLC purified oligonucleotides were cloned into the HindIII site found between the SV40 promoter and luc^+ reporter gene. This cloning site is found outside of the pGL3 control multi-cloning site and was chosen because of its proximity to the functional gene (i.e. the *in vivo* DIE is

located in the 5'-UTR of the cyclin D1 gene). Therefore, the DIE was cloned upstream of the luc⁺ start codon but downstream of the SV40 promoter. These constructs were then co-transfected into young and senescent primary fibroblasts with a *Renilla* luciferase construct (pRL-TK) as a transfection efficiency control. Firefly luciferase expression was measured using an *EG & G Berthold* luminometer and normalized based on pRL-TK expression. Since the firefly and *Renilla* luciferases have dissimilar enzyme structures and substrate requirements they can be selectively discriminated between their respective bioluminescent reactions. Subsequently, when measuring reporter activity the firefly luciferase is analyzed then quenched while the luminescent reaction of *Renilla* luciferase is simultaneously activated. Firefly luciferase converts the beetle luciferin substrate to oxyluciferin in an Mg²⁺-dependent manner while *Renilla* luciferase converts coelenterazine to coelenteramide. Both of these reactions result in light emission that can be easily quantitated using a luminometer.

2.6.6 Poly(ADP-ribose) Polymerase Cleavage

Western blots of lysates from transfected cells were done using mouse α -PARP1 (C2-10, Trevigen Corp.) to identify the cleavage product of poly(ADP-ribose) polymerase, an early marker for the apoptotic caspase cascade (Duriez and Shah, 1997; Soldani and Scovassi, 2002).

2.6.7 Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling

Apoptotic cells were analyzed using an ApopTag® Fluorescein terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) kit (*Intergen*). Briefly, cells were fixed in 1% paraformaldehyde then rinsed with 70% ethanol, washed in PBS, pelleted and resuspended in terminal deoxynucleotidyl transferase (TdT) enzyme and incubated at 37°C for 1 hr. Reactions were stopped, cells were washed and pelleted, resuspended in anti-digoxigenin-fluorescein and incubated for 30 min. at room temperature. After washing in PBS containing 0.1% Triton X-100, DNA was stained with propidium iodide and cells were analyzed using a BD Biosciences FACScan flow cytometer. Cell cycle analyses were performed by harvesting and washing transfected and control cells in parallel. Cells fixed with ethanol and RNase-treated for 30 min. at room temperature were stained with propidium iodide and analyzed using a BD FACScan™ (BD Biosciences) at the University of Calgary Flow Cytometry Core Facility.

2.6.8 Fluorescence Activated Cell Sorting DNA/Cell Cycle Analysis

To prepare adherent cells for FACS cell cycle analysis, cells were trypsinized until partially dislodged and an equal amount of complete media was used to further triturate cells. Samples were then centrifuged at 800 rpm for 10 min at 4°C in 15 ml conical centrifuge tubes. The media was aspirated and the cell pellet was resuspended in 1 ml of ice cold PBS. 1 ml of 95% ethanol was then

added drop wise to permeabilize cells and cells were either stored at -20°C or processed further. Cell suspensions were placed in 5 ml polystyrene round bottom tubes (Becton Dickinson 35-2054) and centrifuged at 800 rpm for 10 min. at 4°C and supernatant was aspirated. Pelleted cells were washed in PBS and centrifuged. The washed cell pellet was then resuspended in 500 μl of PBS and incubated for 30 min. at room temperature with 250 μl of 1 mg/ml RNase A. An additional 250 μl of 50 $\mu\text{g/ml}$ propidium iodide was added to tubes (final volume 1 ml), and samples were incubated in the dark for 20 min. at room temperature. FACS analysis was performed using a BD FACScan™ (BD Biosciences) at the University of Calgary Flow Cytometry Core Facility.

2.6.9 Immunoprecipitation-Western Assays

Primary and transformed cell lines were harvested and lysed under non-denaturing conditions in 10 mM Tris·HCl, pH 7.5/ 1mM EDTA/ 400 mM NaCl/ 10% (vol/vol) glycerol/ 0.5% Nonidet P-40/ 5 mM NaF/ 0.5 mM sodium orthovanadate/ 1 mM DTT and a protease inhibitor mixture (aprotinin, leupeptin and pepstatin). Alternatively, Complete Mini, EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics) were used in cell extraction buffer. For western blots, denatured whole cell lysates ($\sim 30 \mu\text{g}$) in Laemmli sample buffer were electrophoresed (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Detection of ING proteins was done with a panel of monoclonal α -ING1 (CAb) antibodies or with ING1 isoform-specific polyclonal

rabbit antibodies where indicated (Boland et al., 2000). Both rabbit and mouse α -ING1 antibodies recognize native and denatured forms of ING1b, ING1a and the truncated p24^{ING1c} isoform. Immunoprecipitations of non-denatured cell lysates used 5 μ l of polyclonal or affinity purified monoclonal anti-ING1 antibodies, or 50 μ l of concentrated hybridoma supernatant. Additionally, affinity purified ING1b specific rabbit polyclonal antibodies (Product #: KAP-CC025; Stressgen Bioreagents) were used in isoform specific immunoprecipitations. Proteins were visualized in western blots using 1:500 to 1:1000 dilutions of polyclonal or affinity purified monoclonal antibodies (\sim 200 μ g/mL), or with monoclonal hybridoma supernatants diluted 1:1 with the same buffer used to dilute polyclonal antibodies. Horseradish peroxidase (HRP)-conjugated secondary antibodies against the appropriate species that were used for western blotting were from Amersham-Pharmacia Biotech, used at 1:1000 and blots were developed using chemiluminescent substrate.

2.6.10 Immunoprecipitation-HDAC Assays

This project was done in collaboration with Dr. Dallan Young's laboratory at the University of Calgary. A post-doctoral fellow in the laboratory, Dr. Ana Colina, performed the HDAC assays outlined here. HDAC fluorescent activity assays using a *Fluor de Lys* substrate (BIOMOL), were performed according to the manufacturer's instructions using Fluoroskan Ascent Software (Zhou et al., 2001). All reactions were incubated for 30 min. at 22 °C to evaluate histone

deacetylase activity and 1 μ M trichostatin A (TSA) was used to inhibit this reaction as a negative control. Total cell extracts were incubated with 20 μ l of protein G Sepharose and antibody for 4-6 hours at 4 °C. The beads were washed four times with 1 ml of PBS and assayed for deacetylase activity using an HDAC Fluorescent Activity Assay/Drug Discovery Kit (AK-500; BIOMOL Research Laboratories).

2.6.11 Thymidine Incorporation Chromatin Immunoprecipitation Assays

Radiolabelled ChIP assays were done by labeling with $\{^3\text{H}\}$ -thymidine (5 μ Ci ml⁻¹) for 24 hrs. Rabbit polyclonal PKC- δ (Santa Cruz; SC-937), rabbit polyclonal acetylated histone H4 (Upstate; 06-946), and rabbit polyclonal ING1a or ING1b (Boland et al., 2000) antibodies were added and lysates were immunoprecipitated for 4 hours at 4 °C with gentle rocking. ChIP assays and assays of immunoprecipitates were done as described (Hasan et al., 2001). These assays were performed by Dr. Karl Riabowol at the University of Calgary.

2.6.12 Chromatin Immunoprecipitation-PCR Assays

Oligonucleotide sequences of primers for cyclin A, PCNA and β -actin promoters were kindly provided by the laboratory of Dr. Scott Lowe from the Cold Spring Harbour Laboratory (Narita et al., 2003). Immunoprecipitations were done as described previously (Weinmann et al., 2001). Briefly, chromatin was crosslinked using 1% formaldehyde for 10 min at 37°C and quenched using 125

mM glycine for 5 min at 37⁰C. Cells were then lysed in RIPA buffer containing the serine protease inhibitors phenylmethylsulphonylfluoride (PMSF), aprotinin, leupeptin and pepstatin A for 20 min rotating at 4⁰C. PMSF irreversibly inhibits serine proteases by sulfonylation of the serine residue in the active site of the protease, aprotinin is a basic single-chain polypeptide that inhibits numerous serine proteases by binding to the active site of the enzyme, forming tight complexes, leupeptin is a tripeptide aldehyde reversible competitive inhibitor of serine and cysteine proteases and pepstatin A is a pentapeptide derivative, reversible inhibitor of aspartic proteases. Chromatin was sheared by sonication on ice for 4 intervals of 10 sec each. Sonication efficiencies were measured by running diagnostic agarose gels with small volumes of each sample. Five percent of each sample was used as an input control. Normalized extracts were pre-cleared using 60 µl of 1:1 PGS slurry at 4⁰C for one hour. 10 mg/ml BSA was added to 1% in each sample as a blocking agent. Appropriate antibodies (2 µg) were added to pre-cleared extracts and incubated overnight at 4⁰C with rocking. PGS and mouse IgG negative controls were incubated overnight in parallel at 4⁰C. The following day, PGS beads were added and incubated for 4 hrs with rocking at 4⁰C. Beads were pelleted, washed in low and high salt wash buffers and bound protein-DNA complexes were eluted using fresh elution buffer (1% SDS in 0.1 M NaHCO₃). Samples were then de-crosslinked by adding NaCl to 200 mM and incubating at 65⁰C overnight. DNA was recovered proteinase K digestion followed by a phenol/chloroform extraction and ethanol precipitation. Precipitated

DNA was resuspended in 30 μ l of 10 mM Tris buffer. Subsequently, aliquots were diluted 100 fold and 2–3 μ l of diluted samples were used in each PCR reaction.

Chapter 3
RESULTS

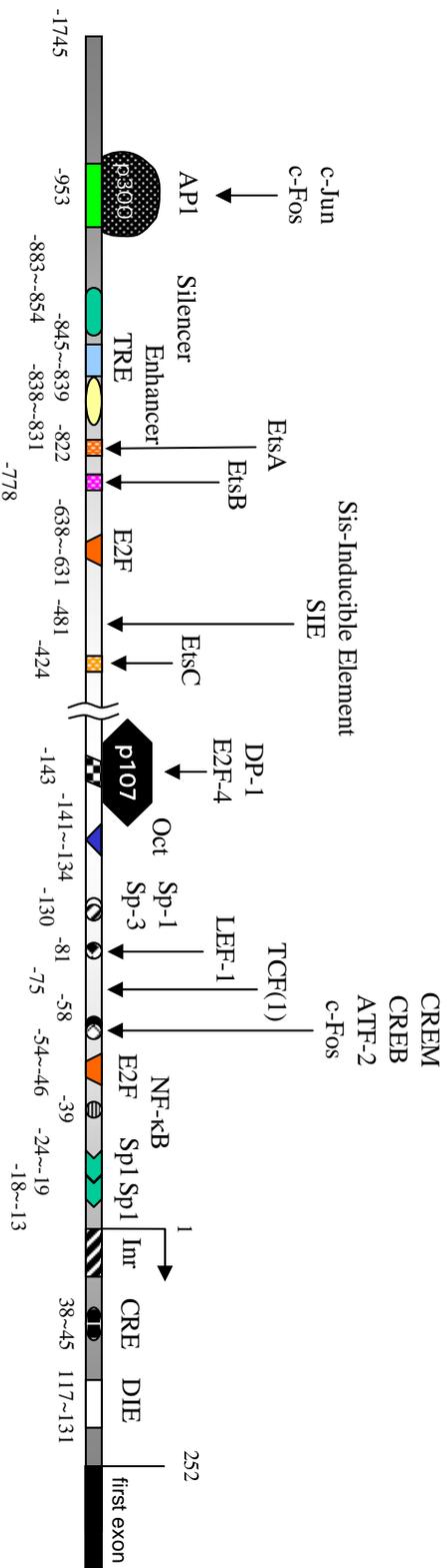
Chapter 3: Results

3.1 Senescence-associated cyclin D1 gene regulation

3.1.1 Background

The cyclin D1 gene promoter is influenced by the binding of several growth factors and oncogenes (Figure 8). Its induction through Ras and Rac requires ERK-independent signalling pathways however the ERK pathway is also capable of directly activating cyclin D1. The 5'-regulatory region of the cyclin D1 gene has been well characterized and the regions responsible for serum-inducible transcription have been identified (Motokura and Arnold, 1993; Herber et al., 1994). However, the regulation of cyclin D1 gene expression is not well understood under conditions of senescence-associated growth arrest where induction is equal to that seen in response to serum. The increased expression of cyclin D1 is specific for senescence-associated growth inhibition in primary fibroblasts and is not apparent in contact-inhibited or serum-deprived cells arrested at a similar place in the cell cycle (Lucibello et al., 1993; Atadja et al., 1995b). This is in contrast to the closely related cyclin D2 where expression is increased in senescent, quiescent and contact inhibited cells (Meyyappan et al., 1998). Thus, unique transcriptional mechanism(s) may strongly contribute to cyclin D1 expression during senescence.

Figure 8: The annotated cyclin D1 promoter. Cyclin D1 is a key regulator of G1-to-S phase progression of the cell cycle. The cyclin D1 gene encodes a regulatory subunit of a holoenzyme that phosphorylates and inactivates the tumor suppressor protein Rb (retinoblastoma) resulting in release of the Rb binding proteins and transcription factors, E2F. Several cyclin dependent kinase inhibitors (CDKI) block the activity of cyclin D1/cdk complexes, however at low levels, as is the case for p21^{CIP1}, CDKIs can actually facilitate holoenzyme assembly and kinase activity. As a consequence of being a very labile protein, in many circumstances cyclin D1 abundance is rate limiting in passage through the G1-phase of the cell-cycle. Several different growth factors and oncogenes have been shown to induce cyclin D1 expression through its well annotated promoter. Mitogenic stimulation typically acts through a mitogen-activated protein kinase Ras-mediated pathway independent of Erk, however the extracellular-signal-regulated kinase (Erk) pathway is also capable of directly activating cyclin D1. The Ets DNA binding domain family of transcription factors functions to modulate proximal promoter activity. We have identified a novel regulatory element in the cyclin D1 5'-untranslated region called the cyclin D1 inhibitory element (DIE). This element plays an important role in the differential expression of cyclin D1 during cellular replicative senescence.



Cyclin D1

FIGURE 8

3.1.2 *Consequences of increased cyclin D1 expression*

Normal human primary fibroblasts that have reached the end of their *in vitro* life span (senescent cells) express three-fold higher levels of cyclin D1 protein than low passage cells (Atadja et al., 1995b). Individual cells in mass culture that fail to initiate DNA synthesis in response to serum addition have several fold higher levels of this cyclin than proliferation competent cells (Atadja et al., 1995c). It has been shown that cyclin D1 overexpression may inhibit this entry into S phase through binding to proliferating cell nuclear antigen (PCNA) and cyclin dependent kinase 2 (Cdk2) (Pagano et al., 1994; Atadja et al., 1995c; Fukami-Kobayashi and Mitsui, 1999). Cyclin D1 dysregulation and gene amplification have been implicated in a variety of cancers (Lammie et al., 1991; Hunter and Pines, 1994), suggesting that deregulated expression of cyclin D1 contributes to abnormal cell proliferation.

Ectopic overexpression of cyclin D1 in normal human diploid fibroblasts (HDFs), the mammary epithelial cell line MCF-7, Dami megakaryotic cells and rat embryo fibroblasts inhibits DNA synthesis and cell growth (Pagano et al., 1994; Atadja et al., 1995a; Han et al., 1995; Wilhide et al., 1995; Han et al., 1996; Meyyappan et al., 1998). Cyclin D1 has also been shown to be upregulated in numerous nonproliferative differentiated cell types (Burger et al., 1994; Horiguchi-Yamada et al., 1994; Yan and Ziff, 1995; van Grunsven et al., 1996; Gao and Zelenka, 1997) and during apoptosis (Freeman et al., 1994) lending further evidence that it may also provide growth suppressor function. In senescent

fibroblasts, the cyclin D1 mRNA and protein levels are constitutively upregulated by approximately 3 to 5-fold compared to serum stimulated young cells (Dulic et al., 1993; Atadja et al., 1995c; Fukami et al., 1995; Fukami-Kobayashi and Mitsui, 1998).

In order to guarantee rapid degradation in the absence of appropriate mitogenic stimuli, the D-type cyclins are very labile proteins. Cyclin D1 proteolysis is regulated by glycogen synthase kinase-3 β (GSK-3 β), which is inactivated by a pathway that sequentially involves Ras, phosphatidylinositol 3-kinase (PI3K), and Akt (Diehl et al., 1998). Briefly, GSK-3 β phosphorylates cyclin D1 on the carboxyl terminus, specifically on threonine 286 (T286), thereby changing its subcellular localization (Figure 5A). Once phosphorylated, cyclin D1 is shuttled from the nucleus to the cytoplasm and targeted for proteolysis by ubiquitin-mediated pathways. Paradoxically, active GSK-3 β was recently shown to be accumulate in the nucleus of senescent WI-38 fibroblast cells (Zmijewski and Jope, 2004), suggesting that cyclin D1 degradation is regulated by additional pathways.

Since cyclin proteins were identified and named according to their ability to oscillate in overall protein levels through the cell cycle (Nurse, 1975), I initially tested whether cyclin D1 protein levels were altered dramatically through the cell cycle. Cyclins are synthesized and assembled with cdk4 and/or cdk6 in response to growth factor stimulation, thereby generating active holoenzymes that help inactivate the growth-suppressive function of the Rb protein by phosphorylation.

In order to evaluate if this holoenzyme assembly occurs in a cell cycle dependent manner as a result of increased cyclin D1 protein levels I first synchronized cells using either serum deprivation or aphidicolin treatment (Figure 9). Aphidicolin is a fungal metabolite and a specific inhibitor of DNA polymerase- α therefore it effectively inhibits DNA synthesis from starting and blocks cells at the G1/S transition. However, due to the strong response of the cyclin D1 promoter to growth factors, when analyzing cell lysates harvested from various time-points (0, 12, 18.5, 22 and 27 hrs) for cyclin D1 protein levels, I did not see consistent cycling of cyclin D1 protein levels as previously reported (Winston and Pledger, 1993). This suggested that cyclin D1 plays an additional role to regulating cell cycle progression since it was not always upregulated at the expected late G1 phase of the cell cycle and is very responsive to external mitogenic stimulation.

Figure 9: Primary human fibroblast cell cycle traverse and synchronization.

In order to elucidate the role of cell cycle regulatory genes *in vitro*, primary human fibroblasts were synchronized using mitogenic/serum deprivation or aphidicolin treatment. **A.** Fluorescence activated cell sorting (FACS) was used to determine cell cycle progression over various time-points for normal human fibroblasts (HS-68). Over a series of several experiments it was determined that greater than 95% of cells serum starved for no less than 5 days would accumulate in G1. Due to the robustness of these cells no apoptosis was evident during this time-frame. These cells were typically released from arrest using DMEM medium supplemented with 20% FBS. **B.** Several experiments designed to synchronize primary fibroblast cells were done using serum deprivation for 5 days followed by 20% FBS stimulation and average values were plotted on the graph shown. Arrows highlight the time-points at which cells were typically harvested in order to isolate cells in various cell cycle phases as shown in panel A. **C.** Other synchronization strategies were also explored for their efficacy in maintaining cells in a particular cell cycle state. These included supplementation of normal cell growth medium with 1 $\mu\text{g}/\text{mL}$ of the fungal metabolite and antibiotic aphidicolin. As shown, the synchronization of cells using serum deprivation was as effective as aphidicolin treatment therefore the former strategy was used. Time points for cell cycle analyses were harvested as follows: 0 hrs – G0, 12 hrs – G1, 18.5 hrs – G1/S, 22 hrs – S and 27 hrs – G2 (panel A).

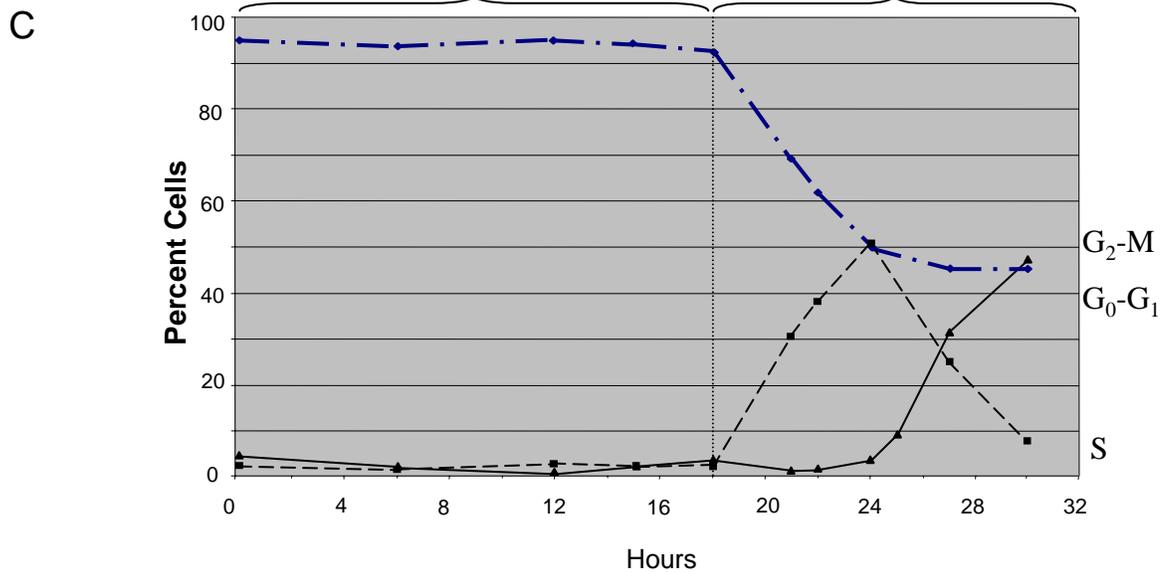
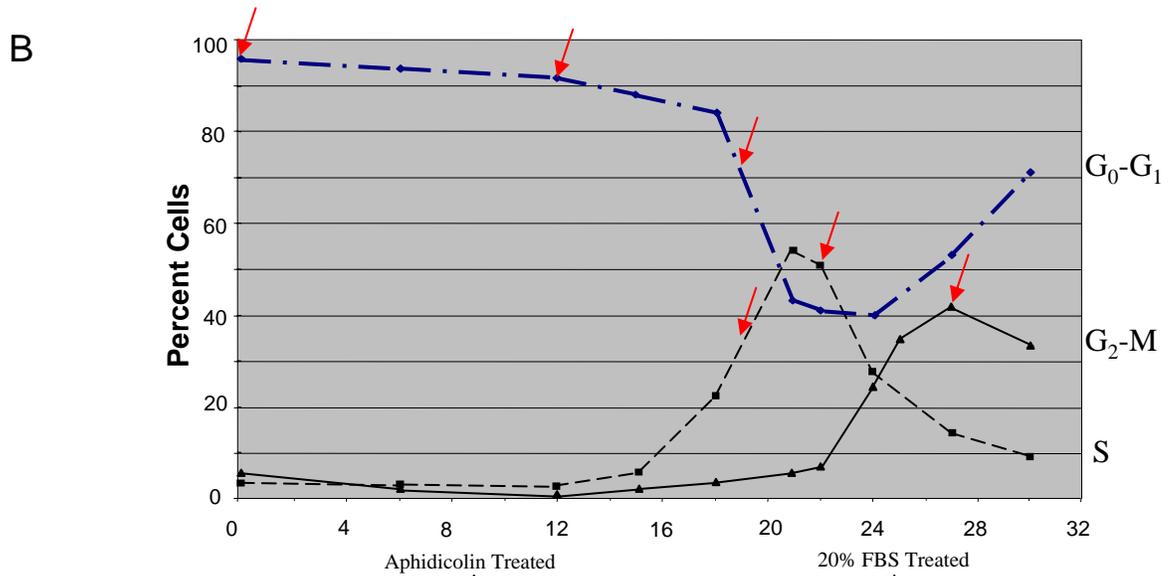
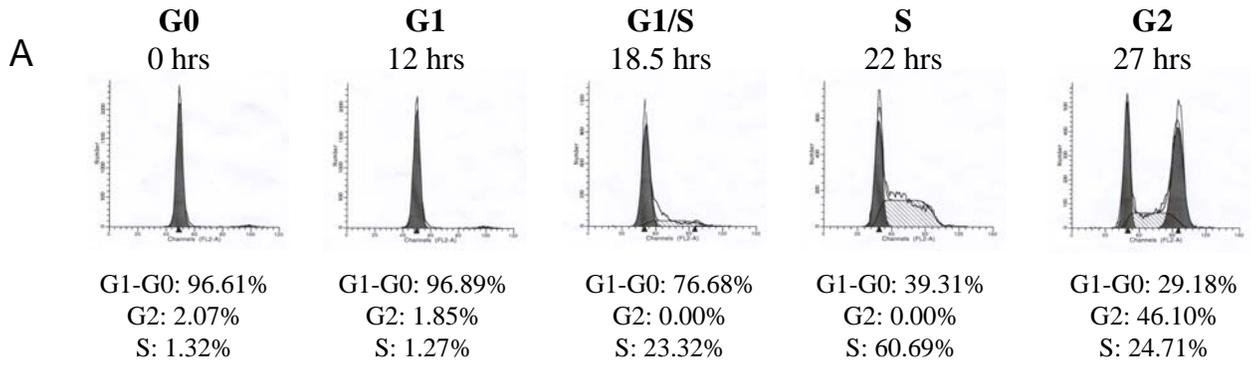


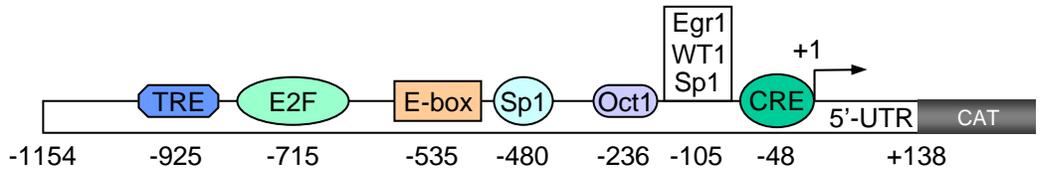
FIGURE 9

3.1.3 The 5'-untranslated region of cyclin D1 differentially binds protein extracts from young and senescent fibroblasts

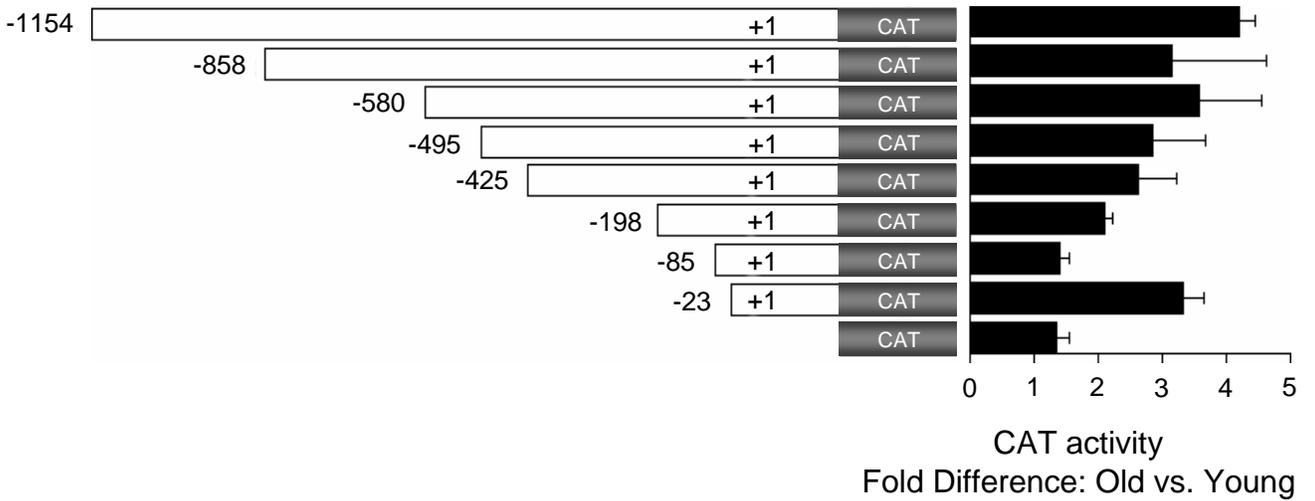
Senescence depends on a number of pathways that together result in a permanent and irreversible cell cycle exit. Since the molecular machinery that controls cell cycle progression is based on the sequential activity of cyclins and cyclin dependent kinases (CDKs), we initially investigated the regulation of cyclin D1 by examining the potential regulatory regions and mechanisms that may be responsible for the upregulation of cyclin D1 seen in aging primary fibroblasts. Analysis of the cyclin D1 promoter by transient transfections of truncated promoter sequences and nested promoter deletions driving a chloramphenicol acetyltransferase (CAT) reporter gene into young and senescent fibroblasts enabled us to identify a region in the cyclin D1 promoter that was important for its differential expression (Figure 10 and 11). This element is located in the 5'-UTR of cyclin D1 and binds a low molecular weight protein in a sequence-specific manner more avidly in young cells compared to senescent cells (Figure 12, 13 and 14). This suggests that loss of binding to this element in senescent cells contributes to the increased expression of cyclin D1 seen during cellular aging. To address this further we tested whether the 5'-UTR of the cyclin D1 promoter had an effect on two different reporter genes when transiently transfected into young and senescent fibroblasts.

Figure 10: 5'-deletion analysis of the cyclin D1 promoter. **A.** Schematic representation of simplified transcription factor binding sites in the cyclin D1 promoter. Abbreviations used: TPA response element (TRE), immediate-early growth response gene (Egr1), Wilms tumour suppressor gene product (WT1), cyclic adenosine monophosphate response element (CRE), transcription factor E2F binding site (E2F), promoter-specific transcription factor (Sp1), Octamer-like transcription factor binding site (Oct1), insulin-responsive region (E-box). **B.** A series of 5'-truncations of the cyclin D1 promoter in pBluescript vector was generated by exonuclease III digestion, and the resulting deletions were subcloned into the pBLCAT3 vector for transient transfections into young and senescent fibroblasts (left). Bar graphs on the right represent the fold difference in chloramphenicol acetyltransferase (CAT) activity in old versus young cells after normalizing for transfection efficiencies by measuring activity from co-transfected β -galactosidase constructs. Results represent CAT activity from three independent transfections with the standard error indicated.

A



B

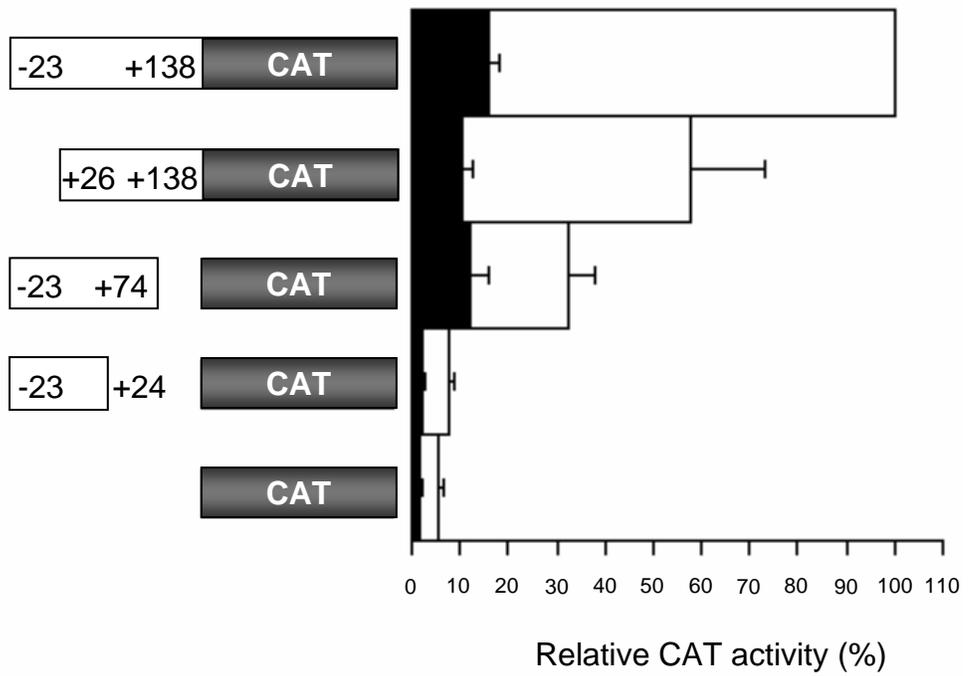


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FIGURE 10

Figure 11: 5' and 3' nested deletion analysis of the -23 to +138 region of the cyclin D1 promoter. **A.** Further analysis of the 5' and 3' deletions of the cyclin D1 promoter (left) and the resulting CAT activity in young (black bars) and old (white bars) cells plotted after normalizing for β -galactosidase activity (right). **B.** Summary of fold difference in CAT activity in old versus young cells from panel A. The -23 to +138 and +26 to +138 showed the biggest fold difference (old/young) in reporter expression while the -23 to +74 shows the least difference. This suggests that a regulatory element found in the +75 to +135 region (labeled -23 to +74) is responsible for the differential activity of the cyclin D1 promoter in old versus young cells.

A



B

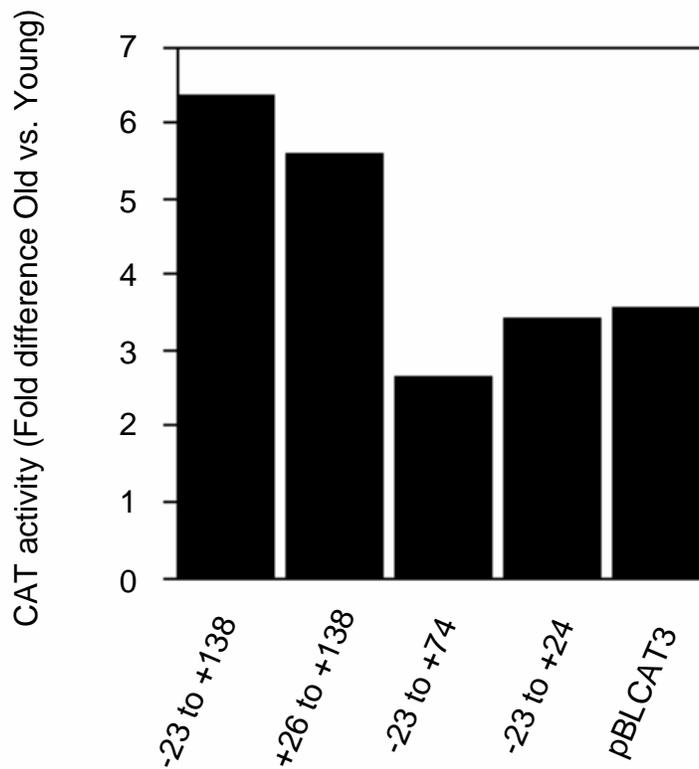
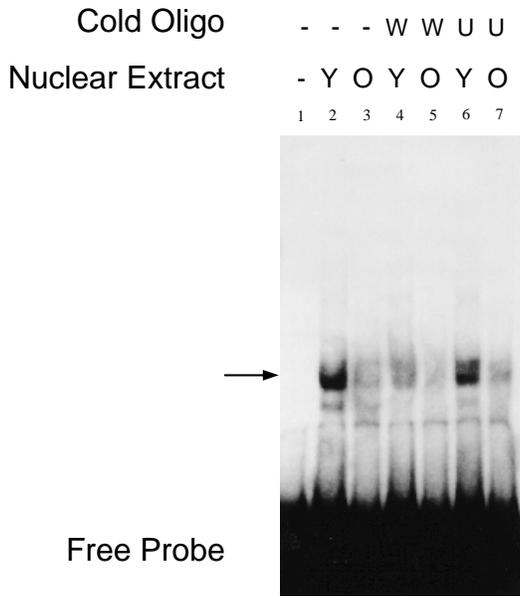


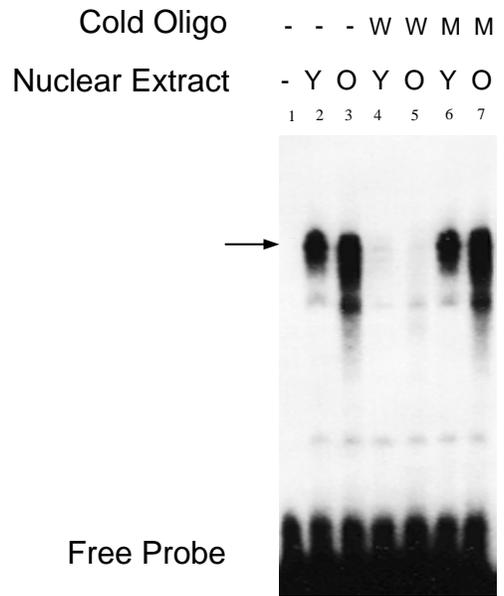
FIGURE 11

Figure 12: DNA binding activity of the cyclin D1 5'-untranslated region (5'-UTR). **A.** Nuclear extracts from asynchronous young (Y) and old (O) HS-68 HDFs were incubated with an oligonucleotide corresponding to the 64-bp sequence of the cyclin D1 5'-UTR (lanes 2, 3). The arrow points to the major specific DNA-protein complex. Lane 1 corresponds to the reaction with the labelled oligonucleotide in the absence of nuclear extracts. Lanes 4 and 5 show the reaction with the 64-bp oligonucleotide in the presence of a 100-fold excess of unlabelled (cold) wild type (W) oligonucleotide or in the presence of a 100-fold excess of cold unrelated (U) CRE oligonucleotide (lanes 6, 7). **B.** To rule out the possibility that the nuclear extracts from old HS-68 fibroblasts were deficient in their ability to bind DNA through some non-specific mechanism, we performed gel shift studies using the same extracts described in (A) with an oligonucleotide containing the CRE present in the *c-fos* promoter. Lane 1 is a mock control showing probe alone in the absence of nuclear extract. Lanes 2 and 3 show complexes formed on the CRE when young and old nuclear extracts were added. Lanes 4 and 5 show the reaction in the presence of a 100-fold cold CRE wild type oligonucleotide (W) or a 100-fold cold CRE mutant oligonucleotide (M) (lanes 6 and 7). **C.** Electrophoretic mobility shift assays using independently isolated extracts from young growing cells (Y), young quiescent cells (Q) and senescent subconfluent cells (O). The first three lanes show oligonucleotide in the absence of extract and the following lanes (4-12) show the degree of binding activity of the extracts of the extracts to the 64-bp oligonucleotide, a subdomain (5'-UTR-4) and a mutant subdomain (5'-UTR-4d) of the oligonucleotide as shown in Fig. 13.

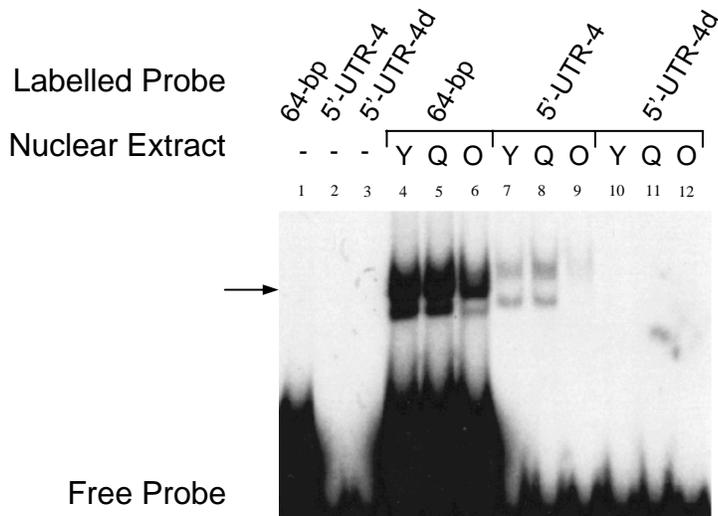
A



B



C



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FIGURE 12

Figure 13: Identification of the DIE using overlapping oligonucleotides. A. A portion of the cyclin D1 5'-UTR showing a 64-bp region divided into five overlapping 22-bp oligonucleotides (5'-UTR-1 to 5'-UTR-5). Bolded letters from +117 to +131 show the highest binding affinity in nuclear extracts as shown in panel (B). **B.** Electrophoretic mobility shift assay of the overlapping oligonucleotides shown in (A) with young (Y) and old (O) cell nuclear extracts. Lanes 1 to 7 are control reactions in the absence of nuclear extract. Detection of complex formation was evident in young cell nuclear extracts and weaker in old cell extracts when using 5'-UTR-4 and 5'-UTR-5 as a probe (lanes 14 to 17) but signal was not detected when 5'-UTR-1 or 5'-UTR-3 was used as a probe (lanes 8, 9 and 12, 13). A very weak complex was detected preferentially in young cell extracts when 5'-UTR-2 was used as a probe (lanes 10, 11). Complex formation on the 64-bp and control CRE oligonucleotide is also shown (lanes 18 to 21). **C.** The 5'-UTR-4 and 5'-UTR-5 oligonucleotides were used as probes for competition experiments either alone or with the other unlabelled probes at a 100-fold excess as indicated, to determine the specificity of binding.

Figure 14: Mutations of oligonucleotides in the 5'-UTR-4 region. A. To determine the effect of various base pair changes on protein binding activity, four sets of mutant oligonucleotides from the 5'-UTR-4 fragment of the cyclin D1 untranslated region (5'-UTR-4a to 5'-UTR-4d) were generated and used in mobility shift assays. These nucleotide substitutions within the 5'-UTR-4 oligonucleotides are shown here and are labeled 4a, 4b, 4c and 4d. **B.** Electrophoretic mobility shift assays with young and old cell nuclear extracts using the mutant 5'-UTR-4 oligonucleotides shown in (A). Lanes 1 to 5 are control lanes in the absence of nuclear extracts. Introduction of 2 or 4-bp changes inhibited binding activity (lanes 8 to 13), while an 8-bp change within the DIE nearly abolished binding activity (lanes 14, 15) compared to the wild type 5'-UTR-4 oligonucleotide (lanes 6, 7).

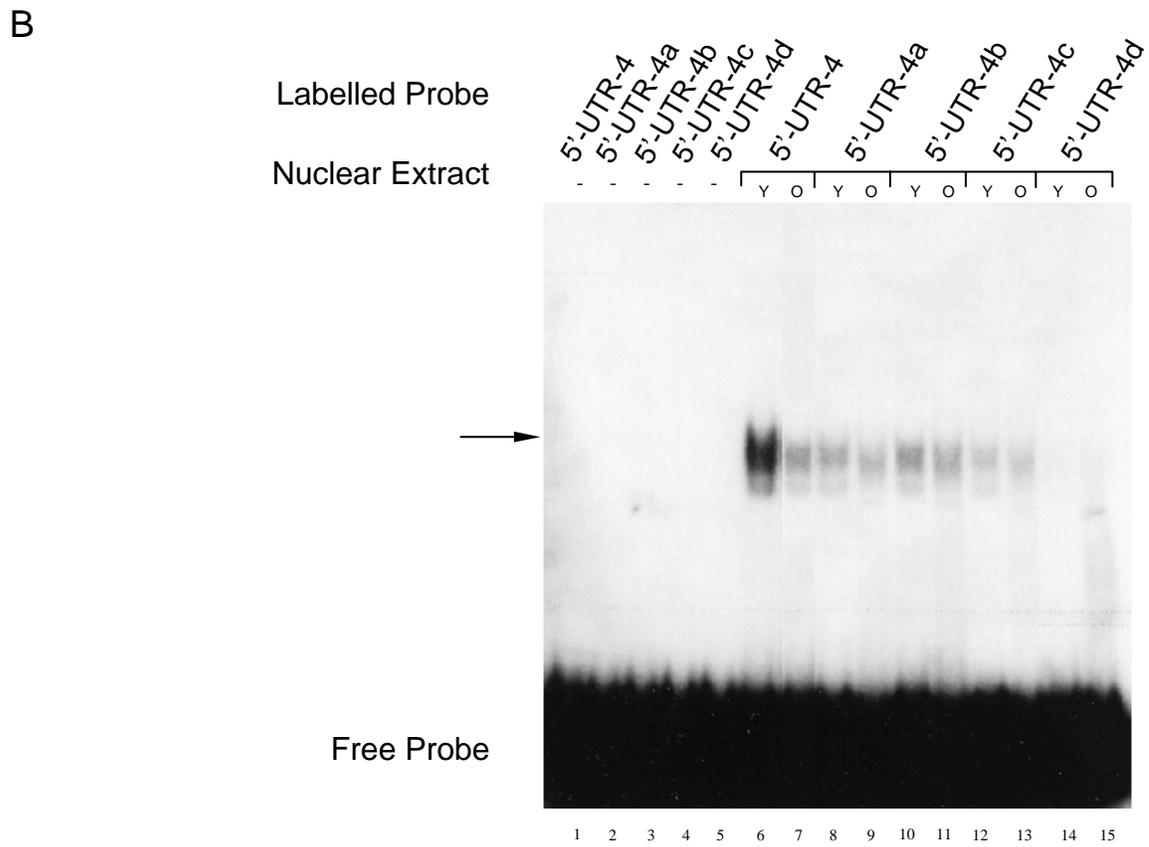


FIGURE 14

3.1.4 Removal of a 64 bp region in the 5'-UTR of cyclin D1 abolishes increased promoter activity in senescent cells

To determine the basal activity of the cyclin D1 promoter in young and senescent cells, a 1.3 kb cyclin D1 promoter fused to a CAT reporter gene was used in transient transfection studies. Briefly, CAT reporters were used because of the inherent advantages in selecting an enzymatic activity to monitor promoter function (Gorman et al., 1982). Chloramphenicol acetyltransferase (CAT) is a bacterial enzyme that inactivates the antibiotic chloramphenicol by acetylation (formation of mono- and diacetylated derivatives). Since its activity is not normally found in mammalian cells and is completely distinguishable from any endogenous activities there is no interference from other enzymatic activities which could compete for the substrate or cofactors. This coupled with the ease and reproducibility of CAT assays makes CAT activity a good reporter candidate. Therefore cell extracts are incubated in a reaction mix containing ^{14}C -chloramphenicol and n-butyryl Coenzyme A. CAT transfers the n-butyryl moiety of the cofactor to chloramphenicol. The radiolabelled, butyrylated chloramphenicol can then be detected using a liquid scintillation counter.

Various 5' deleted cyclin D1 promoter-CAT reporter constructs were generated and transiently co-transfected with a CMV-driven β -galactosidase construct (as internal control) into young and senescent cells. CAT activity due to the full length cyclin D1 promoter was 4 to 5 fold greater in senescent than in young cells (Figure 10B), consistent with endogenous levels of cyclin D1

expression in those cells (Atadja et al., 1995c). Deletion of segments from the 5'-end from -1154 to -85 progressively reduced the expression difference between young and senescent cells to levels observed with the minimal promoter pBLCAT3 vector (Figure 10B). However, the smallest deletion construct containing -23 to +138 of the cyclin D1 promoter retained 3 to 4 fold higher expression levels in senescent compared to young cells indicating that this region is sufficient to confer increased expression in senescent cells.

We then generated additional 5' and 3' nested deletions of the -23 to +138 region to further define the region involved in the differential regulation of cyclin D1. As shown in figure 11A, removal of the transcription initiation site resulted in approximately equivalent levels of inhibition of CAT activity in young and senescent cells. However, when 64 bp (+75 to +138) of the 5'-UTR was deleted, it resulted in a further 44% decrease in senescent cells but the CAT activity in young cells remained relatively unchanged, thus reducing the fold difference in CAT activity to levels observed with the control vector (Figure 11B, compare -23 to +138 with -23 to +74). Further removal of the 5'-UTR did not have any differential effect on CAT activity as it resulted in approximately 77% decrease in young and 82% decrease in senescent cells. These results indicate that together, other regions of the promoter are likely to contribute as strongly to the differential expression of cyclin D1 in young and senescent cells. Therefore, the 64-bp sequence between +75 and +138 appeared to be a candidate regulatory region that

was responsible for the differential expression of cyclin D1 during cellular senescence.

3.1.5 Decreased DNA binding activity in extracts of senescent cells to the +75 to +138 region of the cyclin D1 promoter

To determine if any proteins could bind this 64-bp region, electrophoretic mobility shift assays (EMSA) with young and senescent cell nuclear extracts were performed. Specific complexes were formed with young cell extracts while the levels were dramatically reduced with senescent cell extracts (Figure 12A; lanes 2, 3). Specificity of the complexes was confirmed by incubation with a 100-fold excess of unlabelled oligonucleotide which effectively competed with the labelled probe (Figure 12A; lanes 4, 5) whereas a 100-fold excess of unlabelled, unrelated CRE oligonucleotide did not compete efficiently (Figure 12A; lanes 6, 7).

To rule out the possibility that the nuclear extracts from senescent HS-68 fibroblasts were deficient in their ability to bind DNA through some non-specific mechanism, we performed EMSA studies using the same extracts with an oligonucleotide containing the CRE present in the *c-fos* promoter (Atadja et al., 1994). As shown in figure 12B, CRE-binding activity was actually about 50% higher in senescent cell extracts than in young (lanes 2, 3). As expected, 100-fold competition with unlabelled oligonucleotide bearing the same CRE sequence competed with the complexes formed on the labelled CRE (Figure 12B, lanes 4 and 5) while mutant sequences did not (Figure 12B, lanes 6 and 7). Given previous reports that CRE binding activity is about equal in young and senescent

cells, specific binding activity of the complex in figure 12A may actually be even more dramatically reduced in extracts from senescent compared to young cells (Atadja et al., 1994).

Although extracts from young cells showed more binding activity than extracts from senescent cells, it was possible that reduced binding in senescent cells was due to cells exiting the cell cycle when senescent, rather than to a senescence-specific event *per se*. For example, cyclin D2 is up-regulated as primary fibroblasts approach their replicative capacity, but increased expression is also seen upon serum withdrawal or contact inhibition-induced cell cycle arrest (Meyyappan et al., 1998). To test whether loss of binding was senescence-specific, we incubated the 64-bp element with extracts from young proliferating cells, young quiescent cells and subconfluent senescent cells. As shown in figure 12C, reduced binding was not seen in young quiescent cells (lanes labelled Q) with either the 64-bp element or with subdomains of this element, indicating that loss of binding is senescence-dependent rather than growth-dependent.

3.1.6 Identification of a 15-bp cyclin D1 inhibitory element (DIE) in the 5'-UTR with overlapping oligonucleotides

DNase I foot-printing assays were attempted but did not prove useful in identifying sequences within the 64-bp region that were differentially protected using young and senescent cell nuclear extracts. This was likely a consequence of weak binding affinity of the protein with its consensus binding motif resulting in no 'footprint' being observed. An alternative approach using overlapping

oligonucleotides was undertaken in an attempt to better define the region involved in protein binding. As shown in figure 13A, five double stranded 22-bp oligonucleotides designated 5'-UTR-1 to 5'-UTR-5 were synthesized and used in EMSAs using young and senescent cell nuclear extracts. No detectable binding activity was observed when the 5'-UTR-1 and 5'-UTR-3 oligonucleotides were used (Figure 13B; lanes 8, 9 and 12, 13) whereas a very weak complex was detected in young cell extracts when the 5'-UTR-2 probe was used (lanes 10, 11). Complexes were readily detected when using 5'-UTR-4 and 5'-UTR-5 as probes with young cell but not with senescent cell extracts (lanes 14 to 17). The migration of the complexes with these oligonucleotides was slightly slower than the migration of the complexes with the 64-bp oligonucleotide (lanes 18, 19), probably reflecting the smaller charge-to-mass ratio with the shorter oligonucleotides. Complexes with the shorter oligonucleotides were also less readily detectable than those with the 64-bp oligonucleotide, perhaps reflecting stabilization of the complexes by peripheral sequences present in the 64-bp oligonucleotide. Since both 5'-UTR-4 and 5'-UTR-5 share a 15-bp overlap as shown in figure 13A, the region involved in protein binding was mapped to this site in the 5'-UTR that we have termed the cyclin D1 Inhibitory Element (DIE).

In order to determine if this interaction was specific, the labelled 5'-UTR-4 and 5'-UTR-5 probes were incubated with a 100-fold excess of unlabelled 5'-UTR-4, 5'-UTR-5, 64bp or 5'-UTR-3 as a negative control. As shown in figure 13C, incubation with unlabelled oligonucleotides containing the DIE (lanes 3 to 8

and lanes 13 to 18), but not the 5'-UTR-3 oligonucleotide (lanes 9, 10, 19, 20), competed with the labelled probe for binding proteins and the degree of competition varied when using different unlabelled probes.

3.1.7 *Mutation of the DIE abolishes DNA binding activity*

To identify the bases within the DIE that were responsible for protein binding, oligonucleotides of 5'-UTR-4 containing various mutations (5'-UTR-4a to 5'-UTR-4d) were generated as shown in figure 14A. In each case, purines and pyrimidines were exchanged for non-complementary pyrimidines and purines respectively. EMSAs of young and senescent cell nuclear extracts with these oligonucleotides showed that the mutations in 5'-UTR-4a, -4b and -4c reduced binding in young cell extracts significantly compared to 5'-UTR-4 (Figure 14B, compare lanes 8 to 13 with lanes 6 and 7), and that 5'-UTR-4d which contains all eight nucleotide mutations nearly abolished binding in both young and senescent extracts (lanes 14, 15). Densitometric scanning of different exposures of the lanes in figure 14B indicated that the binding to these oligonucleotides was reduced by greater than 90% in the case of the most mutated (5'-UTR-4d). Using the *Discovery Series Quantity One 1-D Analysis Software* digital densitometry software (Biorad), the relative binding values for the oligonucleotides were 5'-UTR-4 (100%), 5'-UTR-4a (53%), 5'-UTR-4b (42%), 5'-UTR-4c (22%), 5'-UTR-4d (9%). Therefore, the base changes in the 5'-UTR-4d oligonucleotide were used for site-directed mutagenesis of the DIE in the context of the full-length cyclin D1 promoter.

3.1.8 Mutation of the DIE increases cyclin D1 promoter activity in young cells

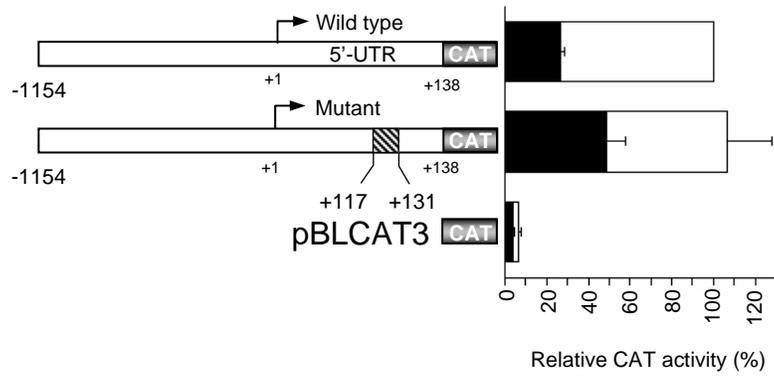
An 8-bp change in the full length cyclin D1 promoter-CAT reporter construct from +117 to +131, corresponding to the mutations in 5'-UTR-4d, was introduced by site-directed mutagenesis. The 1.3 kb full-length cyclin D1 promoter-CAT reporter construct and the mutated construct were transfected into young and senescent cells and the CAT activity resulting from each construct was measured after normalizing for β -galactosidase activity. Normalization is important in all reporter assays in order to adjust for different transfection efficiencies between various cells (i.e. young vs. senescent). As shown in figure 15A, mutation of the DIE in the 5'-UTR resulted in nearly a doubling of CAT activity in young cells relative to senescent cells, compared to the activity of the wild-type construct. In contrast, the CAT activity from the mutant construct was similar to that from the wild-type construct in senescent cells. Figure 15B shows the results of the same experiment plotted as fold difference in CAT activity in senescent versus young cells. Mutation of the DIE resulted in a nearly 50% decrease in the fold difference in CAT activity compared to the wild type control. These results suggest that the DIE within the 5'-UTR of cyclin D1 constitutes a binding site for a potential transcriptional repressor in young cells. Since the activity and/or levels of the DIE binding protein are dramatically reduced in senescent cells, this may strongly contribute to the differential cyclin D1 expression seen in senescent cells.

Figure 15: CAT activity in young versus old cells using a cyclin D1 promoter containing a mutant DIE. The 8-bp change corresponding to 5'-UTR-4d (see figure 14A) was introduced into the full-length cyclin D1 promoter by site-directed mutagenesis and the construct was transfected into young and old cells.

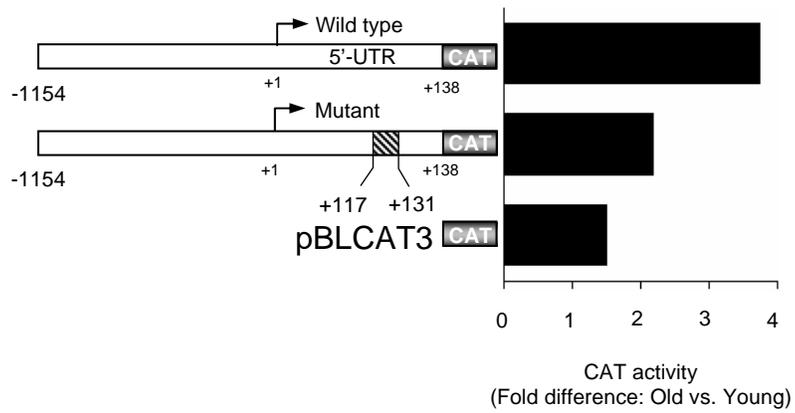
A. The graph on the right of panel A represents CAT activity from young (*black bars*) and old (*overlapping white bars*) cells after normalizing for transfection efficacy using co-transfected β -galactosidase construct. The wild type cyclin D1 promoter shows a greater differential expression than the mutant cyclin D1 promoter. In each experiment, all samples were normalized to wild type cyclin D1 promoter expression in old cells.

B. Results from panel A plotted as fold difference in CAT activity in old versus young cells. Averages of three independent transfections were used to calculate total fold difference in CAT activity. The greatest fold difference between CAT activities was observed when using the wild type cyclin D1 promoter as shown here.

A



B



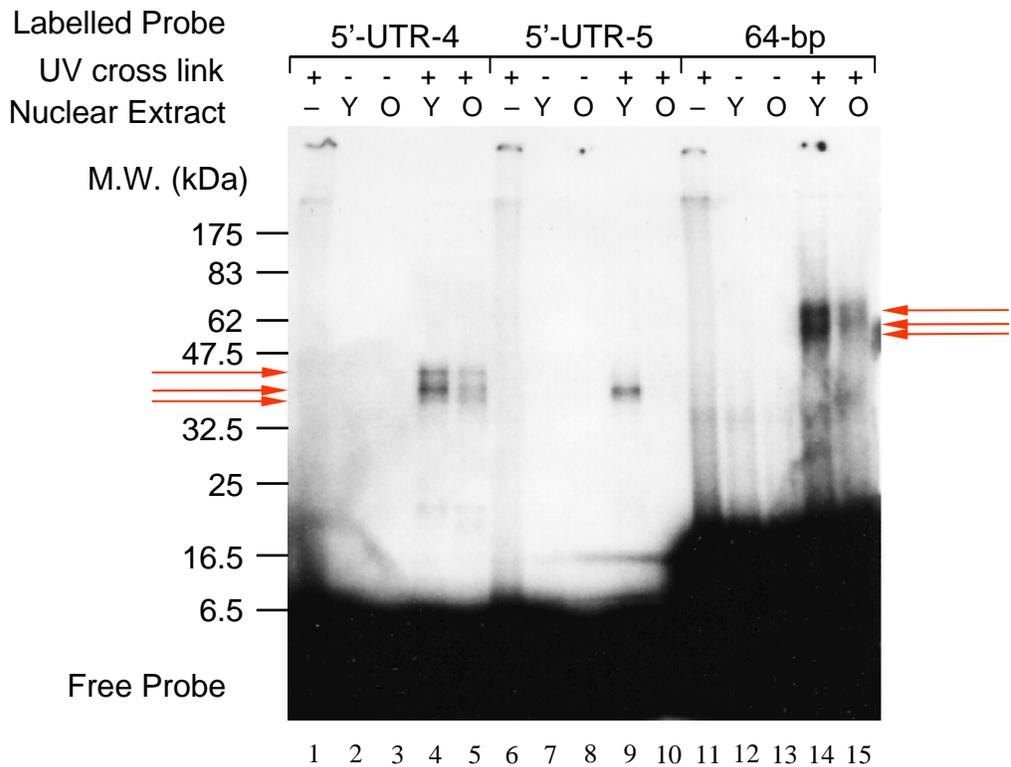
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FIGURE 15

3.1.9 Low molecular weight proteins bind the DIE

DNA-protein interactions are critical to the structure and function of genes. In order to estimate the molecular weight of any protein(s) that bound specifically to the DIE, nuclear extracts from young and senescent cells were incubated with 5'-UTR-4, 5'-UTR-5 or the 64-bp oligonucleotide and were subjected to UV crosslinking. Following resolution by SDS-polyacrylamide gel electrophoresis (PAGE), complexes were apparent in young cell extracts that were decreased in senescent cell extracts (Figure 16). Three different complexes were formed with the 5'-UTR-4 suggesting that this sequence might contain a more complete binding site(s) than 5'-UTR-5 with which only two complexes were seen (compare lane 4 with lane 9). Three complexes were also apparent with the 64-bp oligonucleotide (lanes 14, 15). After subtraction of the molecular weight of the labelled probe (i.e. 649 Da per bp), an estimated molecular weight range of 20 to 45 kDa was calculated for proteins bound to both the 22-bp and 64-bp oligonucleotide, depending upon the amount of DNA protected by UV crosslinking.

Figure 16: Determination of the approximate molecular weight of protein(s) binding to the DIE. *In vitro* binding reactions using the 5'-UTR-4, 5'-UTR-5 and a longer 64-bp oligonucleotide probes with young and old cell nuclear extracts were subjected to UV crosslinking, digested with DNase I, and subsequently analyzed by SDS-PAGE. The lanes without UV crosslinking served as negative controls. The *arrows* identify various DNA-protein complexes.



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FIGURE 16

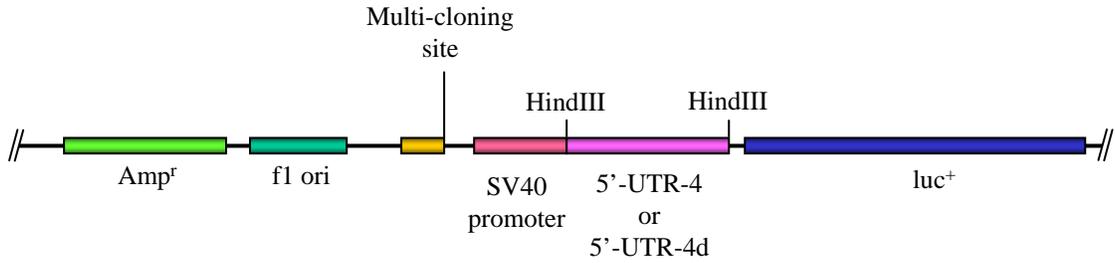
3.1.10 The DIE preferentially inhibits gene expression in young fibroblasts

In order to test the activity of the 15-bp wild-type and mutant DIE elements, luciferase reporters containing these elements (Figure 17A) were transfected into young and senescent HS-68 fibroblasts. Transfection efficiencies of 55% and 40% in young and senescent fibroblasts, respectively, were obtained using Lipofectamine 2000. Transfection efficiencies were normalized based on *Renilla* luciferase (pRL-TK) expression. *Renilla reniformis* (Sea Pansy) is a species of soft coral. The organism is a colony of polyps each of which is bioluminescent at the sites identified by the characteristic green fluorescence. *Renilla* luciferase uses coelenterazine and its derivatives to catalyze the oxidative decarboxylation of coelenterazine to produce coelenteramide and light. The *Photinus pyralis* (North American firefly) luciferase protein oxidizes the beetle luciferin substrate in an ATP, Mg²⁺ and O₂ dependent manner. Consequently, the luciferin is transformed to an excited state oxyluciferin molecule and carbon dioxide, each containing one oxygen atom. Visible light emission results from the rapid loss of energy of the excited state oxyluciferin molecule by a fluorescence pathway.

Figure 17: Dual-luciferase reporter assays using wild type and mutant DIE sequences. 26-bp sequences containing wild type or mutant forms of the 15-bp DIE (5'-UTR-4 and 5'-UTR4d) were cloned into pGL3-control firefly luciferase reporter plasmids are shown in the sense orientation. Assays show differential inhibition by the wild type *versus* mutant DIE on luc⁺ expression. Young HDFs show more than 80% reporter inhibition while senescent HDFs show less than 30% inhibition. Mutant constructs show little to no inhibition of reporter expression.

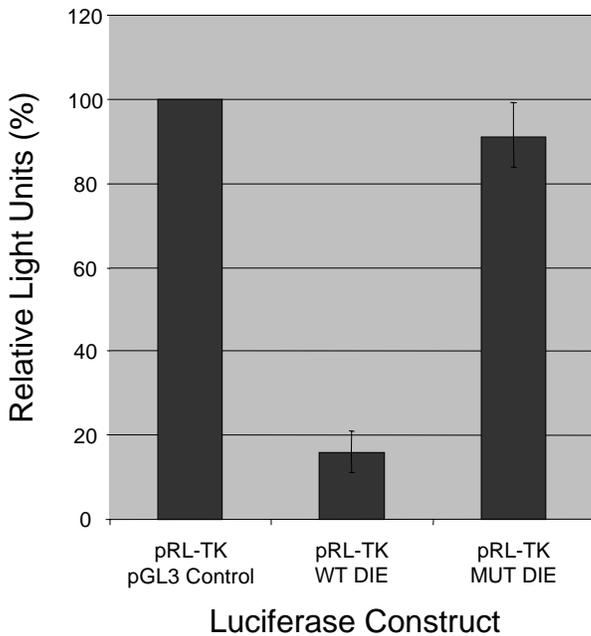
A

pGL3-Control Vector (5256-bp)



B

**Dual Luciferase Assay:
Young Human Diploid
Fibroblasts (Hs68)**



**Dual Luciferase Assay:
Senescent Human Diploid
Fibroblasts (Hs68)**

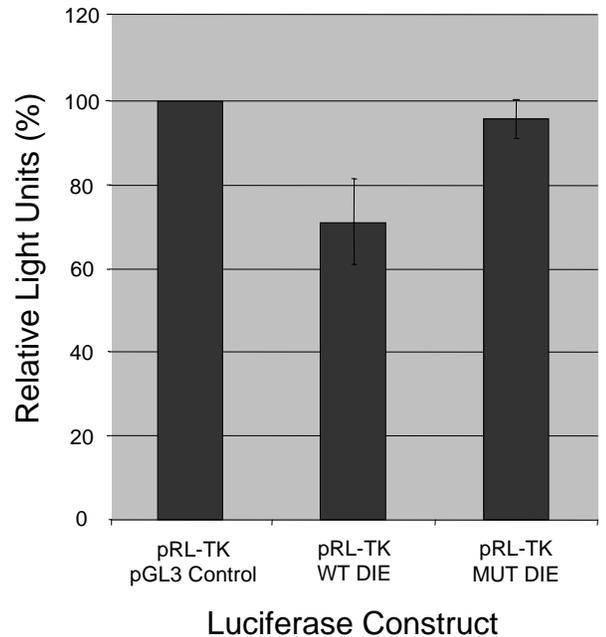


FIGURE 17

As shown in figure 17B, young HDFs showed more than 80% reporter inhibition by the wild-type DIE compared to the mutant element, while senescent HDFs were inhibited approximately 30%. Therefore, although the DIE element had an inhibitory effect upon transcription in both young and senescent cells, the inhibitory effect was markedly greater in young cells (i.e. 4 to 5 times greater inhibition of reporter expression in young cells compared to senescent cells), even though the assay is done with a single DIE element (compared to several seen in most genes) outside the context of the native cyclin D1 promoter.

3.1.11 The 15 bp cyclin D1 inhibitory element (DIE) is conserved in key cell cycle regulatory genes

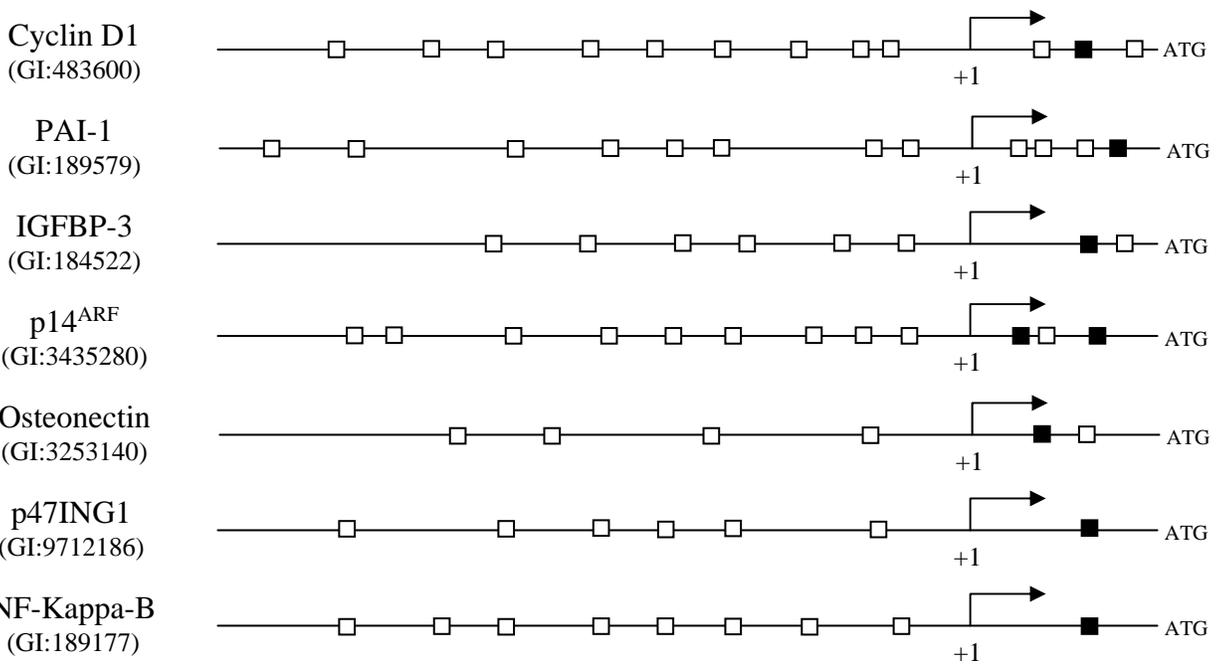
We next examined whether sequences similar to the DIE were present in promoters of other genes upregulated during cellular senescence. Well characterized genes whose expression is increased in senescent cells include **1**) plasminogen activator inhibitor type-1 (PAI-1) (Goldstein et al., 1994) **2**) insulin-like growth factor binding protein-3 (IGFBP-3) (Goldstein et al., 1993; Moerman et al., 1993) **3**) p14^{ARF} (Wei et al., 2001) **4**) ING1 (Garkavtsev and Riabowol, 1997; Garkavtsev et al., 1998) **5**) NF-kappa-B (Smith and Pereira-Smith, 1989; Supakar et al., 1995) and **6**) osteonectin (Murano et al., 1991). As controls, the “housekeeping” genes glyceraldehyde phosphate dehydrogenase (GAPDH), expressed similarly in young and senescent fibroblasts (Wong and Riabowol, 1996), and phenylalanine tRNA synthetase, whose expression decreases during cellular senescence (Lee et al., 1999) were used. The 15-bp DIE was searched for

in the promoters of these genes using the FINDPATTERNS program (see Materials and Methods). As shown in figure 18A, sequences with similarity to the DIE were found to be clustered within 400-bp of the transcription initiation site (often within the 5'-UTR) of the genes that are upregulated in aging cells, whereas the frequency of DIE-like sequences in the control genes was much lower. Alignment of the DIE with the sequences detected in the other promoters showed the presence of six perfectly conserved bases in the group of genes upregulated during cellular senescence, whereas only two bases were conserved in the controls (Figure 18B). Furthermore, p14^{ARF} had a second DIE that was very well conserved. Taken together, these results suggest that the regulatory function of the DIE may not be restricted to cyclin D1 gene expression, but also to a selected number of genes that are overexpressed in senescent cells.

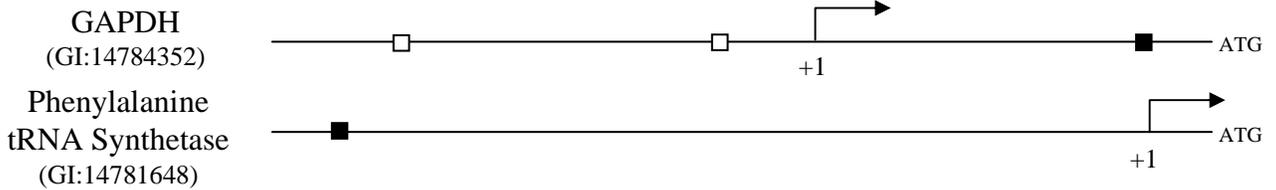
Figure 18: DIE elements in the promoter regions of other genes transcriptionally upregulated during replicative senescence. A. Schematic representation of the promoter regions of genes well established to be upregulated with *in vitro* age. White boxes indicate all DIE-like elements with at least 66.6% (10/15) homology to the DIE. Black boxes indicate the DIE-like elements most similar to the cyclin D1 DIE that are expanded in panel B. **B.** DIE-like elements from the 5'-UTR of the indicated genes from panel (A) showing closest sequence homology to the cyclin D1 DIE.

A

Experimental



Controls



B

Cyclin D1	+117:	C A G G A C C C A C A G C C C C	:+131
PAI-1	+103:	C A G G A C G A A C C G C C A	:+117
IGFBP-3	+88:	C T G C G C G C C C A G C C T	:+102
p14 ^{ARF}	+382:	G G G G G C C A G C T G C C T	:+367
Osteonectin	+170:	C A G G G C T C A G A G C C G	:+155
p33 ^{ING1}	+143:	A C G G T C T C C C C G C C T	:+128
NF-Kappa-B	+211:	C C G T T C C C T C C G C C G	:+196
GAPDH	+292:	G C C G A G C C A C A T C G C	:+306
Phenylalanine tRNA Synthetase (FARS1)	-897:	G T G C T C T G C C T T C T G	:-912

Berardi et al., 2003

FIGURE 18

3.1.12 DNA affinity chromatography using placental extracts binds non-specifically to proteins

In attempts to isolate sufficient protein for analysis by mass spectrometry a DNA affinity chromatography approach using both wild type and mutant DIE elements was used (Figure 19). Placental nuclear extract homogenate was first run over heparin-agarose ion exchange columns to enrich for DNA-binding proteins. Then a mutant DIE DNA-affinity chromatography pre-column was run to eliminate non-specific DNA binding proteins and excess poly (deoxyinosinic-deoxycytidylic) acid was added to the eluent to competitively bind DNA end-binding proteins. After fractions were eluted from mutant and wild type DNA affinity columns using high salt (~ 5 mL bed volume), samples were run on SDS-PAGE gels and silver stained. Silver staining revealed no unique bands from mutant or wild-type columns indicating a failure to enrich for DIE-specific binding proteins. Since transcription factors make up approximately 0.01% of total nuclear proteins, the potential difficulty in identifying DIE-binding proteins was recognized, especially when using the mosaic of cells typically found in placental tissues. This was also seen by running additional EMSAs with placental nuclear extracts as shown in figure 20. Here again we observed non-specific binding of proteins to DNA ligand when using similar conditions to original EMSAs using fibroblast lysates (Figure 20).

Figure 19: Flow chart of DNA affinity chromatography approach. In order to purify the DIE-binding protein (DIE-BP) a multi-step DNA affinity chromatography approach was attempted using placental nuclear extracts. A heparin-agarose ion exchange column was initially used to enrich for DNA-binding proteins. After a high-salt elution (2 M NaCl), a pre-column using a mutant DIE sequence was run to reduce non-specific DNA binding proteins. Poly(dI-dC) was added to the pre-column eluent and a final column using a wild type DIE sequence was run. Total eluent was pooled and run over wild type and mutant DIE coupled columns and collected in fractions. Samples were harvested in 9 fractions and run on a 12.5 % SDS-PAGE gel and silver stained. No differential banding pattern was observed.

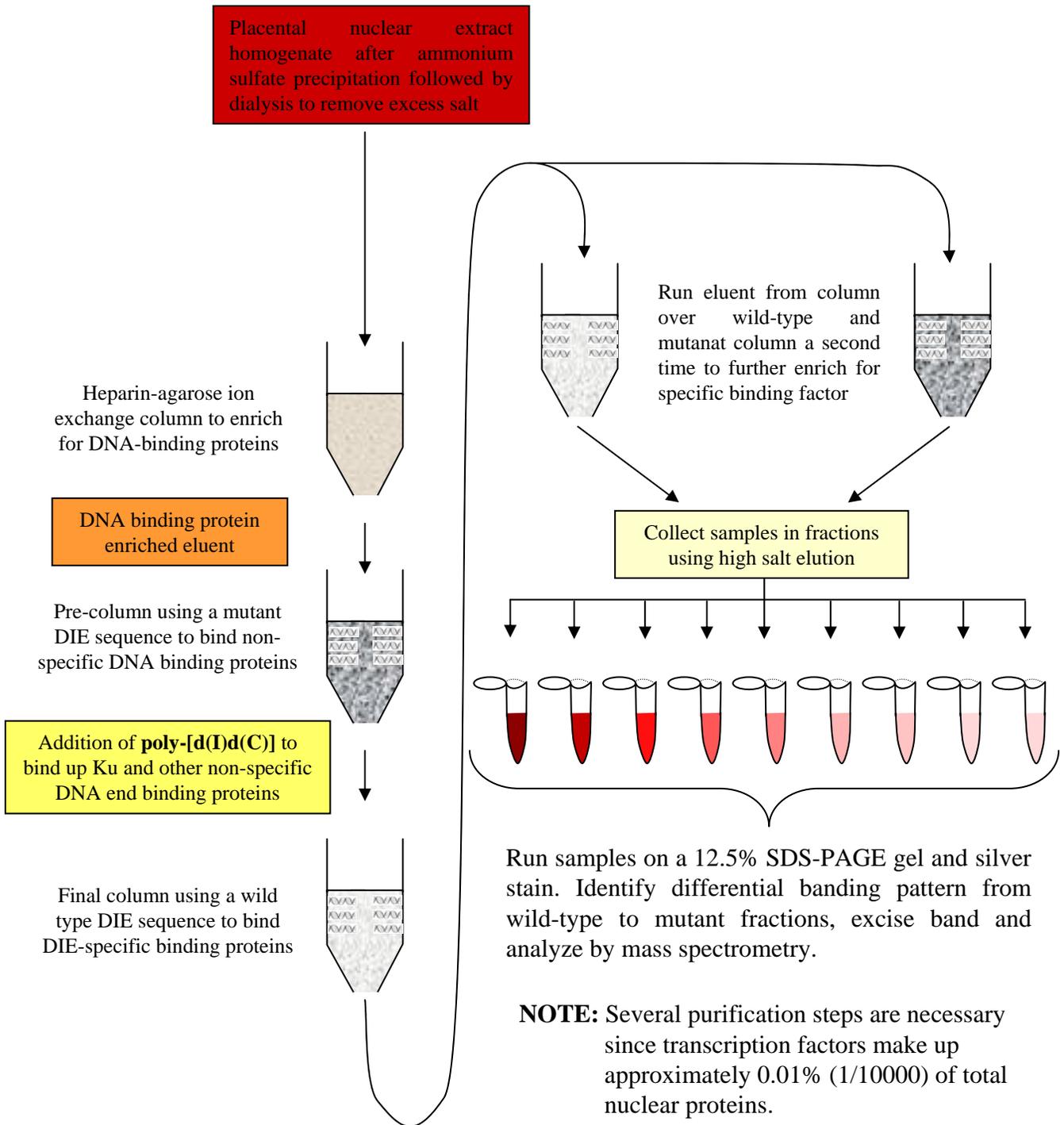


FIGURE 19

Figure 20: Electrophoretic mobility shift assay using placental extracts. In order to test whether the placental extracts were a good source of protein for DNA affinity chromatography, EMSAs were performed using nuclear extracts from fresh placentas. As shown in the top panel using the DIE mutant oligonucleotide (5'-UTR-4d) as the radiolabelled probe with decreasing placental extracts (20 μ g – 2 μ g), there is non-specific binding at high protein concentrations. Therefore, shorter incubation times were attempted with 5 μ g of placental nuclear extracts. As shown in the lower panel, non-specific binding to mutant DIE persisted suggesting that placental extracts are not adequate for DIE-BP purification.

3.1.13 Summary of results in section 3.1

Senescent human diploid fibroblasts are unable to initiate DNA synthesis following mitogenic stimulation and adopt a unique gene expression profile distinct from young or quiescent cells. In this study, a novel transcriptional regulatory element was identified in the 5'-untranslated region (5'-UTR) of the cyclin D1 gene. We show that this element differentially suppresses cyclin D1 expression in young versus senescent fibroblasts (i.e. represses reporter expression 4 to 5 fold more in young cells than in senescent cells). Electrophoretic mobility shift assays revealed abundant complexes forming with young cell nuclear extracts compared to senescent cell nuclear extracts. Binding was maintained in young quiescent cells, showing that loss of this activity was specific to senescent cells and not an effect of cell cycle arrest. Site-directed mutagenesis within this cyclin D1 Inhibitory Element (DIE) abolished binding activity and selectively increased cyclin D1 promoter activity in young, but not in senescent cells. Sequences with homology to the DIE were found in the 5'-UTRs of other genes known to be upregulated during cellular aging suggesting that protein(s) that bind the DIE might be responsible for the coordinate increase in transcription of many genes during cellular aging. This study provides evidence that loss of transcriptional repressor activity contributes to the increased expression of cyclin D1, and possibly additional age-regulated genes, during cellular senescence.

3.2 Senescence-associated chromatin remodeling by the inhibitor of growth-1

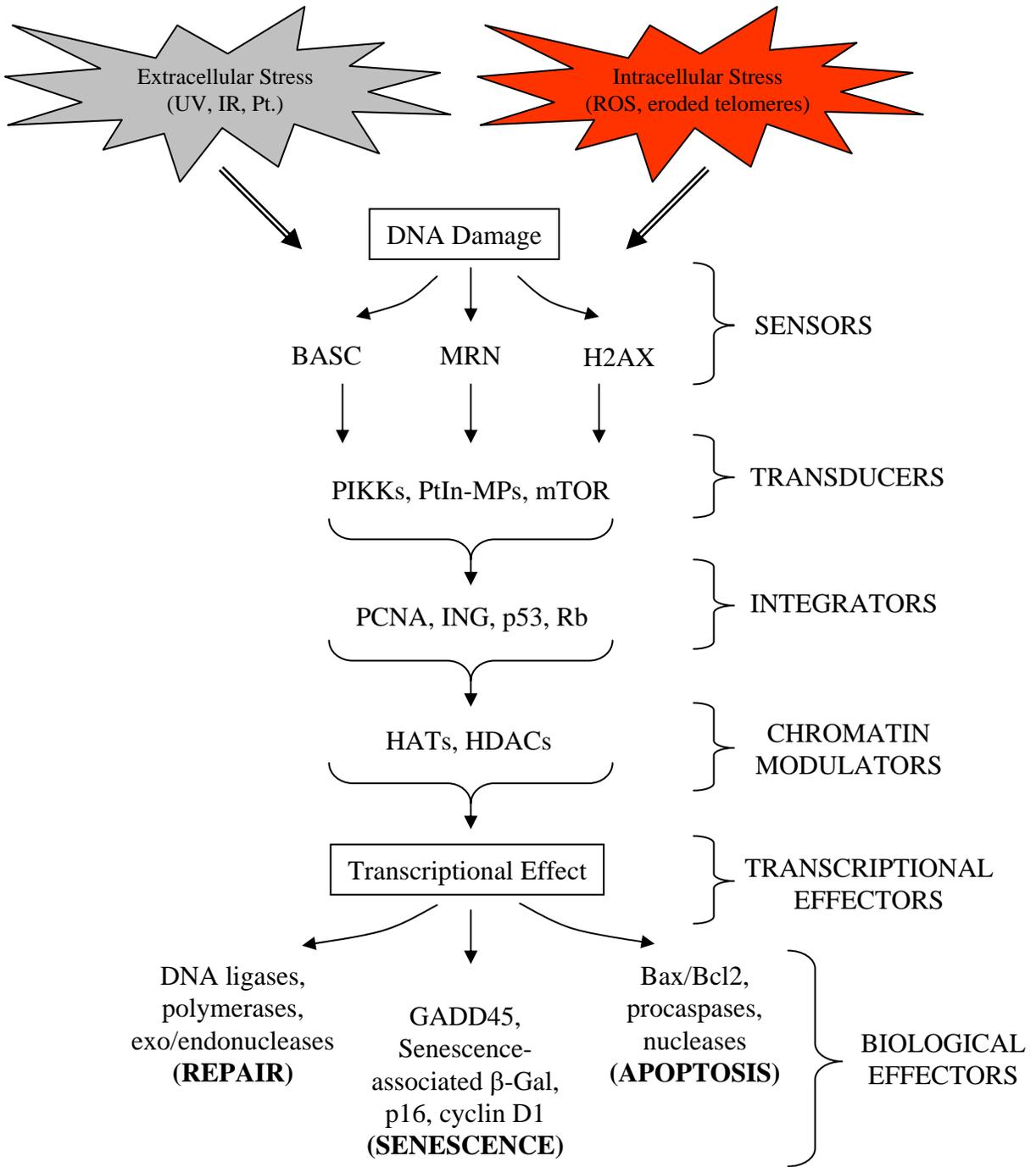
3.2.1 *Background*

Initially identified in the budding yeast *Saccharomyces cerevisiae*, three classes of mammalian histone deacetylases have been identified: class I HDACs, class II HDACs and Sir2p which are the class III HDACs that use NAD⁺ as a cofactor (also called Sirtuins). It has recently been shown that both ING1a and ING1b can associate with HDAC complexes, while ING1b shows a strong preference for binding p300/CBP HAT complexes. Furthermore, the ING1b isoform of ING1 has been shown to associate with the human Sir2 type III HDAC (Kataoka et al., 2003), with other HAT complexes (Vieyra et al., 2002a), and with proliferating cell nuclear antigen (PCNA), an essential component of DNA polymerase δ complexes that are active in DNA replication and repair (Scott et al., 2001). Thus, ING1 proteins are believed to functionally link DNA repair and apoptosis to the alteration of gene expression by altering localized chromatin conformation as shown in figure 21 (Berardi et al., 2004).

3.2.2 *Senescent fibroblasts show altered morphology and DNA staining patterns*

Senescent cells used in this study expressed elevated levels of cyclin dependent kinase inhibitors p21^{WAF1}, p16^{INK4a} (Hara et al., 1996; Wong and Riabowol, 1996), cyclin D1 (Dulic et al., 1993; Berardi et al., 2003) and senescence-associated β -galactosidase activity (SA- β -gal) (Dimri et al., 1995) (Figure 22). Furthermore, it was shown that senescent cells accumulate a globular

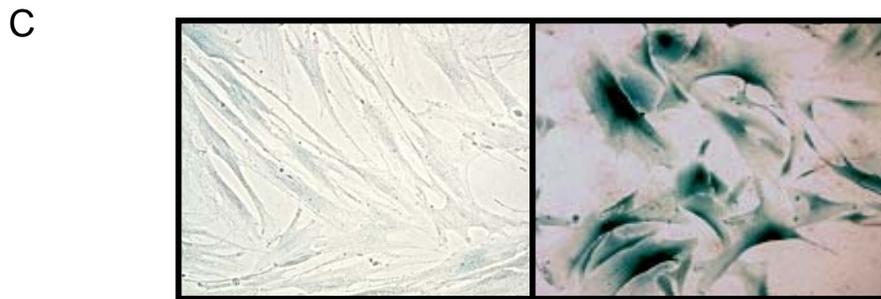
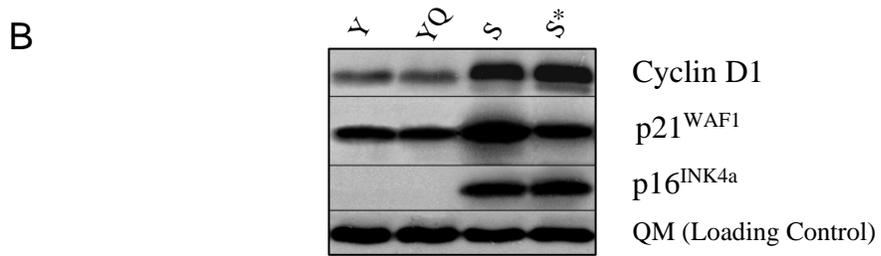
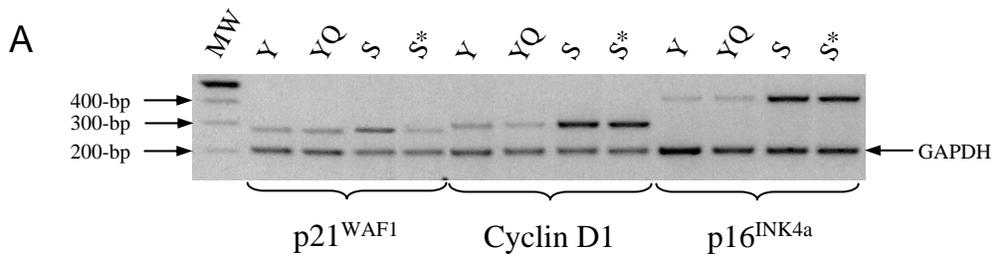
Figure 21: ING1 as an integrator of stress signal transduction. Cellular responses to DNA damage, telomere erosion or extracellular stress all impinge upon common regulators of transcription. Both external stresses such as UV or chemical toxins and internal stresses such as reactive oxygen species (ROS) activate phosphatidylinositol 3-kinase-related kinases (PIKK). This pathway is common to many DNA damage signalling models in that it involves *sensor*, *transducer* and *effector* molecules. Here we propose the addition of *integrators* such as PCNA, ING1, p53 and Rb that target chromatin modifying proteins to particular regions of chromatin to activate or repress particular genes. The activation of ING family proteins is likely mediated in part by the inducible relocalization and binding of phosphatidylinositol monophosphates (PtIn-MPs). These ultimately impinge upon the transcriptional activity of key *biological effectors* that induce DNA repair, senescence or apoptotic pathways.



Berardi et al., 2004

FIGURE 21

Figure 22: Identification of senescent human diploid fibroblasts. **A.** Primer-dropping multiplex PCR was used to examine gene expression profiles in young (Y), young quiescent (YQ), senescent (S, less than 5% of cells incorporate $\{^3\text{H}\}$ -thymidine in 24 hr) and senescent primary fibroblasts cultured an additional 4 weeks after reaching senescence (S*). Primers used to examine p21^{WAF1}, cyclin D1 and p16^{INK4a} gene expression in the presence of the GAPDH internal control are described in materials and methods. **B.** Normal primary fibroblast cell cultures grown and harvested in parallel were immunoblotted for p21^{WAF1}, cyclin D1 and p16^{INK4a}. The transcription factor QM was used as a loading control since its expression level does not change in a cell growth dependent manner. **C.** Young and senescent HDFs were fixed and stained on cover slips in parallel to test for the presence of senescence associated β -galactosidase activity under acidic conditions (pH 6).



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FIGURE 22

form of actin in their nuclei that can be visualized using phalloidin (Kwak et al., 2004). Briefly, phalloidin is a fungal metabolite from *Amanita phalloides* that binds actin and prevents actin filaments from depolymerising. Comparison of young and senescent fibroblasts in figures 23D and 23H highlights these morphology changes. Senescent cell nuclei also accumulate senescence-associated heterochromatic foci (SAHF) (Figure 23E) and both SAHF (indicated by punctate staining in senescent cell nuclei; see arrows in panel 20E) and nuclear staining of actin were seen specifically in senescent fibroblasts. Note that senescent cells have larger nuclei, show typical flattened cell morphology and when images of DNA staining with DAPI (blue), actin staining with phalloidin (green) and ING1 staining with monoclonal antibodies (red) are merged, nuclei from senescent cells stain more yellow, indicating a relatively greater contribution from actin staining. This is consistent with the previously reported increase in nuclear globular actin in senescent cells (Kwak et al., 2004).

3.2.3 *ING1 pre-mRNA is differentially spliced during cell senescence*

To measure ING1 mRNA levels in young versus senescent cells, semi-quantitative RT-PCR using isoform specific primers (Figure 24A) was done with GAPDH primers as an internal control for mRNA integrity and amplification efficiency using the primer dropping method (Wong et al., 1994). This observation was made independently by a research technician in the Riabowol laboratory, Svitlana Pasteryeva.

Figure 23: ING1 staining in normal human fibroblasts. Young (panels **A – D**) or senescent (panels **E – H**) cells were stained with DAPI to visualize DNA (blue), with ING1 monoclonal antibodies (Boland et al., 2000) to visualize ING1 (red) and with phalloidin to visualize actin (green). A composite image of the different staining patterns was then prepared. Arrows in panels E - H highlight bright staining foci in senescent cells that resemble senescence-associated heterochromatic foci (Narita et al., 2003). Strong actin stain in senescent nuclei is a result of increased nuclear globular actin accumulating during senescence (Kwak et al., 2004) and results in less blue nuclei in merged images. The bars represent 20 μ M.

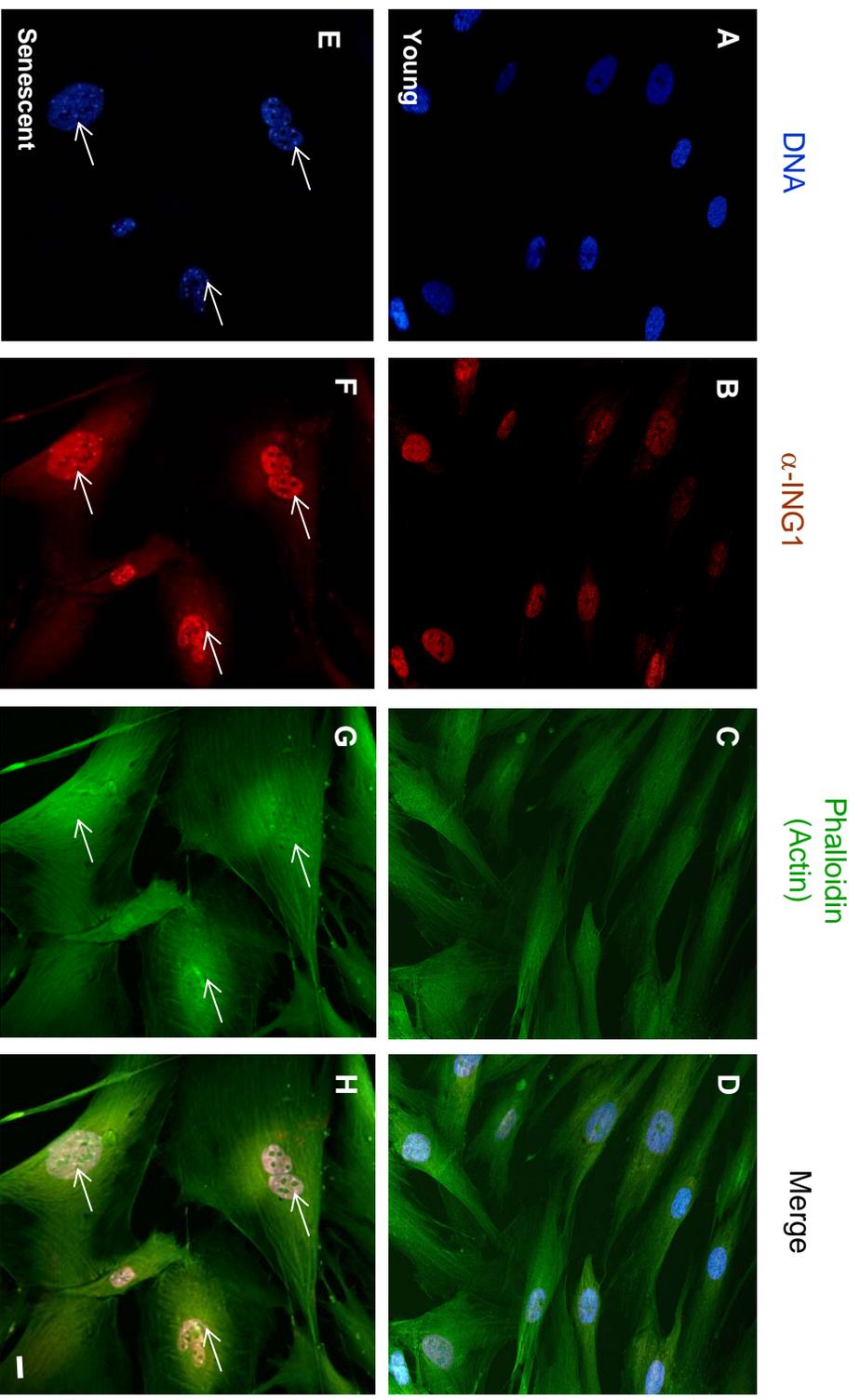


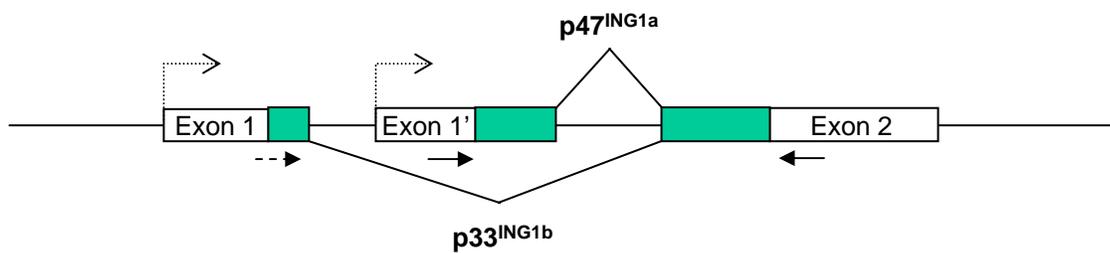
Figure 23
 Berardi et al., 2005

As seen in figure 24B, levels of the ING1b isoform message decrease in senescent cells as previously reported (Vieyra et al., 2002b). In contrast, we noted that the level of the ING1a transcript increases in senescent fibroblasts. Measuring five independent semi-quantitative RT-PCR reactions using the steady-state GAPDH internal control to normalize values showed that the average decrease in ING1b message in senescent cells was to one third the level seen in young proliferating cells, while the levels of ING1a message increased approximately ten-fold in senescent cells (right panel of figure 24B).

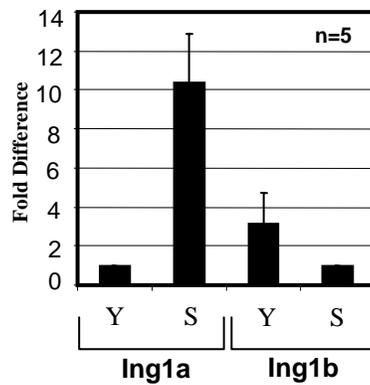
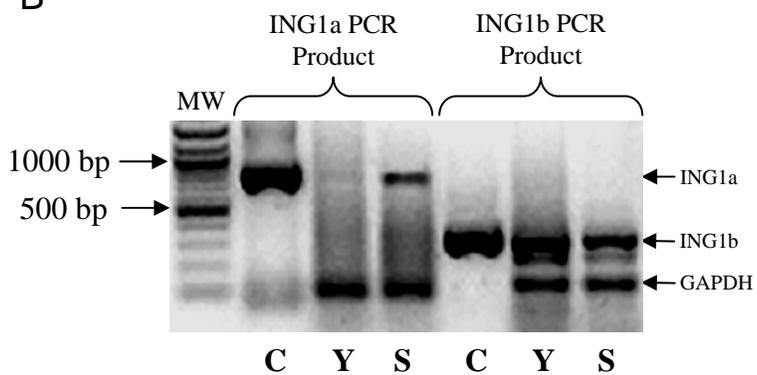
A panel of anti-ING1 monoclonal antibodies (Boland et al., 2000) was then used to examine ING1 protein levels and to test whether transfection of ING1b into senescent cells would decrease the endogenous levels of the ING1a isoform (Figure 25A). Little, if any effect on ING1a protein levels was seen in response to increased ING1b (Figure 25A, lanes 7-9) suggesting that regulation of the ING1 isoforms is not responsive to protein feedback mechanisms. Scanning of multiple equally loaded western blots and analyzing by densitometry indicated that ING1b protein levels decrease greater than 3-fold in senescent cells, while ING1a levels increase by at least 7-fold in senescent cells, consistent with results from RT-PCR. Senescent cell expression profiles are summarized in figure 25B.

Figure 24: ING1 gene structure and differential expression. **A.** The two predominant isoforms of ING1 (p47ING1a and p33ING1b) possibly arise from alternative splicing by exon skipping of three exons in the ING1 gene locus (Exon 1, Exon1' and Exon 2), or by unique promoters driving the expression of two transcripts ultimately resulting in the multiple ING1 isoforms. Primers designed to specifically amplify ING1 isoforms are shown below exon 1 (dashed arrow) and below exon 1' (solid arrow). These were used in conjunction with a primer recognizing the common exon (Exon 2). Dashed arrows above the gene are to show potential alternative promoters that may be acting to regulate ING1 isoform expression. Green boxes are indicative of coding regions while white boxes are the untranslated regions of the mRNA transcript. **B.** Total RNA from young (Y) or senescent (S) HS-68 fibroblasts was reverse-transcribed and the cDNA was analyzed by PCR to determine relative amounts of ING1 transcripts in harvested samples. GAPDH was used as an internal control to quantitate RNA isolation and PCR amplification efficiency. Dilutions of ING1 expression plasmids were used as positive controls to verify amplification products (C lanes). Scanning densitometry of bands from independent experiments (n = 5) was used to generate the bar graph showing relative levels of ING1a and ING1b in young and senescent cells. Absolute levels of ING1a relative to ING1b cannot be determined from these data due to different amplification efficiencies.

A



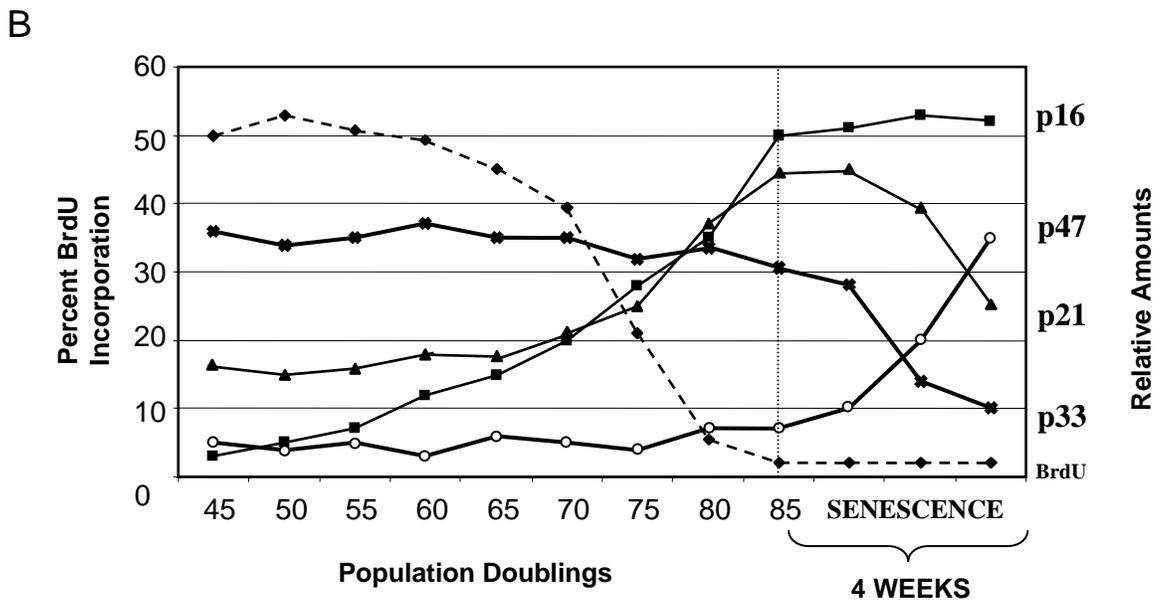
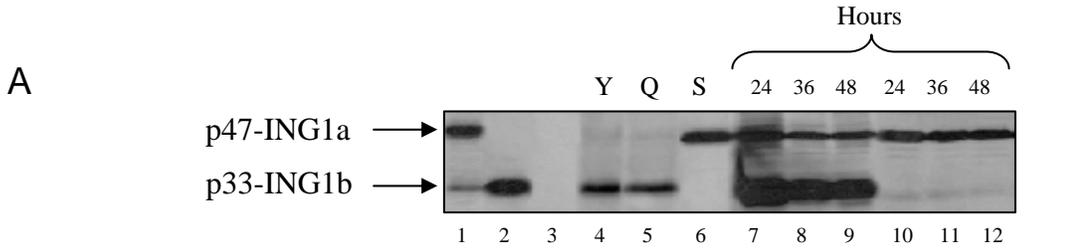
B



Berardi et al., 2005

FIGURE 24

Figure 25: ING1 protein levels in young and senescent fibroblasts. A. Western blots using control lysates from HS-68 fibroblasts transfected with expression constructs encoding each of the ING1 isoforms (lanes 1-2) and using lysates from untransfected young log phase (Y), serum-deprived quiescent (Q) and senescent (S) HDFs (lanes 4-6) and using lysates from senescent HS-68s transfected with an ING1b expression vector (lanes 7-9) or a control (empty) vector (lanes 10-12). Cells in lanes 7-12 were harvested at the times indicated above gel after transfection. **B.** Protein levels of ING1 isoforms p33, p47 and cyclin dependent kinase inhibitors p16 and p21 known to be differentially expressed during cellular senescence. Each time point represents the average of three independently determined values. HS-68 cells failed to divide beyond 85 MPDs but were given fresh growth medium weekly for 4 weeks. Incorporation of the thymidine analog bromodeoxyuridine was measured in parallel to quantitate total DNA synthesis (dashed line).



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FIGURE 25

3.2.4 *ING1 isoforms differentially associate with HATs and HDACs in senescent cells*

ING1b associates with HAT complexes that contain p300/CBP components with a greater affinity than does ING1a, and ING1a has been shown to associate with HDAC complexes that contain HDAC1 (Vieyra et al., 2002a). After formation, these complexes may be recruited to specific regions of DNA through the ability of ING1b proteins to interact with the chromatin-bound insoluble portion of PCNA in response to cellular stress (Scott et al., 2001). As shown in figure 26A using normal human foreskin fibroblasts (HS-68), ING1a does not coimmunoprecipitate with CBP, but it is abundant in HDAC1 immunocomplexes. Furthermore, the interaction of ING1a with HDAC1 immunocomplexes is increased several-fold in growth-arrested quiescent and senescent cells (Figure 26A). This may be a consequence of increased binding affinity of proteins or stoichiometric changes due to overall protein abundance changes in various growth states. In contrast, ING1b coimmunoprecipitates with CBP and HDAC1 but shows a relative preference for interacting with CBP, compared to HDAC1 immunocomplexes in young cells. A fraction of the immunoprecipitates were run on parallel SDS-PAGE to ensure that immunoprecipitation efficiency of the CBP and HDAC1 protein complexes was equal between young (Y), quiescent (Q) and senescent (S) lysates (Figure 26A, lower panel). Similar results were obtained using a normal human lung fibroblast cell strain (WI-38) (Figure 26B) suggesting that differential association of ING1

Figure 26: Co-immunoprecipitation of histone acetyltransferases and ING1.

A. Lysates from young log phase (Y), young quiescent (Q) or senescent (S) HS-68 foreskin fibroblast cells were immunoprecipitated with protein G sepharose beads coupled to rabbit α -CBP or rabbit α -HDAC1 under nondenaturing conditions and precipitates were immunoblotted with a panel of mouse α -ING1 monoclonals and mouse α -CBP and mouse α -HDAC1. **B.** Exactly the same IP-westerns were run as in panel A except lysates were from the WI-38 lung fibroblast cells. **C.** The antibody coupling efficiencies to the protein G sepharose beads was measured by aliquoting equal volumes of PGS-antibody complexes, adding sample buffer and running on an SDS-PAGE gel and stained with Coomassie Brilliant Blue stain.

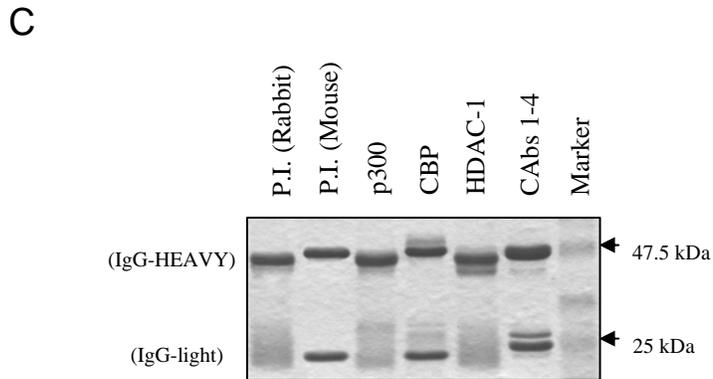
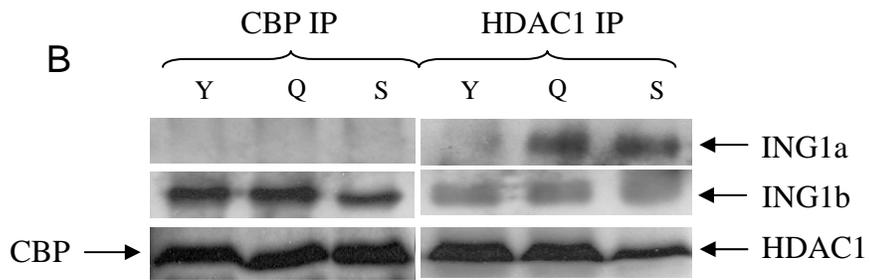
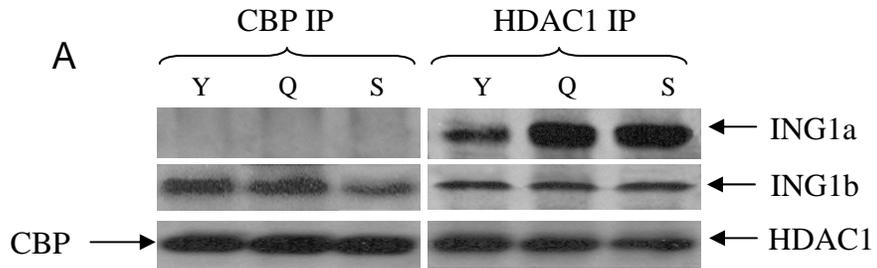


FIGURE 26

proteins with HATs and HDACs is consistent between normal fibroblast cell strains. Reciprocal immunoprecipitations were also performed to confirm ING1 association with CBP and HDAC1, and although they confirmed associations as noted previously (Vieyra et al., 2002a), it was not possible to determine ING1 isoform specificity due to antibody limitations. Briefly, the isoform specific antibodies are only available in a rabbit polyclonal whole serum mix that cannot be used in immunoprecipitation/western blot applications because of native immunoglobulins (IgGs) found in whole serum. Consequently, the mouse monoclonal panel of ING1 antibodies derived from hybridoma cells grown in serum free media are used in these assays and are directed against all ING1 isoforms (Boland et al., 2000). Control Coomassie brilliant blue stain in figure 26C demonstrates similar coupling efficiency of various rabbit and mouse antibodies to protein G sepharose beads (*Amersham Biosciences*) used in these immunoprecipitation assays.

3.2.5 Increased HDAC activity in ING1a immunocomplexes from quiescent and senescent cells

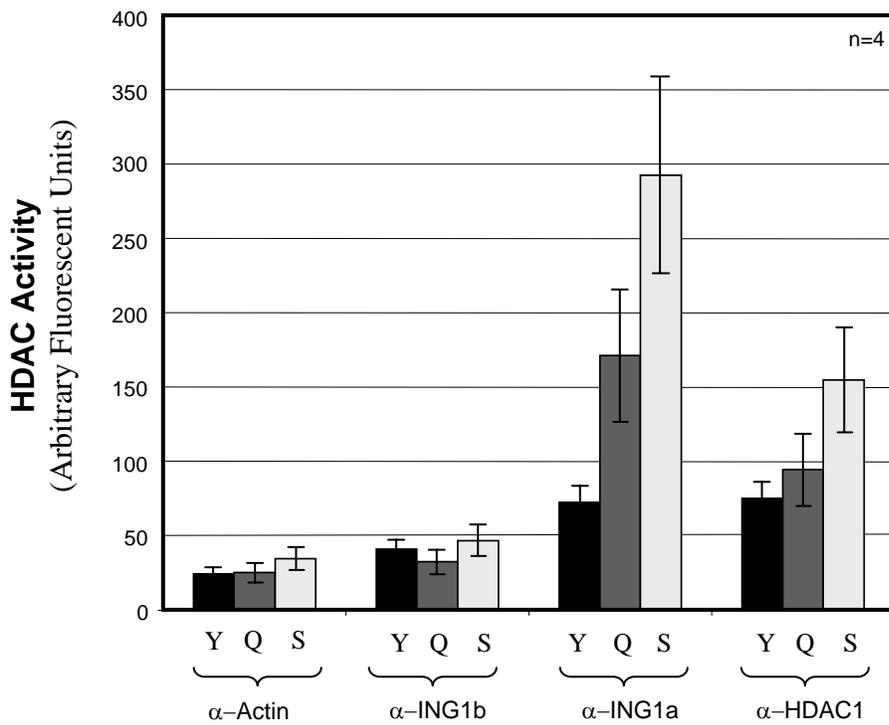
To confirm whether the increased levels of ING1a seen in senescent cells bound to chromatin we performed chromatin immunoprecipitation (ChIP) experiments with extracts of cells labelled with $\{^3\text{H}\}$ -thymidine. This assay was performed by Dr. Karl Riabowol at the University of Calgary. When normalized to the control α -PKC antibody, senescent cells showed much higher levels of ING1a cross-linked to chromatin than young cells. We next tested whether the

increased levels of chromatin-bound ING1a in cell cycle arrested quiescent and in senescent fibroblasts showed altered HDAC activity as measured by *in vitro* deacetylation assays (Figure 27). This work was done in collaboration with Dr. Dallan Young's laboratory and experiments were performed by a post-doctoral fellow in the lab, Dr. Ana Colina. The *Fluor de Lys* HDAC fluorimetric assay (Biomol) is a sensitive and convenient alternative to protocols utilizing radiolabelled, acetylated histones or peptide/HPLC methods for the assay of histone deacetylases. It is based on the unique *Fluor de Lys* (Fluorimetric Histone deAcetylase Lysyl) substrate and developer combination and provides an assay that can be carried out in two simple mixing steps, all on the same 96-well plate. First, the *Fluor de Lys* Substrate, which comprises an acetylated lysine side chain, is incubated with the immunoprecipitated protein complexes containing HDAC activity. Deacetylation of the substrate sensitizes the substrate so that, in the second step, mixing with the *Fluor de Lys* developer generates a fluorophore.

Immunoprecipitates from young (Y), quiescent (Q) and senescent (S) cell lysates using a rabbit polyclonal α -ING1b isoform specific antibody recovered background levels of HDAC activity, similar to the control actin antibody, while immunoprecipitates with HDAC1 antibodies (rabbit polyclonal) recovered approximately two-fold greater HDAC1 activity from senescent, compared to young cells. In contrast to α -ING1b, isoform specific rabbit polyclonal α -ING1a immunoprecipitates contained high levels of HDAC activity in quiescent cells and even higher levels in senescent cells (Figure 27). Furthermore, ING1a

Figure 27: Histone deacetylase (HDAC) activity of ING1a immunocomplexes.

Lysates from young growing (Y), quiescent (Q) or senescent (S) cells were immunoprecipitated with the indicated antibodies under nondenaturing conditions and the protein complexes were analyzed for *in vitro* HDAC activity. ING1a immunoprecipitates treated with 1 μ M of TSA were used as a negative control and showed complete elimination of HDAC activity. Experiments were done four times resulting in the noted standard deviations.



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FIGURE 27

immunoprecipitates showed higher levels of HDAC activity than did immunocomplexes isolated using α -HDAC1 antibodies (Figure 27). This could be due to the association of ING1a with multiple HDAC species (Gao et al., 2002; Kataoka et al., 2003) or to activation of HDAC activity by the presence of ING1a as suggested by previous studies (Vieyra et al., 2002a).

3.2.6 *ING1a induces a senescent cell morphology*

To test whether ING1a induced features similar to those seen in replicative senescence and to ask if overexpression of ING1a could affect the morphology of normal low passage fibroblasts, we co-transfected cells with either ING1a or ING1b expression constructs and a GFP expression construct. As shown in figures 28A-28D, overexpressed GFP showed both nuclear and cytoplasmic subpopulations as expected and had no effect upon morphology. Cells transfected with ING1b expression constructs accumulated nuclear apoptotic bodies and showed gross morphologic changes in both the nucleus and cytoplasm, typical of cells undergoing apoptosis (Figures 28E-28H). In contrast, cells overexpressing ING1a for 36 hrs acquired a senescent-like flattened phenotype and SAHF typical of senescent cells (Figures 28I-28L). ING1a transfected cells also expressed senescence associated β -galactosidase activity, consistent with a senescent morphology. However, overexpression of ING1b also showed senescence associated β -galactosidase activity probably as a result of the normal stress response elicited by ING1b in primary fibroblasts (Kurz et al., 2000). This is a manifestation of an increase in the classic acid lysosomal β -galactosidase as a

result of cellular stress induces by ING1b. These findings, coupled with previous reports showing that inhibiting ING1 gene expression allows primary fibroblasts to undergo several additional population doublings when approaching senescence (Garkavtsev and Riabowol, 1997), support the idea that the ING family of HAT/HDAC modulators contribute to regulating the senescent phenotype.

Senescent cells accumulate SAHF (Figure 28N) and SAHF formation and maintenance is linked to Rb and to the activity and expression of E2F and E2F target genes (Narita et al., 2003). SAHF are not sites of active transcription and can be easily visualized by staining DNA. Comparison of DAPI stained nuclei from young (Figure 28M) and senescent (Figure 28N) primary fibroblasts highlights the enlarged nuclei and accumulation of SAHF typical of senescent cells. When ING1a was transiently overexpressed in young, proliferation competent cells, nuclei enlarged and developed SAHF, resembling those seen in senescent cells (compare panels 25N and 25O). In contrast, transient overexpression of ING1b in young fibroblasts caused nuclei to deform, shrink and acquire characteristics of apoptosis as shown in figure 28P. These observations are consistent with ING1a blocking cell cycle progression with a phenotype similar to cellular senescence while ING1b promotes progression into apoptosis. Formation of heterochromatin (SAHF) in cells overexpressing ING1a increased with time after transfection suggesting that these phenotypic changes occur in a time and/or dose-dependent manner.

Figure 28: Morphology of cells overexpressing ING1a versus ING1b. Young, proliferation-competent HS-68 cells were transfected with GFP (**A – D**), GFP plus ING1b (**E – H**) or GFP plus ING1a (**I – L**), fixed and stained with DAPI (blue). Panels A, E and I show GFP fluorescence, B, F and J show DNA staining and C, G and K show merged images. Panels D, H and L show representative nuclei stained with DAPI at 3X higher magnification. Panels M – P show representative nuclei from young, senescent and transfected cells respectively. White arrows indicate senescence associated heterochromatic foci in panel N and ING1a induced heterochromatin formation in panel O. The bars represent 20 μ M.

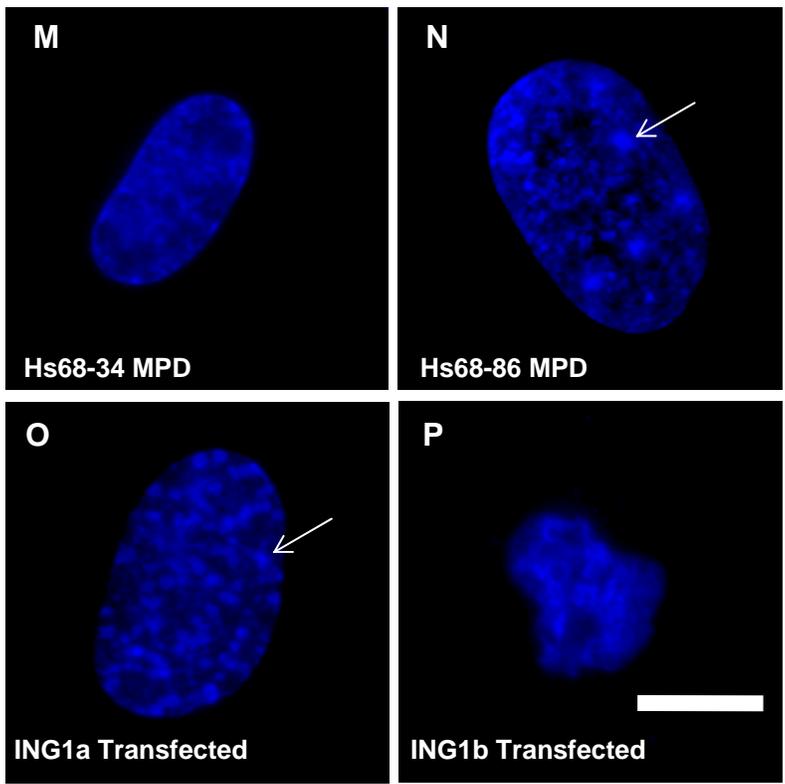
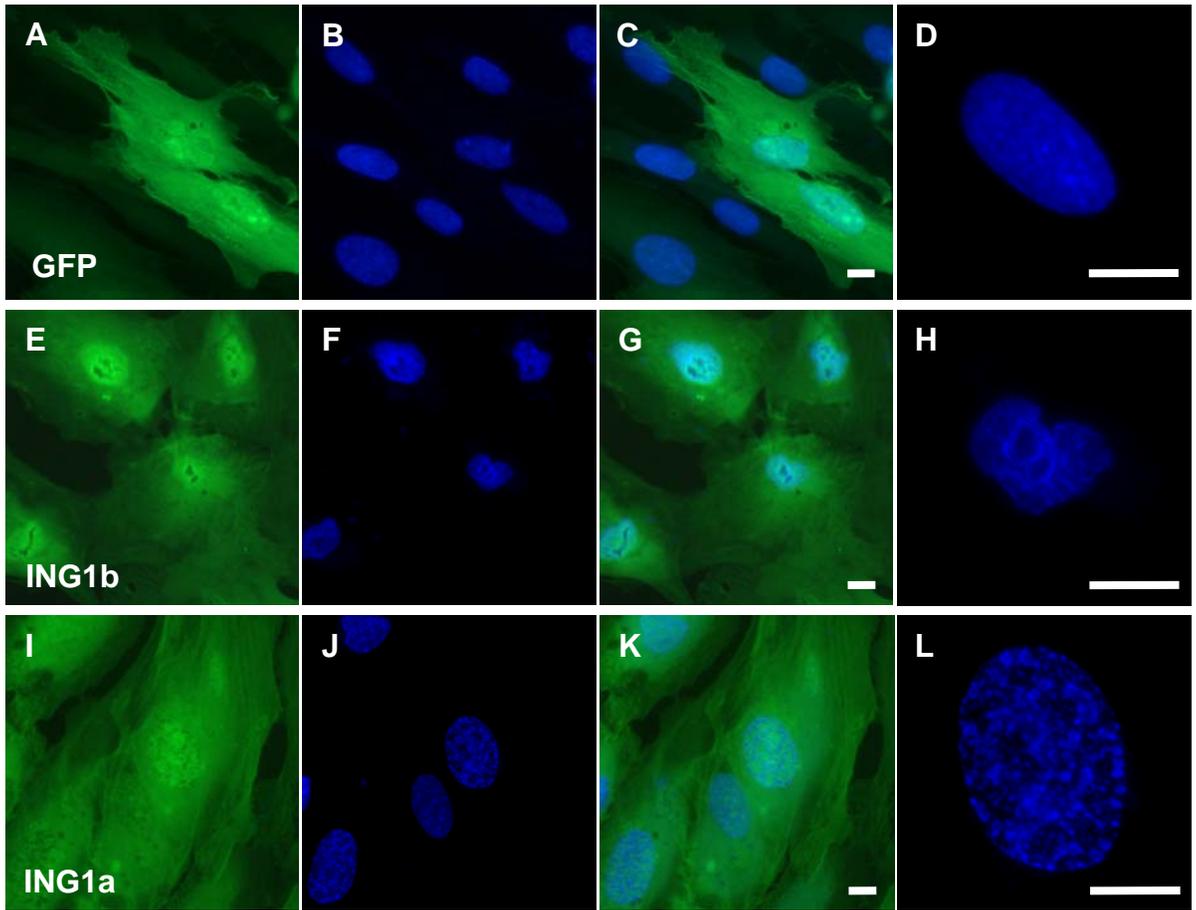
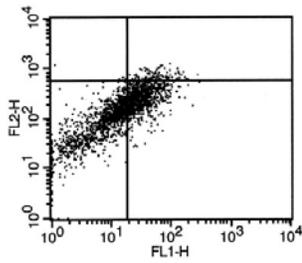


FIGURE 28

3.2.7 *ING1 isoforms differentially affect apoptosis and cell cycle progression*

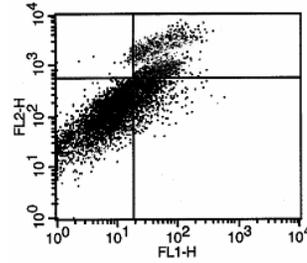
Although ING1a and ING1b showed clearly different effects on primary cell morphology, we wished to corroborate these observations using independent methods. Using an *Annexin V-FITC* assay we were able to determine that ING1a did not induce apoptosis by 48 hrs post-transfection whereas ING1b strongly induced apoptosis as early as 12 hrs post-transfection (Figure 29). To determine if ING1 isoforms had a cell cycle effect, we transfected primary fibroblasts with expression constructs similar to what we did with the *Annexin V* assay and analyzed cell cycle progression (Figure 30). This clearly demonstrated the ability of ING1a to induce cell cycle arrest (Figure 30A) but failed to show a sub-G1 population of cells indicative of apoptosis in ING1b transfected cells (Figure 30B). Subsequently, this data was summarized with other corroborative data to show that by *Annexin V* that ING1b induced apoptosis (Figure 31A) while by FACS cell cycle analysis ING1a induced cell cycle arrest (Figure 31B). Apoptosis was not induced by ING1a in this time frame, although ING1b was able to induce cell cycle arrest of a subset of cells, consistent with previous reports (Garkavtsev and Riabowol, 1997). These biochemical data were consistent with effects upon cell morphology and since senescent cells are known to be resistant to the induction of apoptosis, it suggested that ING1a and ING1b might antagonize each other functionally. To test this idea, increasing amounts of ING1a expression constructs were co-transfected with a constant amount of ING1b expression

Figure 29: ING1b induced apoptosis. *Annexin V-FITC* (BD Biosciences) was used to verify ING1b induced apoptosis in HS-68 fibroblast cells. Cells were transiently transfected with ING1a expression constructs and were harvested at 12, 24, 36 and 48 hours. After all time points were harvested, cells were incubated with Annexin V-FITC and propidium iodide for 10 minutes in the dark followed by analysis by flow cytometry. As shown in the left column, ING1a overexpression minimally induced apoptosis by 48 hrs. However, the ratio of viable to apoptotic cells decreases by greater than 20 fold over a 48 hr time course in cells overexpressing ING1b (right column). A GFP control was also included as a negative and transfection efficiency control. GFP overexpression did not induce apoptosis to levels above background and approximately 58 % of fibroblasts were successfully expressing plasmid. In individual FACS analyses, the abscissa (X-axis) represents fluorescein isothiocyanate (FITC) end-labeled DNA and the ordinate (Y-axis) is a propidium iodide (PI) counterstain of all nuclei.



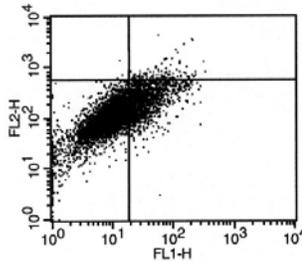
pCI-ING1a: 12 hrs

Viable: 86.65%
Apoptotic: 0.66%



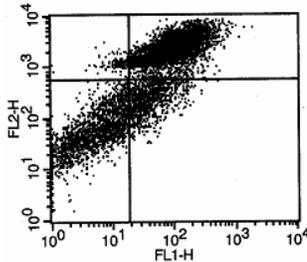
pCI-ING1b: 12 hrs

Viable: 78.45%
Apoptotic: 8.66%



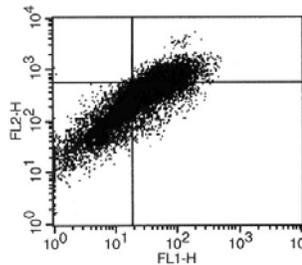
pCI-ING1a: 24 hrs

Viable: 85.35%
Apoptotic: 1.02%



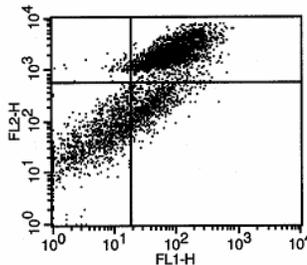
pCI-ING1b: 24 hrs

Viable: 35.45%
Apoptotic: 49.02%



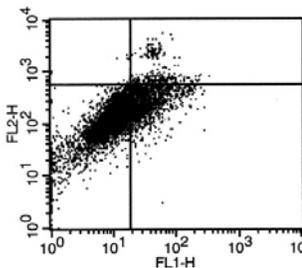
pCI-ING1a: 36 hrs

Viable: 80.87%
Apoptotic: 2.53%



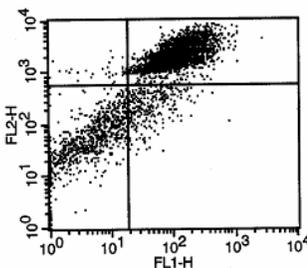
pCI-ING1b: 36 hrs

Viable: 30.07%
Apoptotic: 55.43%



pCI-ING1a: 48 hrs

Viable: 80.05%
Apoptotic: 4.63%



pCI-ING1b: 48 hrs

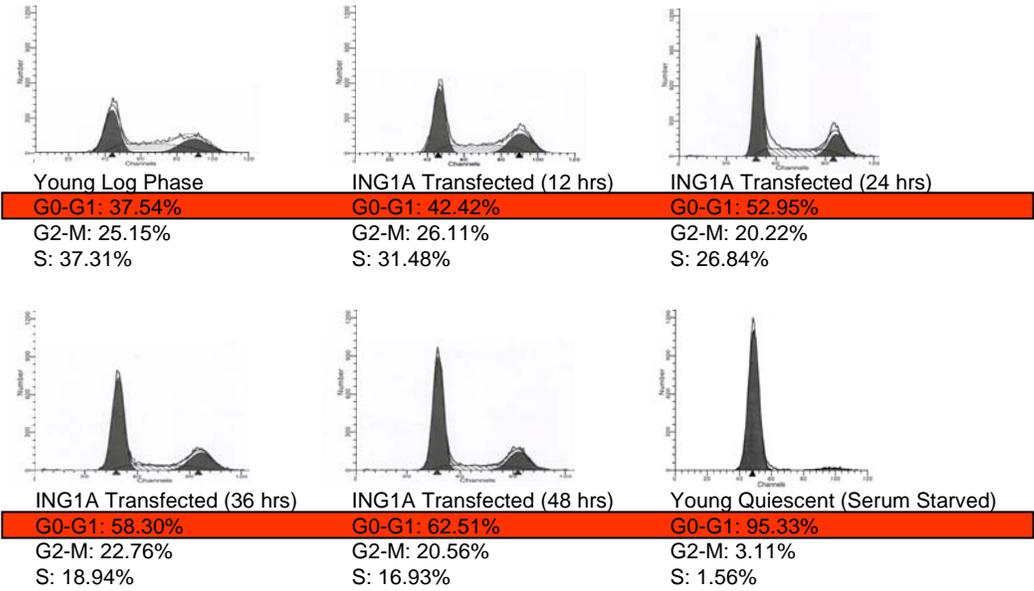
Viable: 26.53%
Apoptotic: 60.63%

FL2-H: Propidium Iodide
FL1-H: FITC

FIGURE 29

Figure 30: ING1a induced cell cycle arrest. In order to decipher ING1 isoform function, FACS analysis was performed on log phase HS-68 fibroblast cells to evaluate DNA content using propidium iodide staining. ING1 plasmids were transiently transfected into 60 mm dishes and harvested over a 48-hr time course. **A.** As shown here, ING1a overexpression in HDFs results in a G0-G1 accumulation (boxed values shown in red). Untransfected young log phase HDFs were used as a negative control (1st panel) and serum starved young quiescent HDFs were used as a positive control (6th panel). **B.** Similar analysis using ING1b expression plasmids did not show same level of G0-G1 accumulation, but also failed to show a robust sub-G1 population of cells indicative of apoptosis. Immortalized mouse embryonic fibroblasts (MEF) were serum starved for 5 days and analysed by FACS analysis as a positive control for apoptosis induction (sub-G1 DNA content).

A



B

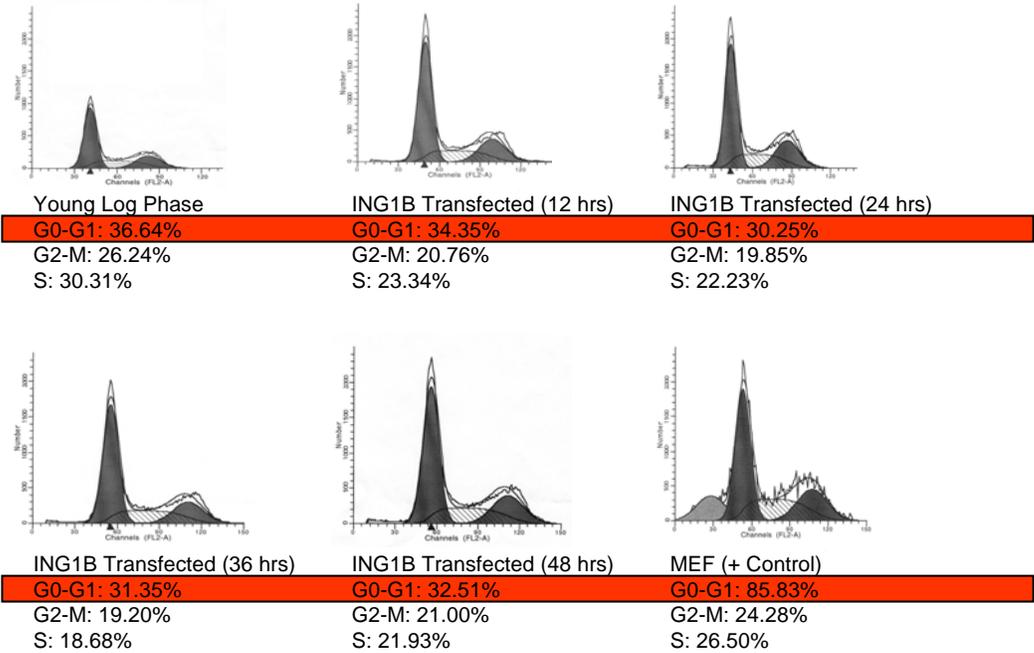
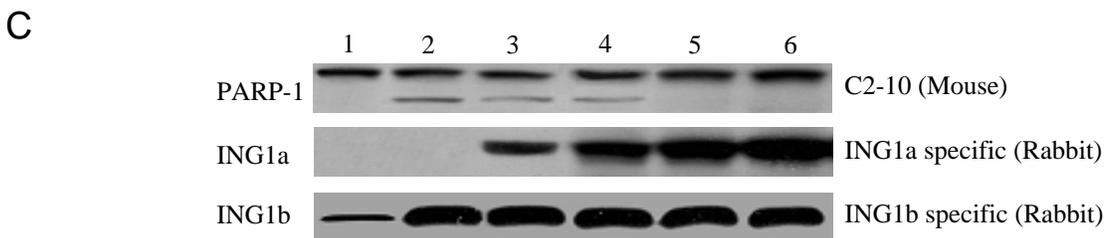
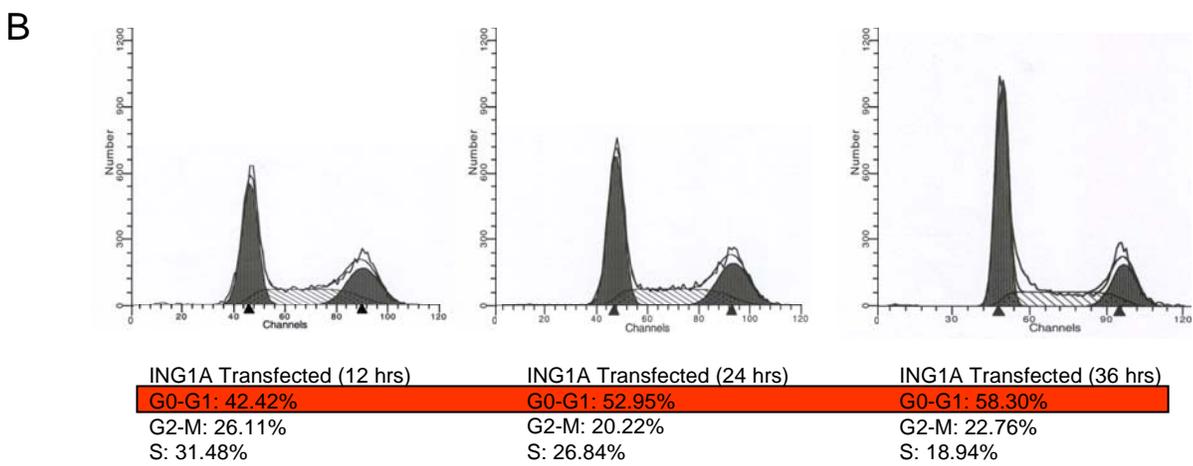
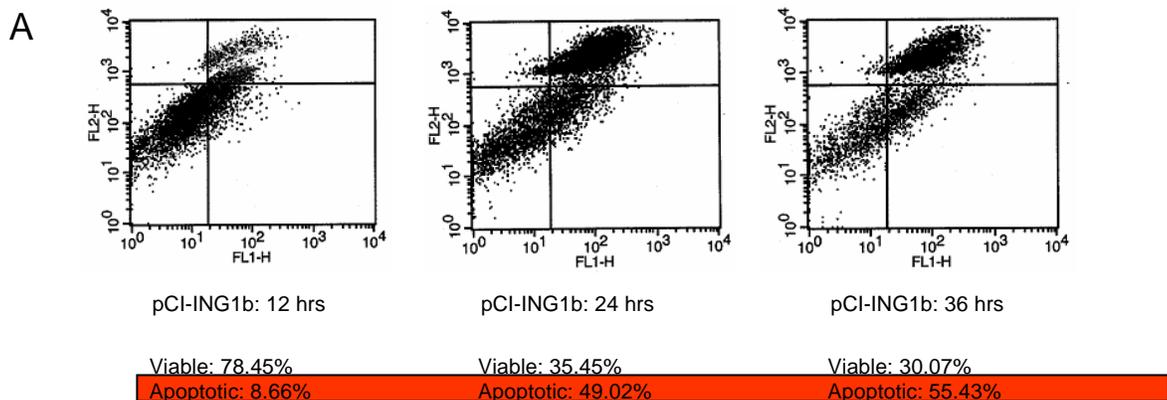


FIGURE 30

Figure 31: Differential effects of ING1 isoforms on cell growth and apoptosis.

A. *Annexin V-FITC* showing time-dependent induction of apoptosis by ING1b overexpression (summary of figure 29 – right column). **B.** Propidium iodide staining of HS-68 cells overexpressing ING1a analyzed for DNA content. Percentages of cells in different parts of the cell cycle are indicated below the graphs (summary of figure 30A). **C.** Young HS-68 cells (36 MPD) were transfected with combinations of control (empty vector), ING1a and ING1b expression constructs and were harvested 24h after transfection. Cell lysates were sequentially analyzed by western blotting with mouse C2-10 antibody to visualize PARP1, with polyclonal α -ING1a and with polyclonal α -ING1b. The lower band in the panel using the PARP1 monoclonal antibody represents PARP1 cleaved by caspase and is a measure of activation of an apoptotic proteolytic cascade. No staining for ING1a is seen in the absence of transfection since this ING1 isoform is expressed at very low levels in proliferating cells.



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FIGURE 31

construct that was capable of inducing apoptosis. As shown in figure 31C, ING1a was capable of blocking the ability of ING1b to induce apoptosis, in a dose-dependent manner as estimated by PARP1 cleavage. These data corroborate effects upon morphology and upon the induction of apoptosis by ING1b, but not ING1a (Vieyra et al., 2002b).

3.2.8 *ING1 isoforms differentially affect PCNA levels by altering transcription*

In order to test if ING1 proteins bound chromatin with higher affinity during cellular senescence we performed radiolabelled chromatin immunoprecipitations (ChIP). This assay was performed by Dr. Karl Riabowol at the University of Calgary as described in Hasan *et al.* (Figure 32) and simply demonstrates the ability of α -ING1a antibodies to immunoprecipitate ^3H -thymidine labelled DNA from senescent cells (Hasan et al., 2001). However, a dramatic change in this ability is not seen when compared to young cells since DNA synthesis is necessary to incorporate radiolabel.

Since ING1a is associated with HDAC activity (Figure 27), ING1a overexpression induced heterochromatin formation in the nuclei of primary fibroblasts (Figure 28), and heterochromatic foci form at the PCNA promoter as a result of altered histone acetylation (Narita et al., 2003), we asked if ING1a altered PCNA expression. This could possibly occur through ING1 protein binding to specific gene promoters and targeting recruitment of chromatin modifying proteins to these regions (Kataoka et al., 2003).

Figure 32: Chromatin binding affinity of ING1 increases during senescence.

HDFs at the indicated passage were incubated with 1 $\mu\text{Ci/ml}$ of $\{^3\text{H}\}$ -thymidine, fixed with 1% formaldehyde to cross link DNA to protein in chromatin and equal counts of labelled DNA were immunoprecipitated with the indicated antibodies as described in Hasan *et al.* (Hasan et al., 2001). The α -PKC antibody recognizes a cytoplasmic protein and it serves as a negative control to account for background binding. A major limitation to this approach in assigning ING1 binding affinity to chromatin in proliferating versus senescent cells is that senescent cells do not synthesize DNA to the same extent as proliferation competent young cells. Therefore, incorporation efficiency of $\{^3\text{H}\}$ -thymidine will not be uniform across samples. Samples were normalized by analyzing the α -PKC antibody immunoprecipitates (negative control) radioactivity by liquid scintillation counting. This experiment was performed by Dr. Karl Riabowol at the University of Calgary.

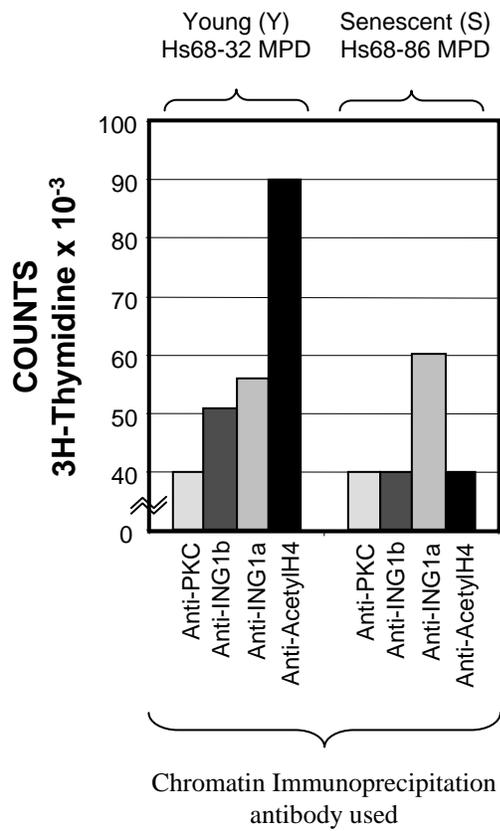
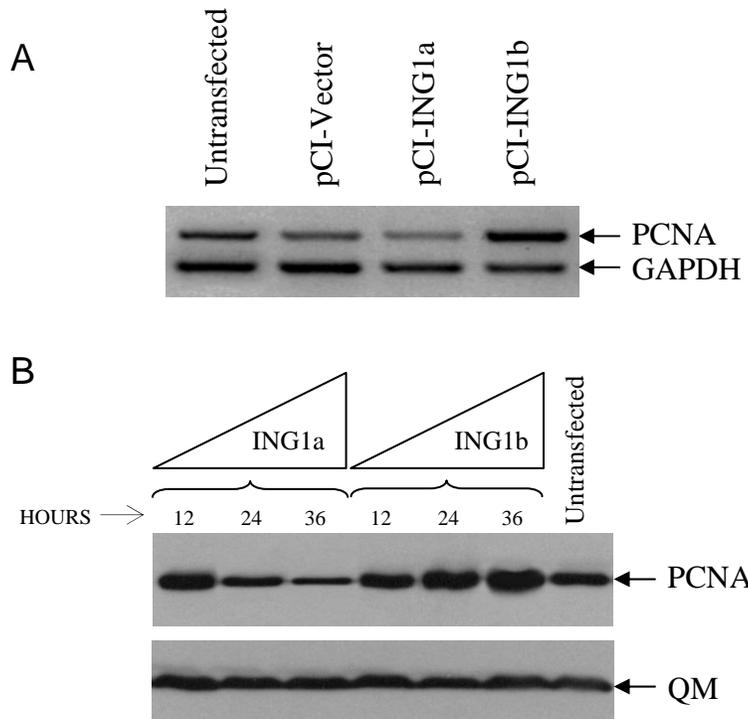


FIGURE 32

As shown in figures 33A and 33B, overexpression of ING1a decreases, and ING1b increases the amount of PCNA RNA and protein, consistent with ING1a targeting HDAC activity to the PCNA promoter. To test this idea further, ChIP assays were done using chromatin from young (Y), quiescent (Q) and senescent (S) primary fibroblasts (Figure 34). Figure 34A illustrates the efficient shearing of chromatin to help ensure that immunoprecipitations were successfully pulling down DNA sequences relatively close to protein binding regions. Since ING1a levels increase during senescence and increased binding of ING1 to chromatin is seen during senescence (Figure 32), the prediction was that increased levels of ING1a would result in increased binding of ING1 to the PCNA promoter in senescent cells. Figure 34B shows decreased binding of acetylated histone H3 (acH3) and increased binding of Rb and p130 to the PCNA and cyclin A promoters in senescent cells as reported previously (Narita et al., 2003). Protein G sepharose beads with no antibody was used as a negative control and total chromatin input served as a positive control for amplification. Figure 34B also demonstrates that there is increased p130 binding to the cyclin A promoter during quiescence and senescence (Narita et al., 2003) and an additional negative control in which chromatin was immunoprecipitated with non-specific mouse IgG. Precipitates using ING1 antibodies show that ING1 preferentially binds to the PCNA promoter in senescent cells similar to the increase seen for Rb binding. Although some binding of ING1 to the cyclin A promoter may occur, signals were variable and considerably weaker than for the p130 control suggesting that

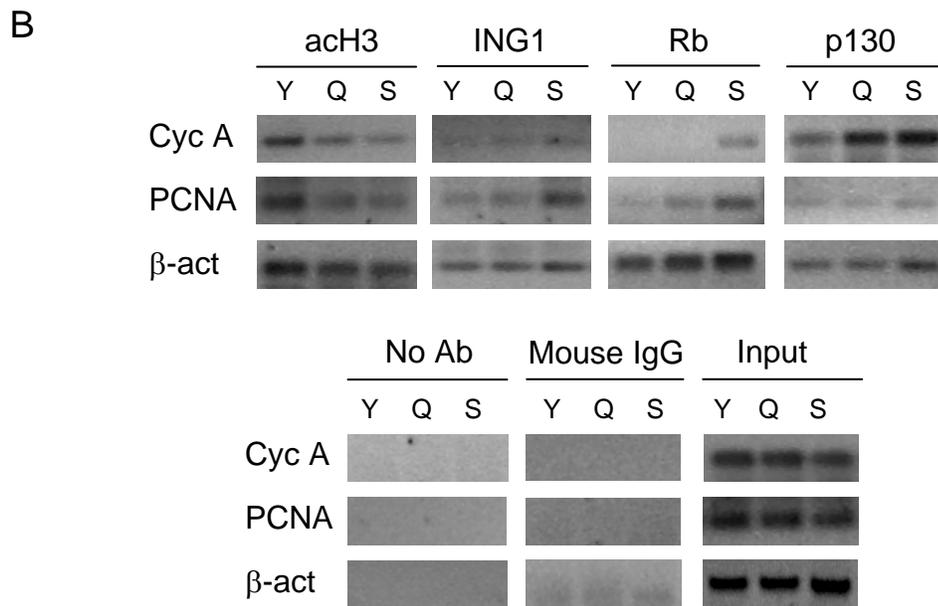
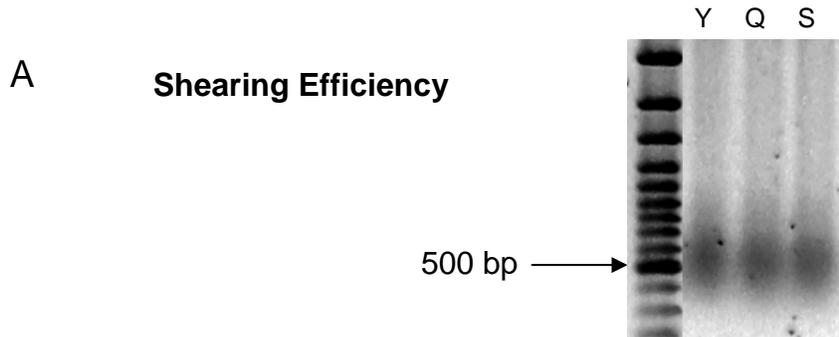
Figure 33: PCNA is differentially regulated by ING1 isoforms. **A.** Normal young (36 MPD) primary cells overexpressing individual ING1 isoforms differentially affect the expression of PCNA. Multiplex PCR was performed on cDNA harvested 36 hrs after transient transfection of HS-68 fibroblasts. Empty pCI-vector was included as negative control and GAPDH was included as an internal PCR control. **B.** A time course of PCNA protein levels in lysates harvested from ING1a and ING1b overexpressing cells. Cells overexpressing ING1a or ING1b were harvested at 12, 24 and 36 hr time points after transfection and analyzed by SDS-PAGE. An untransfected control was included to determine basal PCNA levels in log phase fibroblast cells. QM was used as a loading control.



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FIGURE 33

Figure 34: Chromatin immunoprecipitation of PCNA promoter by ING1. A. Shearing efficiency of sonication of young (Y), quiescent (Q) and senescent (S) fibroblast lysates was measured in order to insure approximate fragmentation of chromatin. As shown, DNA was fragmented in pieces of approximately 500 bp in size. **B.** Chromatin immunoprecipitation (ChIP) using an α -acetylated H3 (acH) shows that the cyclin A and PCNA promoters are more rigorously deacetylated during quiescence and senescence than is the β -actin promoter. Furthermore, Rb immunoprecipitations show that Rb is associated with cyclin A and PCNA promoters in a senescence-dependent manner. Binding affinities of p130 and ING1 to cyclin A and PCNA promoters is different in Y, Q and S cells. Protein G sepharose (PGS) beads alone (labeled as No Ab) were used as a negative control to evaluate non-specific binding of chromatin to beads and a normal mouse IgG control was included to eliminate the possibility of non-specific protein binding to experimental antibodies of the same isotype. An additional input control of 1/20th of total lysates was included to ensure equal starting material in each ChIP.



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FIGURE 34

unlike Rb which shows increased binding for both the cyclin A and PCNA promoters in senescent cells, ING1 preferentially targets the PCNA promoter in this assay, consistent with its effects on PCNA expression. Therefore our working model of the effects of ING1 on chromatin structure and nucleosome homeostasis during cellular replicative senescence is outlined in figure 35.

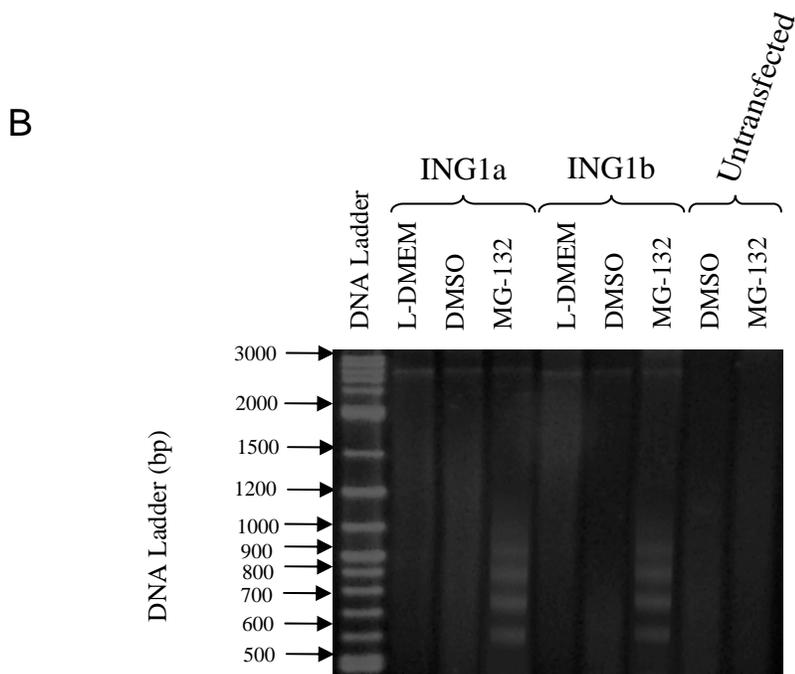
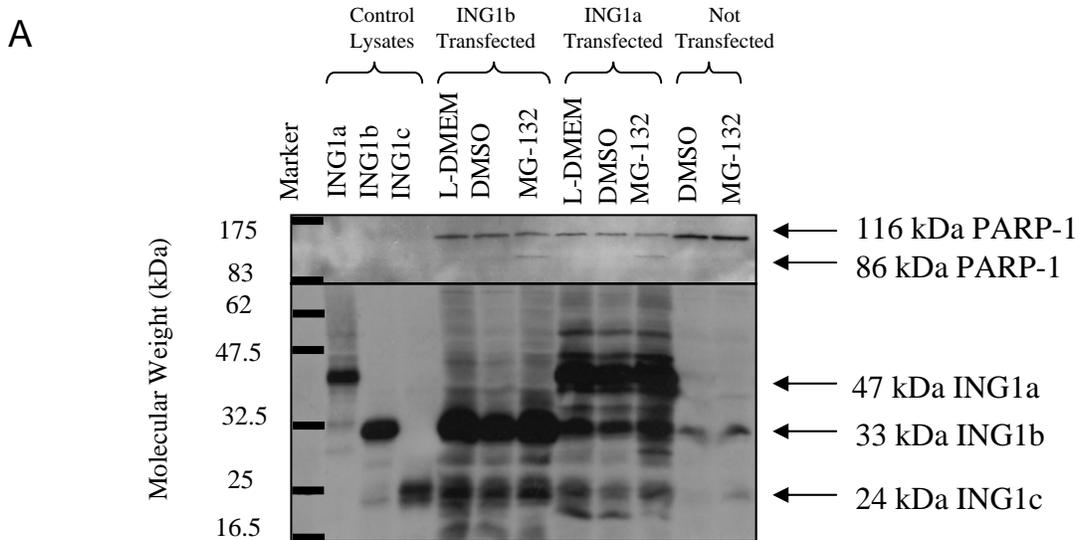
Figure 35: Model of chromatin modification in senescent cells. Members of the PI3-kinase related kinase (PIKK) family are thought to recognize a form of DNA damage signal from the shortened telomeres of senescent cells, with constitutively active p53 and Rb playing a central role in this process. Due to its sub-telomeric location, or to general alterations in splicing isoform machinery, the ratio of p47ING1a:p33ING1b changes by >20-fold, targeting HDAC activity to the chromatin. Rb is constitutively activated in senescent cells due to increased levels of p16 generated by alternative splicing from the INK4a locus. It is targeted to specific sites in chromatin, selectively inhibiting the expression of growth-associated genes and contributing to the formation and maintenance of senescence associated heterochromatic foci (SAHF). Activated p53 induces growth inhibitory genes such as p21WAF1 that contribute to blocking cell cycle progression, and p53 may also serve to help target HDAC complexes to chromatin. Together, the effects of these tumour suppressors, which are activated in senescent cells, and p47ING1a which is activated by increased expression in senescence, may serve to coordinately alter transcription and set chromatin structure into a state that strongly inhibits further cell cycle progression, DNA repair or entry into apoptosis, limiting senescent cells to a restricted basal metabolic activity.

3.2.9 *Proteasome inhibitor MG-132 enhances ING1-induced apoptosis*

Since the mechanism by which ING1 proteins are metabolized is unknown and inhibition of the proteasome induces a senescent-like phenotype in primary fibroblasts (Chondrogianni and Gonos, 2004), we wanted to test whether ING1a accumulation in senescence could be a compound effect of both increased expression and reduced degradation. Since PHD fingers have been reported to having E3 ubiquitin ligase activity (Lu et al., 2002) and there is evidence that ING1 isoforms homo- and heterodimerize (personal communication with Mr. Wei Gong in the Riabowol laboratory), we tested if inhibition of the proteasome resulted in ING1 accumulation (i.e. possibly by a negative feedback loop resulting in ING1 protein auto-ubiquitination). Since ubiquitination of conserved lysine residues can target proteins for degradation by the proteasome, inhibition of the proteasome should result in these proteins accumulating. Interestingly, analysis of protein levels of cells treated with 10 – 20 μ M of carbobenzoxy-L-leucyl-L-leucyl-L-leucinal ($C_{26}H_{41}N_3O_5$; MG-132) did not result in substantial protein accumulation but did enhance induction of apoptosis (Figure 36). MG-132 is a potent and reversible inhibitor of the chymotrypsin-like activity of the 26S proteasome (Lee and Goldberg, 1998). Apoptosis was measured using PARP-1 cleavage assay (Figure 36A) and ligation mediated PCR as shown in figure 36B (Staley et al., 1997). Since this data was inconclusive it was not pursued any further.

Figure 36: Proteasome inhibition facilitates ING1 induced apoptosis.

Inhibition of proteasomal degradation with 20 μ M MG-132 augments the apoptotic effect of ING1b and ING1a. **A.** The top panel shows apoptosis as measured by PARP-1 cleavage **B.** The bottom panel measures apoptosis using a ligation mediated DNA laddering approach. These data suggest that ING1 either induces apoptosis through the activation of pro-apoptotic genes that are normally degraded by the proteasome or that ING1 acts to auto-ubiquitinate itself by homo- or heterodimerization thereby targeting it to the proteasome.



All samples are shown after 29 cycles of PCR.
 24 bp adaptor: 5'-AGCACTCTCGAGCCTCTCACCGCA-3'
 12 bp adaptor: 5'-TGCGGTGAGAGG-3'

FIGURE 36

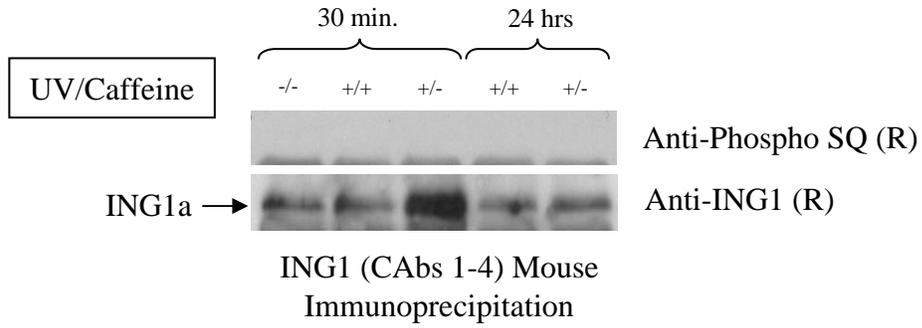
3.2.10 Caffeine treatment induces ING1a accumulation after UV-induced DNA damage

Several DNA damage and stress response proteins are phosphorylated and stabilized by stress activated kinases such as ATM, ATR and DNA-PK (Yang et al., 2003). Interestingly, this stress response pathway has also been reported to be activated by telomere erosion as a consequence of serial cultivation (d'Adda di Fagagna et al., 2003). Since this stress/senescence induced phosphorylation event often results in protein stabilization, as is the case with p53, resulting in increased activity, we wanted to test if ING1a was phosphorylated on one of its SQ-motifs by a PIKK in a stress inducible manner. Caffeine is a known non-specific PIKK inhibitor at 20 mM and ING1 has been shown to be responsive to UV-induced genotoxic stress. Therefore, an experiment was designed to evaluate ING1 protein levels in response to UV (60 J/m²). As shown in figure 37A, ING1 immunoprecipitates are not recognized by an α -phospho SQ rabbit polyclonal antibody (a generous gift for Dr. Susan Lees-Miller). This suggests that ING1 is not phosphorylated on any of its SQ motifs in response to UV irradiation. However it was noted that there was an early ING1a accumulation in the UV-treated cells that did not have caffeine to inhibit PIKK activity. This suggests that UV induces the accumulation of ING1 in a PIKK dependent manner since the UV/caffeine treated cells do not show this accumulation. Since it is known that p53 can be phosphorylated on serine 15 (SQE) and serine 37 (SQ), we analyzed p53 immunoprecipitates with the α -phospho SQ rabbit polyclonal antibody (Figure

37B). Here we see clear accumulation of p53 by 30 minutes when cells are treated with both UV and caffeine and an increased signal when cells are treated with UV in the absence of caffeine.

Figure 37: Caffeine treatment induces ING1a accumulation after UV-induced DNA damage. A. Since it can be argued that senescence is a stress response state, UV treating young human primary fibroblasts was used to measure ING1 response to genotoxic damage. To test if ING1a was phosphorylated on one of its SQ-motifs by a PIKK in a stress inducible manner we treated cells with 20 mM caffeine which is known to inhibit the kinase activity of DNA-PK, ATM and ATR ($IC_{50} = 10$ mM for DNA-PK, $IC_{50} = 10$ mM for ATM, $IC_{50} = 3$ mM for ATR). ING1 immunoprecipitates were then probed with an α -phospho SQ rabbit polyclonal antibody (a generous gift for Dr. Susan Lees-Miller). Although these experiments suggested that ING1a was not phosphorylated on an SQ motif, it did indicate that ING1a accumulated in cells in a PIKK dependent manner within 30 min of UV irradiation (60 J/m^2). **B.** Since it is known that p53 can be phosphorylated on SER 15 (SQE) and SER 37 (SQ), we next analyzed p53 immunoprecipitates with the α -phospho SQ rabbit polyclonal antibody as a proof of principle. Here we see clear accumulation of p53 by 30 minutes when cells are treated with both UV (60 J/m^2) and caffeine (20 mM) and an enlarged signal when cells are treated with UV in the absence of caffeine. The top band in western blots (upper panel of figure 37B) represents phosphorylated p53, while the lower band represents unphosphorylated p53.

A



B

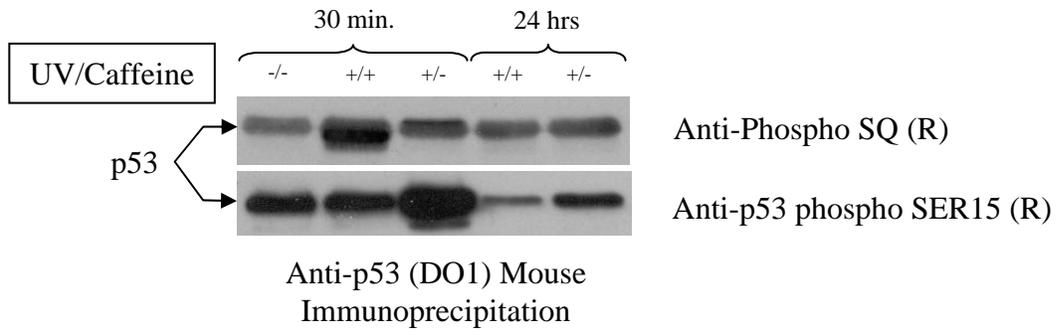


FIGURE 37

Figure 38: FACS analysis of senescent cells. FACS analysis of senescent cells reveals that the G1 arrest that typifies senescence is a result of vigorous biochemical analysis and not by DNA quantification using propidium iodide staining. Young quiescent and log phase cells were first analyzed as positive and negative controls respectively. Quiescent cells serum starved for 4 days show a greater than 95 % accumulation in G0/G1 while log phase cells show a relatively even distribution in various phases of the cell cycle. Interestingly, senescent cells show an accumulation in G0/G1 as expected, but show a considerable G2/M population (> 30 %). This is likely a result of the genomic instability and multi- and micro-nuclear accumulation that typifies senescence thereby resulting in total DNA concentration of greater than 2N in spite of being arrested in G0.

Young serum-starved quiescent Hs68 fibroblasts.

G0 – G1: 95.33%

G2 – M: 3.11%

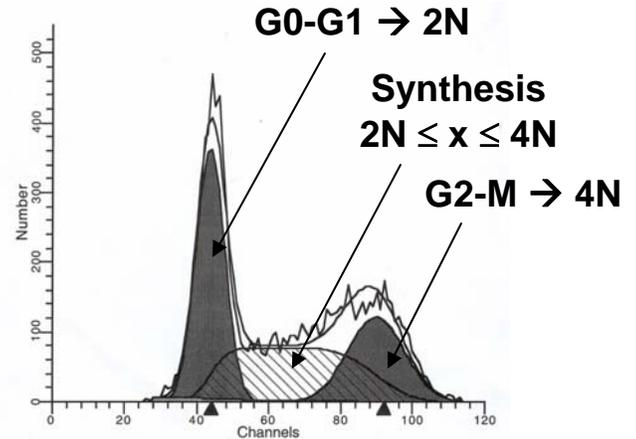
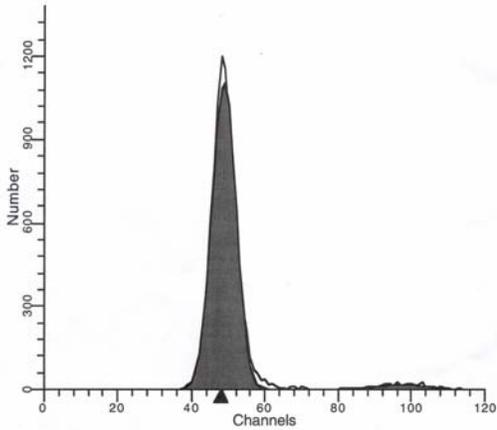
S Phase: 1.56%

Young log phase Hs68 fibroblasts.

G0 – G1: 37.54%

G2 – M: 25.15%

S Phase: 37.31%



Senescent (2 weeks post-plating) Hs68 fibroblasts.

G0 – G1: 54.33%

G2 – M: 30.82%

S Phase: 14.85%

Senescent (4 weeks post-plating) Hs68 fibroblasts.

G0 – G1: 51.65%

G2 – M: 33.36%

S Phase: 14.99%

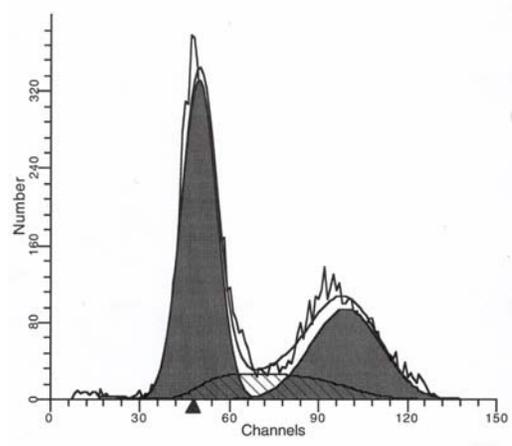
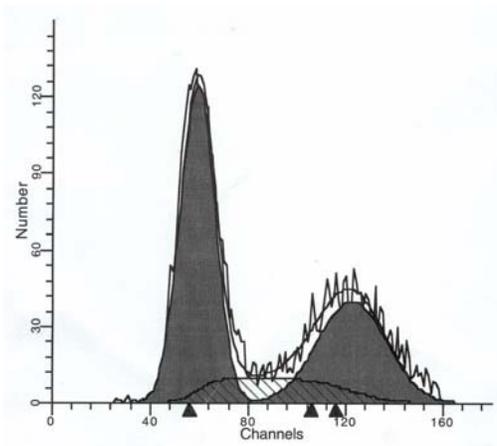


FIGURE 38

3.2.11 Summary of results in section 3.2

The ING family of PHD proteins inducibly bind phosphatidylinositol monophosphates in response to genotoxic stress and affect cell growth, apoptosis and DNA repair by interacting with proliferating cell nuclear antigen (PCNA), p53, and acetylation/deacetylation complexes. Here we show that as a result of senescence-associated ING1 alternative splicing or use of alternative promoters ING1a increases, altering the ING1a:ING1b ratio by >20-fold. ING1a, which inhibits histone acetylation, binds chromatin with high affinity in senescent cells and ING1 immunocomplexes from senescent cells contain several fold higher levels of HDAC1 and deacetylase activity than immunocomplexes from low passage replication-competent cells. Overexpression of ING1a induces formation of senescence associated heterochromatic-like foci, a G1-phase cell cycle arrest and a senescence-like phenotype while overexpression of the ING1b isoform induces apoptosis. Finally, chromatin immunoprecipitates using α -ING1 antibodies are enriched with PCNA promoter DNA in a cell growth dependent manner and ING1 isoform overexpression differentially affects PCNA expression levels. These data demonstrate a role for the ING1 proteins in regulating senescence-associated chromatin remodeling and support the idea that altered ratios of the functionally antagonistic ING1 isoforms contribute to establishing the senescent phenotype through altering PCNA gene expression by HDAC and

HAT complex-mediated alteration of chromatin structure and by suppression of apoptosis.

Chapter 4
DISCUSSION

Chapter 4: Discussion

4.1 Transcription regulating gene elements in cellular senescence

The initial objective of my project was aimed at defining a genetic regulatory element responsible for the increased expression of cyclin D1 in senescent cells. This work was based upon initial efforts by a previous student, Dr. Muthupalaniappan Meyyappan. Ultimately, it resulted in the identification of an element embedded in the 5'-untranslated region (5'-UTR) of the first exon of the cyclin D1 promoter that selectively represses cyclin D1 gene expression in young cells (Berardi et al., 2003). This is an intriguing observation because the increase in cyclin D1 expression relies on a decrease in the binding affinity and/or overall abundance/availability of a particular protein to an inhibitory element during cellular senescence. This is in contrast to the hypothesis that the increased availability and binding of proteins to transcription-activating elements, as opposed to transcription-inhibiting elements, in senescent states results in the activation of senescence-associated genes.

This study focused on cyclin D1 because it has been characterized as an essential component in the activation of the G₁ kinases cdk4 and cdk6, and subsequently targets the Rb protein for phosphorylation and inactivation (Fu et al., 2004). Rb can suppress S-phase entry and cause a transient G₁ arrest following DNA damage and is also critical in establishing terminal cell cycle withdrawal in cells exposed to differentiation or senescence-inducing signals (Narita et al., 2003). Therefore, cyclin D1 has been implicated in oncogenesis *in vivo* because of

its role in Rb inactivation resulting in cell cycle progression. Not surprisingly, cells lacking all the D-cyclins (cyclin D1, D2, D3) display reduced susceptibility to oncogenic transformation (Kozar et al., 2004). However, the observation of reduced oncogenic susceptibility in cells lacking D-cyclins (*i.e.* indicating that the D-cyclins act in an pro-oncogenic capacity) does not accurately reflect the entire role of the D-type cyclins, since mouse fibroblasts deficient for all the D-type cyclins proliferate relatively normally but show increased requirement for mitogenic stimulation in cell cycle re-entry (Kozar et al., 2004). More specifically, cyclin D1-deficient mice have been shown to display developmental abnormalities, hypoplastic retinas, and pregnancy-insensitive mammary glands (Geng et al., 1999). This suggests that cyclin D1 plays a role in proliferation, but has important independent functions of the canonical Rb-mediated cell cycle traverse that is typically described (Arnold et al., 1989; Motokura et al., 1991; Rosenberg et al., 1991). It also brings to light the kinase-independent mechanisms cyclin D1 has on interfering with proliferation arrest and senescence because cells lacking these proteins continue to proliferate (Geng et al., 1999; Kozar et al., 2004).

It is becoming increasingly apparent that cyclin D1 regulates a multitude of additional transcription factors independent of the cdk4 and cdk6 driven liberation of E2F-family transcription factors from Rb. These Rb-independent functions of cyclin D1 include control of the activity of nuclear receptors such as the androgen receptor (AR) and estrogen receptor α (ER α) (Ewen and Lamb,

2004). Therefore as a gene with multiple roles in the context of cell cycle progression and senescence, we established that it would be an appropriate model to understand the regulation of senescence-associated differential gene expression.

4.1.1 Identification of a novel regulatory element in the cyclin D1 gene

The age-related increase in cyclin D1 transcripts observed in senescent cells could be the result of specific transcriptional and/or post-transcriptional regulation. To address this possibility, our laboratory initially showed that cyclin D1 transcripts are similarly stable in both young and senescent cells. This was done by inhibiting transcription with actinomycin D (derived from *Streptomyces parvullus*, actinomycin D binds DNA and blocks the action of RNA polymerase II), followed by harvesting RNA over a 16-hour time course and performing a PCR analysis to assess percentage of cyclin D1 RNA transcript remaining. This provided the initial evidence that age-related accumulation of the cyclin D1 transcripts occurs at the level of transcription, since the degradation rate as measured by PCR was equal between young and senescent cells (Atadja et al., 1995b). This observation was extended and we identified an element in the cyclin D1 promoter that strongly contributes to selectively repressing cyclin D1 gene expression in proliferatively active cells. In other words, protein(s) more robustly bind this regulatory element in replicatively competent (young) cells and consequently inhibit cyclin D1 expression. Since binding of this element is lost in

replicatively exhausted (senescent) cells, cyclin D1 expression increases. As will be discussed in section 4.3, this repressive mode of growth-dependent transcriptional regulation has been demonstrated for other cell cycle regulatory gene products that act to reinforce the senescent state (Jacobs et al., 1999; Itahana et al., 2003; Park et al., 2004). While deletion of most of the cyclin D1 promoter sequence affected reporter gene expression to a similar extent in young versus senescent cells, deletion of a 64-bp sequence from +75 to +138 of the 5'-UTR selectively increased expression in young cells. This was initially observed using sequential truncation mutants of the cyclin D1 promoter driving a chloramphenicol acetyltransferase (CAT) reporter. As shown in figure 10, the fold difference in CAT activity between senescent and young cells is consistently reduced with progressive truncations of the cyclin D1 promoter until the -23 fragment (Figure 10B). This suggested to us that there was an element within the -23 to +138 region of the cyclin D1 promoter largely responsible for its differential expression seen during senescence.

To further address this observation a series of nested deletions of the -23 to +138 fragment were generated and additional CAT reporter assays performed (Figure 11A). Figure 11B summarizes the average fold difference in CAT activity from figure 11A. This helped to further define the precise region within the cyclin D1 promoter that acted to differentially inhibit reporter expression in young cells. Notice that the fold difference in CAT activity between senescent and young cells (Figure 11B) remains elevated in the -23 to +138 and +26 to +138 truncation

mutants but decreases sharply when the 64-bp region between +75 to +138 (labelled -23 to +74) is deleted within the reporter construct. This suggested that the element responsible for the differential activity of the cyclin D1 promoter was at least partially within the 64-bp region between +75 to +138 of the cyclin D1 5'-UTR.

Interestingly, nested deletion of the cyclin D1 5'-UTR resulting in increased cyclin D1 promoter activity in young cells was concomitantly made in a separate study by another group focussing on engineering a cyclin D1 promoter-binding ligand in intact cells (Laurance et al., 2001). They made a series of cyclin D1 5'-promoter deletions and cloned them into pGL3-Basic luciferase reporters. Promoter activities for the 5'-deletion constructs were then compared to that of the full-length (-1745) cyclin D1 promoter following transfection into asynchronous MCF7 human breast carcinoma cells. Indeed, when a region between +10 to +150 of the 5'-UTR was deleted (partially overlapping the +75 to +138 region that we identified), promoter activity increased dramatically. This corroborated our results showing an element between +75 and +138 was able to differentially bind a regulatory protein and was at least partially responsible for cyclin D1 promoter inhibition in young cells.

4.1.2 Differential protein binding to a region of the cyclin D1 promoter

We then tested the ability of this 64-bp sequence to bind proteins differentially in young and senescent nuclear extracts (Figure 12). To address this

we synthesized oligonucleotides corresponding to the 64-bp region of the cyclin D1 5'-UTR and performed electrophoretic mobility shift assays (EMSAs) using different combinations of radiolabelled 64-mer oligonucleotides, unlabelled wild type 64-mer oligonucleotides (W) or unlabelled unrelated oligonucleotides (U). This competition assay clearly shows that in the presence of labelled and unlabelled wild type oligonucleotide there is decreased binding of protein to probe (Figure 12A, lanes 4 and 5). This assay also shows that in the presence of unrelated oligonucleotide, protein binding to the probe remains strong (Figure 12A, lanes 6 and 7), providing the first evidence that protein(s) are able to bind this cyclin D1 5'UTR 64-bp region with high specificity. Figure 12B is an EMSA demonstrating that proteins extracted from young and senescent fibroblasts can bind the cyclin adenosine monophosphate (cAMP) response element (CRE) with similar affinities. This was done only to address the possibility that proteins in general from senescent nuclear extracts maintain their ability to bind DNA, since our initial observation of the cyclin D1 promoter showed loss of protein binding during senescence. These experiments demonstrated that the ability of proteins to bind oligonucleotides in general is not lost in senescent cells (Figure 12B).

To further ensure that this was a senescence-specific event and not simply a consequence of cell growth arrest, we repeated the EMSA using various oligonucleotide probes (defined in figure 13A) with young (Y), quiescent (Q) and senescent (O) nuclear extracts (Figure 12C). These assays also demonstrate that young (Y) and quiescent (Q) cell nuclear extracts bind to radiolabelled DIE probe

differently than senescent (O) cell nuclear extracts (Figure 12C). This is an important proof-of-principle since proteins are able to bind the 64-bp region of the cyclin D1 promoter more in young cells, regardless of their growth status (*i.e.* logarithmically growing young cells or quiescence arrested young cells both show approximately equal protein-DNA complex formation in the EMSA).

By using overlapping oligonucleotides corresponding to fragments of this 64-bp region (Figure 13A), the protein binding region was further defined to a 15-bp sequence (designated 5'-UTR-4) that we named the cyclin D1 inhibitory element (DIE) (Figure 13B, lanes 14 to 17). This was confirmed using competition EMSA assays with 100-fold excess of unlabelled probe (Figure 13C). As shown in figure 13C, the non-radiolabelled 5'-UTR-4 probe (lanes 3 and 4) is best able to compete with the radiolabelled 5'-UTR-4, suggesting that this is the specific DNA binding sequence. Mutation of particular bases within the DIE (Figure 14A), reduced or abolished complex formation as shown by EMSA in figure 14B and selectively upregulated cyclin D1 promoter activity in young cells as measured by CAT reporter assays (Figure 15). Additional EMSA assays using the strongest binding oligonucleotide probes identified in figure 13 (5'-UTR-4, 5'-UTR-5 and 64-bp) were then incubated with young and senescent cell nuclear extracts, irreversibly UV crosslinked and run on an SDS-PAGE gel. After electrophoresis, gels were autoradiographed to visualize approximate migration of protein-DNA complexes. This provided an estimate of proteins ranging from 20 to 45 kDa specifically binding the DIE (Figure 16).

Based on these assays alone, it is impossible to conclude whether the increased level of protein binding to this element in logarithmically growing young or serum-starved quiescent cells (relative to senescent cells) is a consequence of higher affinity of protein for the radiolabelled probe in different growth states or overall abundance of DIE-binding protein in different growth states. Additionally, one must consider the possibility that a combination of higher binding affinity and overall protein levels may be responsible for the differential binding of protein to this element. Further studies must be performed to delineate these matters.

It is tempting to speculate that the differential binding of proteins to this element is a consequence of differing levels of the binding-protein in various growth states and not a changed binding affinity as is the case for some transcription factors (Wheaton et al., 1996). The reasoning behind this speculation is that this element is found in the promoters and 5'-UTRs of genes in a repetitive fashion and it represses transcription in young cells; therefore it may act as an insulator sequence in conjunction with a polycomb repressive complex to induce and maintain transcriptional repression of genes (Gerasimova and Corces, 1998; Jacobs et al., 1999; Chen and Corces, 2001). Briefly, insulators are thought to regulate gene expression by establishing higher-order domains of chromatin organization, although the specific mechanisms by which these sequences affect enhancer-promoter interactions are not well understood (Jacobs and van Lohuizen, 2002). Interestingly, the polycomb group proteins Bmi1 and CBX7,

which bind to insulator sequences and repress transcription, have been shown to play an important role in regulating cellular life span by inhibiting the expression of p16^{INK4a} (Itahana et al., 2003; Gil et al., 2004). Since cyclin D1 and p16^{INK4a} work in the same biochemical pathway ultimately impinging on gene transcription, it would be of no surprise that they share some gene regulatory machinery. Addressing the possibility of the DIE element acting as an insulator sequence in conjunction with a polycomb repressive complex can be easily tested by overexpressing components of the polycomb repressive complex such as Bmi1 or CBX7 and analysing the expression of cyclin D1 and other genes containing the DIE element. Furthermore, additional EMSA analyses can be performed with the inclusion of antibodies directed against proteins such as Bmi1 or CBX7 and run on a non-denaturing polyacrylamide gel to determine if complexes ‘super-shift’ as a consequence of protein-probe-antibody complex formation. However, identifying the exact transcriptional repressor complex that regulates the expression of cyclin D1 remains to be seen since studies have shown concomitant transcriptional activation of cyclin D1 and Bmi1, suggesting that Bmi1 does not repress cyclin D1 gene expression (De Vos et al., 2002).

4.1.3 The effect of the DIE regulatory element on luciferase reporter expression

To further define the requirement of this transcriptional regulatory element in differential cyclin D1 expression, luciferase reporter constructs were made with the wild type or mutant DIE (5'-UTR-4 or 5'-UTR-4d) cloned between the SV40

promoter and the luciferase gene. These constructs were then transfected into young and senescent fibroblasts and luciferase expression/activity was measured (Figure 17A). This effectively demonstrated the ability of this element to differentially regulate transcription in young versus senescent primary fibroblasts (HS-68) as the reporter construct containing the DIE element showed approximately 50% differential reporter expression when transfected into young versus senescent fibroblasts (Figure 17B). As expected, luciferase reporters with the mutant DIE (5'-UTR-4d) showed little, if any, difference in reporter expression/activity between young and senescent fibroblasts. This corroborated previous data using the CAT reporter system showing that mutation within the +117 to +131 region of the cyclin D1 5'-UTR had no effect on reporter expression when transfected into senescent cells (this element does not bind any protein in senescent cells) but resulted in a greater than 2-fold expression change when transfected into young cells (the abrogated binding of protein due to mutant sequence results in no gene repression) (Figure 15). These data suggest that the inhibitory element is only effective in proliferatively competent young cells and not in senescent cells since reporter activity in senescent cells remained constant between the wild type and mutant reporter constructs (Figure 17B). As such, other regions of the cyclin D1 promoter are also likely to contribute to age-related expression, since mutation of the DIE between +117 and +131 of the cyclin D1 promoter did not result in equal reporter expression between young and senescent

cells, as would be the case if this element was solely responsible for differential cyclin D1 expression.

Interestingly, differential DNA-binding activity in young and senescent cells has been reported for other regions of the cyclin D1 promoter, including increase in the binding activity of specific nuclear protein factors to the enhancer element, Sp1, and a decrease in binding to a silencer element in senescent cells (Fukami-Kobayashi and Mitsui, 1998). However, this study did not address whether the changed binding activity of proteins to particular elements within the cyclin D1 promoter had an effect on cyclin D1 expression; therefore no conclusions can be made in regards to the biochemical significance of this altered protein binding. Whether these factors act independently of, or in concert with, the DIE regulatory machinery remains to be seen.

Additional analysis of the inhibitory element identified (DIE) revealed two additional DIE-like sequences with approximately 66.6% homology (matching 10 of 15 bp) within the cyclin D1 5'-UTR (Figure 18A). This included a sequence with limited homology within the 5'-UTR-2 oligonucleotide, with which weak complex formation was observed in mobility shift assays (Figure 13B). Based on our results, it appears that specific interactions of the repressor are stabilized by multiple copies of the DIE such that mutation of one can only partly reverse cyclin D1 gene repression in young cells. This would be consistent with the stronger binding seen using the 64-bp cyclin D1 promoter probe versus the shorter 22-bp oligonucleotides (Figure 12). As discussed in the previous section,

functional compartmentalization of eukaryotic genomes by chromatin insulators or boundary elements is thought to be necessary for the proper regulation of gene expression. Multiple copies of the DIE in functionally related genes is consistent with observations of proteins binding to insulator sequences resulting in repressed gene transcription by limiting access to promoter enhancer regions. This type of gene regulation seems plausible when considering the repetitive nature of the DIE in gene promoters coupled with the inhibitory effect it has on gene transcription (Gerasimova and Corces, 1998).

4.1.4 Additional cell cycle regulatory genes contain DIE-like sequences within their promoters

Sequence similarity comparisons using software made available through the Canadian Bioinformatics Resources (CBR) website revealed that sequences with homology to the DIE were found clustered in the promoters of a subset of genes whose expression is upregulated in senescent cells at a frequency higher than seen in control genes whose expression is unaffected or reduced during replicative senescence (Figure 18). Although regulation of gene expression by the 5'-UTR has not been widely reported, such mechanisms might act to repress transcription more commonly than previously thought (Chen and Corces, 2001). Some recent studies have shown that the 5'-UTR as well as the 3'-UTR act in various capacities to coordinately regulate gene expression of genes that are functionally linked (FitzGerald et al., 2004; Xie et al., 2005). Additional examples of this regulatory strategy include p53 suppression of the expression of B-cell

lymphoma-2 (*bcl-2*) at least partly through a p53 response element located in the 5'-UTR of the *bcl-2* gene (Miyashita et al., 1994). Similarly, a suppressor element has been identified in the 5'-UTR of the androgen receptor gene (Grossmann et al., 1994). Although the exact mechanisms operating to repress transcription are unknown, the results presented here raise the possibility that other genes that are upregulated during cellular senescence could be regulated by a mechanism similar to, if not identical to, the DIE identified here. Indeed, comparison of the DIE to a selected number of genes upregulated during cellular aging revealed the presence of DIE-like sequences in their 5'-UTRs and the absence of these elements in control genes known to be unaffected (glyceraldehyde-3-phosphate dehydrogenase) or down-regulated (phenylalanine tRNA synthetase) during cellular senescence (Figure 18). This reinforces the exciting possibility that the expression of a group of age related genes are coordinately regulated by one principal mechanism, and that it may be possible to influence the expression of many functionally linked genes by targeting a single repressor element or protein. As discussed previously, a similar regulatory mechanism has been shown to occur for stable repression of homeobox-cluster genes during mouse development (Jacobs et al., 1999) and E2F6-responsive genes by association with polycomb transcriptional repressor complexes (Trimarchi et al., 2001).

Comparative genomics both within and across species provides a powerful approach for the systematic discovery of functional elements in the human genome (FitzGerald et al., 2004; Xie et al., 2005). Although the largest and most

conserved elements are readily identified, the vast majority of non-coding functional elements are presumed to remain unknown. The data presented here indicates that a specific transcriptional regulatory mechanism contributes to the increased expression of cyclin D1 during cellular senescence. Interestingly, in another recent study aimed at identifying novel regulatory motifs by aligning promoters and 5'-UTRs, it was found that a greater proportion (58% versus 44%) of novel, short regulatory motifs (like the DIE) were found downstream of the transcription start site (Xie et al., 2005). Therefore, it is becoming clear that a comprehensive inventory of human regulatory motifs will serve as a foundation for understanding the cellular circuitry of gene regulation and its role in health, disease and aging.

Furthermore, searches of transcription factor databases using the DIE, revealed no homologies to other known human transcription factor binding sites. This suggested that either the proteins binding the DIE are uncharacterized, or that the mechanism by which cyclin D1 is repressed is not mediated by a transcription factor. Attempts to purify the putative repressor protein(s) by DNA-affinity chromatography were made, however these efforts were unsuccessful due to excessive non-specific protein binding which obscured any identification of specific proteins as is discussed below (section 4.1.5).

4.1.5 Purification of DIE-binding protein

As shown in figure 19, a systematic approach was taken to isolate the DIE binding protein for analysis by mass spectrometry. Unfortunately, the strategies

used were ineffective as a consequence of several factors outlined here (Figure 20). The insight gained from the previous failure to identify the DIE-binding protein will be useful to future progress of this project. First, it is important to note that transcription factors make up approximately 0.01% of total nuclear proteins, therefore enriching for these very low abundance proteins is inherently challenging (although this element may not act in a transcription factor mediated regulatory fashion as discussed previously). Initial attempts at isolating DIE binding proteins were done using placental *whole-cell* extracts. These attempts were unsuccessful due to a high-level of non-specific ligand binding resulting in an inability to isolate a specific ligand binding protein. It was then suggested to attempt a similar approach using placental *nuclear* extracts and running several pre-columns to enrich for DNA binding proteins.

Initially, placental nuclear extracts were isolated and proteins were differentially precipitated using ammonium sulfate (25% to 50% cut). Since the solubility of proteins depends on, among other things, the salt concentration in the solution, this is the precipitation method of choice because of its simplicity, the maintained stability of most proteins to this protocol and cost effectiveness. At low concentrations, the presence of salt stabilizes the various charged groups on a protein molecule, thus attracting protein into the solution and enhancing the solubility of proteins. However, as the salt concentration is increased, a point of maximum protein solubility is reached, past which protein solubility decreases. Further increase in the salt concentration results in less water being available to

solubilize protein due to monopolization of water by charged species (*i.e.* from salt). In effect, proteins start to precipitate when there are not sufficient water molecules to interact with protein molecules. This phenomenon of protein precipitation in the presence of excess salt is known as *salting-out*.

After salting-out, the placental homogenate was dialyzed at 4⁰C overnight in porous membrane tubing with a molecular weight cut-off of 3500-Da. This is done to remove excess ammonium sulfate and allow further processing of the nuclear extract. Placental nuclear extract homogenate was then run over a heparin-agarose ion exchange column to enrich for DNA-binding proteins. Heparin is a polysaccharide that is highly charged and strongly acidic due to the presence of covalently linked sulfate (-OSO₃H) and carboxylic acid (-COOH) groups. Consequently, proteins that typically bind the acidic DNA molecule *in vivo* will bind to the immobilized acidic heparin molecules in the pre-column. To elute proteins bound to the heparin-agarose column, a high-salt elution (1.5 M NaCl) strategy was used after which an additional dialysis step was performed to remove excess salt. Finally, an excess of poly (deoxyinosinic-deoxycytidylic) acid (poly-d(I)d(C)) was added to the dialyzed homogenate in order to bind non-specific DNA-end binding proteins, such as the DNA-PK regulatory subunit Ku, and the final column was run. After centrifugation of lysates to remove any additional precipitated or complexed proteins, half the lysate was passed over a wild type DIE column and the other half was passed over a mutant DIE column.

After samples were eluted and analyzed for differential binding, no clear DIE-specific binding protein was identified.

Generally, the approach outlined here is successful when isolating DNA binding proteins. Therefore it was reasoned that the placental homogenate might not be the choice protein source for our purposes since it contains a mosaic of cell types and connective tissues. In order to assess this issue further, a series of EMSA analyses were performed using placental protein nuclear extracts in a similar fashion to that which was performed with fibroblast extracts to define the DIE (Figure 20). This proved to be very informative since there was a persistent non-specific protein binding to the mutant 5'-UTR 64-bp oligonucleotide probe. To this end, it was concluded that this experiment would best be performed using nuclear extracts from large volumes of tissue culture cells or purchased nuclear lysates available from Abcam (WI-38 nuclear lysate - *ab14911*) or a service provided by the National Cell Culture Center (NCCC). Noteworthy is the fact that similar gene regulatory mechanisms as the one proposed for the DIE are known to act in a tissue-specific manner (Xie et al., 2005); therefore isolation of the DIE-binding protein may involve the *a priori* analysis of multiple different tissue-types in order to identify the appropriate source-tissue of abundant DIE-binding protein. When the DIE-binding protein is identified, it will reveal important insights regarding the regulation and identification of additional genes containing DIE elements and their role in cellular senescence and aging.

4.2 Epigenetic changes in regulating senescence

Epigenetics (*epi* = upon) refers to factors, other than the primary DNA sequence, that affect the development or function of an organism (Russo et al., 1996). Interestingly, many of these epigenetic signals can be modified by environmental perturbations and this has raised notions regarding neo-Lamarckian evolution (Waterland and Jirtle, 2003; Gorelick, 2004). These epigenetic changes play an important role in gene transcription by altering the accessibility of transcription factors to target promoter elements (Narita et al., 2003). This marks an exciting transition in understanding gene regulation since many biochemists over the last several decades have focused on DNA promoter regulatory elements in the absence of considering the chromatin context. However, the paradigm expansion now becoming more accepted is that genes should be considered more than just DNA; they also include the methylation of cytosine nucleotides and proteins, and other epigenetic signals including lysine acetylation, which are integral in the regulation of genes in their normal cellular contexts (Figure 6). Since the developmentally programmed epigenetic state is most likely subject to modification and/or degradation under the influence of the normal life course, I believe that understanding DNA transcription and repair in the context of the ‘histone code’ is germane to understanding the fundamentals of the aging process. By elucidating the function of chromatin modifying proteins as they pertain to chromatin remodeling and gene regulation during cellular senescence, we will be able to shed light on how normal primary cells naturally limit their proliferative

capacity, thereby understanding what limits cell growth and possibly contributes to the molecular etiology of age-associated diseases.

The ING1 gene was initially identified as a gene associated with suppression of neoplastic transformation and extension of fibroblast proliferative life span when inhibited (Garkavtsev et al., 1996; Garkavtsev and Riabowol, 1997). This was before the identification of multiple ING1 isoforms (Boland et al., 2000; Gunduz et al., 2000) and before the discovery that these isoforms associated with histone deacetylase (HDAC) and histone acetyltransferase (HAT) protein complexes (Loewith et al., 2000; Vieyra et al., 2002a). Therefore, I wanted to first evaluate the expression levels of various ING1 isoforms during cellular senescence because it was previously reported that ING1 RNA and protein levels were 8- to 10-fold higher in senescent cells relative to young, proliferation-competent human fibroblasts (Garkavtsev and Riabowol, 1997). I also wanted to further functionally characterize the ING1 proteins in the context of the senescent state since these proteins had been shown to affect apoptosis in a cell age-dependent manner and alter chromatin structure (Vieyra et al., 2002a; Vieyra et al., 2002b).

This work, done by our laboratory in collaboration with the laboratory of Dr. Dallan Young, identified a new role of the ING1 proteins in *integrating* stress responses with cell cycle arrest and cellular replicative senescence (Figure 21). This was based on the growing evidence that the molecular epigenetic signals of chromatin modulation (including histone lysine residue acetylation), are

influenced throughout the normal life-course and the finding that the two major ING1 isoforms (ING1a and ING1b) are differentially expressed and antagonistically affect histone modification and cell growth (Berardi et al., 2005).

4.2.1 The role of ING1 in stress responses and cell growth/replication

It is now becoming clear that the ING1 candidate tumour suppressor acts as a molecular ‘integrator’ of growth- and stress-mediated signaling pathways on chromatin homeostasis (Berardi et al., 2004). This growing role of ING1 in cell growth and replication and in response to stress can be mediated by ING1 directly binding to DNA (Kataoka et al., 2003) or by being recruited to DNA by interacting with PCNA (Scott et al., 2001). Furthermore, transient overexpression of ING1b induces the hyperacetylation of histones resulting in inappropriate transactivation of cell-cycle regulatory and apoptosis promoting genes such as p21^{Waf1} and Bax (Nagashima et al., 2001; Kataoka et al., 2003). This is consistent with the ability of HDAC inhibitors (promoting histone hyperacetylation) to induce programmed cell death in cancer cells (Glick et al., 1999; de Ruijter et al., 2003) through inhibition of at least eleven known HDACs (Gao et al., 2002).

The ING1b-induced apoptotic effect has been reported to occur in a p53-dependent manner in certain cell types (Garkavtsev et al., 1998; Shinoura et al., 1999; Nagashima et al., 2001; Leung et al., 2002; Nourani et al., 2003) indicating that ING1 is functionally linked to the p53 tumour suppressor. However, overexpression of ING1b induces apoptosis and gene expression in the absence of

wild-type p53 (Helbing et al., 1997; Takahashi et al., 2002; Kataoka et al., 2003; Tsang et al., 2003), indicating that the ING proteins can also function independently of p53. Briefly, the p53 protein has been shown to alter gene expression by:

- 1) directly binding DNA consensus binding sequences (el-Deiry et al., 1992);
- 2) interacting with the basal transcription machinery (Crighton et al., 2003);
- 3) targeting chromatin modifying complexes to gene promoters (Ard et al., 2002)
- 4) acting to globally alter chromatin structure (Rubbi and Milner, 2003).

Interestingly, Rb has also been shown to act like p53 by targeting various protein complexes to E2F-responsive gene promoters (Narita et al., 2003). Therefore, it appears that tumour suppressors such as p53, Rb and ING1 play multi-functional roles in regulating gene expression, including but not limited to, targeted chromatin modification. This is consistent with the fact that p53, Rb and ING1 proteins bind to HAT and HDAC complexes (Skowyra et al., 2001; Zilfou et al., 2001; Ard et al., 2002; Vieyra et al., 2002a; Shiseki et al., 2003) and that many genes regulated by protein complexes containing these tumour suppressors do not contain recognizable consensus binding sites in their promoters (Takahashi et al., 2000; Kataoka et al., 2003; Mirza et al., 2003).

Here we show that although both ING1a and ING1b contain an identical PHD domain that binds phosphoinositide monophosphates in a stress-induced manner (Gozani et al., 2003), expression of ING1a inhibits the ability of ING1b to

induce an apoptosis pathway and appears to antagonize it (Figure 31C). The effects of ING1 on apoptosis are also consistent with the differential effects seen on gene expression (Zeremski et al., 1999; Kataoka et al., 2003; Nourani et al., 2003). Since the ING1 gene locus encodes gene products with functionally antagonistic biological roles and this ratio changes dramatically as cells approach senescence, it is likely that ING1, in concert with other key cell growth regulators, has a profound effect upon chromatin structure and the replicative senescence process. This is also supported by the early observation that inhibiting ING1 gene expression by exogenous expression of complementary antisense RNA against the +942 to +1124 nucleotide region of the ING1 transcript allows normal primary fibroblasts to undergo several additional population doublings when approaching senescence (Garkavtsev and Riabowol, 1997). Interestingly, this antisense approach to inhibiting ING1 gene expression was targeted to the C-terminal region of ING1, which corresponds to the common exon of the ING1 gene. Consequently, the antisense approach taken resulted in the non-specific inhibition of all ING1 protein isoforms, which were not fully characterized at the time of the study (Garkavtsev and Riabowol, 1997).

A recent report in which fragments of ING1b were fused to a DNA binding domain has also implicated the ING1 gene locus in growth arrest and induction of senescence (Goeman et al., 2005). Here we show that while the ING1b isoform can induce an acute G1 phase growth arrest, this arrest is quickly followed by apoptosis (Figure 29 and 30) and ING1a induces a permanent growth

arrest resembling senescence by modulating chromatin structure and inducing the formation of heterochromatic foci (Figure 28M - 28P). Our data also show that the effect ING1 has on growth arrest, apoptosis and senescence is regulated by differential isoform expression, however the factors responsible for this still have not been identified. These factors can include the use of alternative promoters (Gunduz et al., 2000) or the alternative splicing of ING1 pre-mRNA molecules (Haber, 1997; Skowyra et al., 2001).

4.2.2 The ING1 protein alters chromatin structure and is activated in senescent cells

In order to test how ING1 expression was changed in senescent cells, we first needed to serially cultivate cells to their replicative capacity. Senescent fibroblasts were then identified/confirmed according to the classical markers of senescence including upregulation of cyclin dependent kinase inhibitors p21 and p16, senescence associated β -galactosidase staining and accumulation of globular actin within the nucleus (Figure 22 and 23) (Dulic et al., 1993; Dimri et al., 1995; Hara et al., 1996; Kwak et al., 2004). In this study we show for the first time that the relative expression levels of the ING1 isoforms change as cells enter replicative senescence (Figure 23), with the ING1a protein being expressed at over seven-fold higher levels and ING1b protein *decreasing* by ~ 3-fold in senescent fibroblasts (Figure 24). This differential expression results in a significant alteration in the ING1a:ING1b ratio as cells become blocked in the cell cycle (Figure 24 and 25). Furthermore, in senescent lung (WI-38) and foreskin

(HS-68) fibroblasts, increased levels of ING1a associate with HDAC1 protein (Figure 26) and HDAC activity (Figure 27). This data coupled with previous data showing that ING1a can bind to chromatin and decrease histone H3 and H4 acetylation levels (Vieyra et al., 2002a) suggests that the ING1a isoform plays an important role in modifying histones. Overexpression of ING1 isoforms in primary cells was also shown to have a differential effect on cellular/nuclear morphology. Notably, ING1b induces apoptosis and the formation of pycnotic nuclei while ING1a overexpression resulted in cells adopting a senescent-like morphology and accumulation of senescence-associated heterochromatic foci (SAHF) (Figure 28). Since the formation of SAHF is associated with targeted HDAC activity in senescent cells, there is a seemingly new role of ING1a during cellular replicative senescence.

Since the focus of part of this doctoral thesis was ING1 function in senescence associated cell cycle arrest, the pathways leading to a stress-induced permanent cell cycle arrest (SIPS) were not investigated. This would be an interesting avenue of exploration since ING1 possibly acts to alter chromatin structure in a stress-inducible manner thereby providing a new link between stress-responses, apoptosis and senescence. Therefore ING1, along with other chromatin modulating proteins, can act in concert with transcription factors to functionally integrate genotoxic stress to transcription, cell cycle regulation, DNA repair and apoptosis (Berardi et al., 2004).

4.2.3 *ING1 isoforms differentially affect cell growth*

Since ING1a can induce a senescence-like state when ectopically overexpressed (Figure 28I – 28L) and inhibits ING1b-induced apoptosis (Figure 31), the alteration in the ING1 isoform ratio may contribute to the previously noted resistance of senescent cells to apoptosis (Wang, 1995). The mechanism by which cells develop resistance to apoptosis might therefore be related to their state of chromatin condensation since ING1a binds to and activates HDAC complexes (Figure 27) in addition to associating with chromatin as shown in this study (Figure 32). Combined with the previously described activation of p53 during cellular senescence (Atadja et al., 1995a; Vaziri et al., 1997), the alteration of the ING1a:ING1b ratio in senescent cells is speculated to have a significant impact upon chromatin structure. This is also congruent with the recently reported role of the Rb protein in enforcing a heterochromatic state at E2F-responsive gene loci in senescent cells (Narita et al., 2003). Thus, altered gene expression in senescent cells may be the result of a telomere-initiated signal, perhaps from a subset of chromosomes (Baird et al., 2003; d'Adda di Fagagna et al., 2003), activating multiple tumour suppressor pathways in concert (p53, Rb and ING1), to maintain a distinct chromatin state through multiple interactions with chromatin modifying complexes.

From a functional perspective, ING1b is able to induce apoptosis as measured by Annexin V-FITC assays (Figure 29) while ING1a is capable of inducing cell cycle arrest in the absence of induction of apoptosis (Figure 29 and

30). Interestingly, it was also shown that ING1a is able to rescue primary fibroblasts from ING1b induced apoptosis when measured by caspase-mediated PARP1 cleavage (Figure 31). During apoptosis PARP1 undergoes auto-ribosylation and cleavage by the caspase family of proteins resulting in abrogated activity of this polymerase. The specific rate of change in PARP1 activity is dependent upon a variety of factors including cell type, method of induction of DNA damage or apoptosis, and culture conditions. Specific proteolytic cleavage of the 116 kDa full-length PARP1 has been demonstrated to be a reliable marker for apoptosis in a wide variety of cell types generating an 85 kDa and a 31 kDa fragment. The finding that ING1a rescues fibroblasts from ING1b-induced apoptosis as measured by PARP1 cleavage supports previous findings that ING1 isoforms function in an antagonistic manner possibly by heterodimerization or by activating protein complexes with opposite functions.

4.2.4 ING1 affects histone post-translational modifications and alters chromatin structure

Chromatin immunoprecipitations (ChIP) were performed by Dr. Karl Riabowol to test whether ING1 isoforms had a differing affinity for chromatin in young versus senescent fibroblasts (Figure 32). As expected, due to the sheer difference in abundance of the various ING1 isoforms in different growth states, ING1a strongly associates with chromatin during cellular senescence compared to ING1b. However, this is a minimal estimate of ING1 differential chromatin binding affinity since the incorporation efficiency of $\{^3\text{H}\}$ -thymidine is lower in

senescent cells that do not readily synthesize DNA (Goldstein, 1990; Cristofalo and Pignolo, 1993). This ChIP assay also shows a general decline in histone acetylation in senescent cells since the α -acetyl H4 antibody immunoprecipitates far more radioactivity (i.e. ^3H -thymidine labeled DNA/chromatin) in young cells compared to senescent cells. In order to address mechanism, we then screened genes that may be transcriptionally activated or inhibited by ING1 isoforms. Our observations showed that ING1a inhibits PCNA expression resulting in lower steady-state levels of PCNA protein and induces a senescent-like phenotype (Figure 33). In contrast, ING1b is capable of inducing PCNA expression resulting in higher levels of PCNA protein during early time points, but reducing PCNA levels at later time points as a consequence of ING1b apoptosis induction (Figure 33). Therefore, the ING1-PCNA regulatory loop plays a pivotal role in dictating the function of ING1 as an inhibitor of growth. This is consistent with the role of ING1b during DNA synthesis in terms of forming a protein complex with PCNA and DNA polymerases (Garkavtsev and Riabowol, 1997). This is also consistent with the role ING1b plays in promoting histone hyperacetylation (Vieyra et al., 2002a). This data was extended to show that ING1 associates with the PCNA promoter in a cell growth dependent manner (Figure 34B). This provides some evidence for a regulatory feedback loop in terms of PCNA expression, since only ING1b, and not ING1a, binds to PCNA by a canonical PCNA interacting protein motif. Therefore, by binding the PCNA protein and differentially regulating the PCNA promoter, ING1 can both ‘assess’

cellular/nuclear PCNA levels and either inhibit or activate its expression accordingly.

4.2.5 Motifs in the unique region of *ING1a*

In an effort to functionally characterize the various *ING1* isoforms, a series of bioinformatical analyses were performed using tools available through the 'Canadian Bioinformatics Resources' website to identify unique motifs within the various isoforms. As previously published (Scott et al., 2001), in the unique N-terminal region of the *ING1b* isoform there is a PCNA interacting protein (PIP) motif and a potential 14-3-3 protein binding motif (R-x-x-pS-x-P). On the other hand, the unique N-terminal region of the *ING1a* isoform contains an Akt/protein kinase B consensus motif (R-x-R-x-x-S). Since the phosphatidylinositol 3-kinase (PI3K)-Akt pathway is used by many cell types for inhibition of apoptosis and cellular survival and the *ING1* gene plays an important role in both of these processes it seemed plausible that *ING1a* acts downstream of the Akt signaling pathway. However, overexpression studies using a constitutively active Akt expression construct (a generous gift from the laboratory of Dr. Jim Woodgett) proved inconclusive. Additionally, the *ING1a* N-terminal unique region contained three potential casein kinase II (CK-2) and protein kinase C (PKC) phosphorylation sites suggesting that *ING1a* activity is mediated in a phosphorylation-dependent manner. In my opinion, the study of *ING1* post-translational modifications warrants further attention since recent reports have further implicated the *ING* family of PHD proteins in phosphatidylinositol

monophosphate signaling pathways and the ING proteins have long been known to play a role in cell growth, proliferation and apoptosis pathways. Furthermore, preliminary studies done during the course of this dissertation have suggested that ING1 protein activity is altered in a phosphorylation dependent manner (Figure 37)

4.2.6 Senescence-associated alternative isoform expression

Alternative splicing increases protein diversity by allowing multiple, sometimes functionally distinct, proteins to be encoded by the same gene. It can be specific to tissues, stress conditions, and developmental and pathological states, and interestingly is only common in metazoans (Black, 2003). Studies of how stress-induced alternative splicing (Turpin et al., 1999; Hashimoto et al., 2002) and senescence-associated alternative splicing (Haber, 1997; Meshorer and Soreq, 2002; Zhu et al., 2002) are regulated suggest that some factors may be common to these processes. These factors include altered levels of heterogeneous nuclear ribonucleoproteins (hnRNPs) and 3'-processing factors, which mediate this alternative splicing in response to stress. A good demonstration of the importance of this biochemical regulation is the INK4a/Arf gene locus, which encodes the p16^{INK4a} cyclin-dependent kinase inhibitor that functions in cell cycle regulation and cellular senescence (discussed in section 1.3.4) (Robertson and Jones, 1998; Robertson and Jones, 1999; Sharpless and DePinho, 1999). The p16^{INK4a} splicing variant inhibits the cyclin/cdk mediated phosphorylation of Rb resulting in the sequestration of the E2F family of transcription factors and

subsequent silencing of E2F-responsive genes. Alternative splicing of the INK4a/Arf gene transcript has been correlated to a decrease in the ratio of hnRNP-A1 or hnRNP-A2 to the splicing factor SF2/ASF (Zhu et al., 2002), with changes in the hnRNP-A2:SF2 ratio inducing much greater changes in splice site-switching activity (Mayeda et al., 1994; Sakharkar et al., 2004). Whether common splicing machineries process the ING1 and INK4a/Arf transcripts remains to be seen; however, it is tempting to speculate that this type of regulatory mechanism promotes both the transcriptional silencing of growth stimulating gene promoters such as PCNA through the Rb and ING pathways as a result of the formation of heterochromatic foci.

The 5'-terminal exons of an mRNA can also be switched through the use of alternative promoters and to some degree alternative splicing (Black, 2003); although, this is primarily an issue of transcriptional control. Several human genes including ING1 and INK4a/Arf are reported to have isoform specific promoters, which can result in cell type-specific or growth phase-specific expression patterns (Nishio et al., 1994; Gunduz et al., 2000; Nguyen et al., 2001). The ING1 gene locus has been shown to produce four mRNA variants from three different putative promoter regions (Jager et al., 1999; Gunduz et al., 2000; Saito et al., 2000). Studies of how these four mRNA variants are processed are currently underway. Supporting the idea that ING1 isoform expression is being driven by different promoters is the fact that the unique exons of ING1 are 5'-upstream to the common exon (Figure 24A). Therefore, it is conceivable that a promoter

upstream of exon 1 drives the expression of the ING1b isoform and another promoter upstream of exon 1' drives the expression of the ING1a isoform in a senescence or stress dependent manner (Figure 24A). ING1 sequence analysis also reveals the existence of Kozak sequences upstream from exon 1 and exon 1'. Since this short recognition sequence greatly facilitates the initial binding of mRNA to the small subunit of the ribosome it suggests that alternative mRNA transcripts will have a similar capacity to be translated.

Additionally, the ING1 gene and flanking regions are guanine (G) and cytosine (C) rich (*i.e.* the promoters contains several CpG islands), and directed methylation of the different isoform promoter regions may cause the loss of one gene product and concomitant activation of another. The INK4a/Arf gene locus can again offer an example of this regulatory mechanism, since the p14^{Arf}, p15^{INK4b} and p16^{INK4a} gene promoters have been shown to be differentially inactivated by aberrant methylation in various cancer types (Riese et al., 1999; Furonaka et al., 2004). This is an interesting hypothesis since we are now becoming more aware of the non-random nature of cytosine methylation to form 5-methylcytosine in gene promoters and how these epigenetic gene regulatory pathways play an important role in senescence, oncogenic transformation, cellular differentiation, parental imprinting and X-chromosome inactivation (Costello et al., 2000; Black, 2003; Ushijima, 2005). Future work aimed at further annotating the various ING1 isoform promoters would help to clarify whether differential

expression of various ING1 gene products is a consequence of alternative splicing and/or promoter activity changes or both of these processes.

4.3 Genes preferentially expressed in senescent cells

The biochemical signature associated with growth arrest and senescence is slowly being deciphered. With the identification of genes that are differentially expressed and/or spliced during senescence, groups are now attempting to identify commonalities between these genes. Genetic approaches underway, paralleling the approach we've taken with the cyclin D1 promoter analysis, include the bioinformatical clustering/alignment of DNA sequences in human genetic regulatory regions such as upstream promoter elements and 3'-UTRs (FitzGerald et al., 2004; Xie et al., 2005). These studies are delineating the functional properties of genes with regulatory regions that contain consensus DNA sequences. In other words, using bioinformatical analyses of gene promoters and mRNA expression properties, genes involved in related biological processes are being shown to contain, and be regulated by common DNA consensus motifs. Most of this research has focused on functional processes such as housekeeping, or tissue-specific gene expression patterns, or on biological processes such as ribosomal, proteasomal or channel related genes (FitzGerald et al., 2004). However, it would be interesting to extend the observations made within this doctoral dissertation to see if there is a clustering of additional promoter elements in genes that are differentially regulated during cellular replicative senescence. Furthermore, understanding how these elements act in the context of chromatin structure and identifying potential chromatin domains in which these elements are

housed will surely shed light on senescence-associated gene regulation and the adoption of the senescent phenotype by cells.

Like the cyclin D1 inhibitory element identified here, these unique promoter elements can bind proteins that inhibit gene transcription in proliferating cells (Berardi et al., 2003). When cells become senescent, the down-regulation or post-translational modification of these DNA binding inhibitory proteins results in the transcriptional activation of subsets of genes. So rather than having a protein activate a subset of genes in a senescence-associated manner, a protein is losing its ability to repress a subset of genes in a cell growth-dependent manner. Interestingly, reports on the polycomb group proteins CBX7 and Bmi1 (originally identified in mouse as the B-lymphoma Moloney murine leukemia virus insertion region (van Lohuizen et al., 1991) and then in human as the murine leukemia viral (bmi-1) oncogene homolog (Alkema et al., 1993)) have indicated that these oncoproteins work in this repressive manner (Dimri et al., 2002; Itahana et al., 2003; Gil et al., 2004; Itahana et al., 2004; Park et al., 2004). The 37-kDa Bmi1 DNA-binding protein has been described as an ‘insulator’ affecting the enhancer-promoter interactions by affecting chromatin structure (Gerasimova and Corces, 1998; Chen and Corces, 2001). It has been shown that Bmi1 is abundant in proliferating cells and is capable of repressing the expression of the INK4a gene locus. But when cells are senescent, Bmi1 is no longer abundant and the INK4a locus is de-repressed, thereby allowing the p16^{INK4a} cyclin dependent kinase inhibitor to accumulate and induce cell cycle arrest (Dimri et al., 2002; Itahana et

al., 2003). Our findings show a parallel event occurring on the cyclin D1 promoter, in that a protein is binding the element we've identified in young cells resulting in gene transcriptional inhibition. This protein binding is lost in senescent cells resulting in cyclin D1 transcriptional up-regulation. This is an excellent example of how genetic elements must work in concert with epigenetic modifications in order to effectively target genes for activation or inhibition.

From an epigenetic standpoint, there is progress being made in trying to identify senescence-specific gene expression 'fingerprints' or chromatin domains to reveal cell-type-dependent physical clustering of up-regulated chromosomal loci (Zhang et al., 2003). This clustering, which has been shown to be in contrast with the random distribution of genes down-regulated during senescence or up-regulated during reversible proliferative arrest (i.e. quiescence), supports the view that replicative senescence is associated with alteration of chromatin structure acting in concert with transcription factors to induce a phenotypic change. This further supports our finding of ING1 being able to associate with and activate either HAT or HDAC complexes, much like p53 and Rb, and target these complexes to various chromosomal loci thereby changing the growth potential of cells (Zilfou et al., 2001; Ard et al., 2002; Vieyra et al., 2002a). Further investigation of global changes in gene transcription occurring during replicative senescence and ING1 overexpression in various cell types and contexts will further delineate what pathways are impinged upon by ING1-mediated chromosomal changes.

4.4 A molecular basis for cell aging and age-associated diseases

The molecular events that result in growth arrest have been shown to be a consequence of DNA damage response kinase activation as a result of telomere-initiated senescence pathways (d'Adda di Fagagna et al., 2003). This differential kinase activity leads to altered transcription factor activity resulting in the differential expression of genes. Interestingly, these kinases have also been shown to be activated as a consequence of chromatin modulation in the absence of telomere attrition (Bakkenist and Kastan, 2003). This is a higher level in the 'hierarchy' of differential gene expression associated with cellular senescence since chromatin modulation can change accessibility of transcription factors to promoters of cell cycle regulatory genes. Since senescence-associated differential gene isoform expression of ING1 results in the formation of senescence-associated heterochromatic foci (SAHF), ING1 along with other chromatin modifying protein complexes act to augment the stimulation or silencing of chromatin regions associated with cell growth and proliferation.

Noteworthy is the fact that there are examples of genes that normally promote cell growth and proliferation that will induce a senescence-like phenotype when transiently overexpressed in normal primary cells. Oncogenic Ras is one such protein that has been used extensively in studies to elucidate the molecular mechanisms of cell cycle arrest and senescence (Serrano et al., 1997; Benanti and Galloway, 2004). Interestingly, the observation of oncogenic Ras inducing senescence has been shown to be cell specific, further supporting the

hypothesis that there are multiple factors in different cell types within the same organism necessary for the maintained cell cycle arrest observed during cellular senescence (Skinner et al., 2004). The induction of senescence by constitutive Ras activity is likely the consequence of activated cell cycle checkpoints in response to abnormal cell growth signaling. This would be in line with Cuzin, Weinberg and others (Rassoulzadegan et al., 1982; Land et al., 1983; Ruley, 1983) findings that to transform normal rodent cells to a cancerous state, delivery of two oncogenes is required. This finding was extended by the groundbreaking discovery showing that human fibroblast cells need the ectopic expression of the telomerase subunit (hTERT) and inhibition of p53 and Rb pathways using SV40 large T-antigen in addition to constitutive Ras activity to become oncogenically *transformed* (Hahn et al., 1999). Interestingly, in a previous study using human keratinocyte or mammary epithelial cells, it was reported that Rb/p16^{INK4a} inactivation and telomerase activity alone were required to *immortalize* certain epithelial cells (Kiyono et al., 1998). This may be a reflection of the greater immortalization capacity of human epithelial cells and is congruent with the observation that approximately 90% of human cancers are derived from epithelia (Weinberg, 1998).

Worth addressing here is the distinction between transformation and immortalization. Immortalization refers to the act of creating cells that can divide indefinitely in culture (sometimes referred to as establishment), but when these cells are injected into immune-compromised mice they do not form tumours. In

contrast, transformation is essentially the immortalization of cells coupled with the loss of contact inhibition and anchorage-dependent growth. These cells will form tumours when injected into immune-compromised mice and will grow and divide in the absence of survival factors or mitogenic signals (Hahn, 2002).

Not surprisingly, there are a number of viral oncoproteins, such as the adenovirus E1B 55 kDa protein, human papillomavirus (HPV) E6, and simian virus 40 large T-antigen, bind to and repress the biological functions of p53 and Rb (Lane and Crawford, 1979; Linzer and Levine, 1979; Scheffner et al., 1990; Werness et al., 1990). This inhibitory activity occurs to ensure that cell cycle checkpoints are not activated in host cells as a result of viral infection. The E6 proteins of highly oncogenic HPV types 16 and 18 (HPV16 and HPV18) associate with p53 and target it for ubiquitination and subsequent degradation (Scheffner et al., 1990). Simian virus 40 large T-antigen binds to the sequence-specific DNA binding domain of p53 (Tan et al., 1986). This interaction interferes with sequence-specific DNA binding of p53 and therefore inhibits p53-mediated transcriptional transactivation (Bargonetti et al., 1991; Farmer et al., 1992). Inhibition of p53 transactivation function is thought to be the key step in cell transformation induced by adenovirus (Yew and Berk, 1992). The transforming function of adenovirus maps to the early region 1 (E1) of the 36 kb adenovirus genome (Geisberg et al., 1995). The E1 region encompasses two independent transcription units, E1A and E1B. E1A encodes two major polypeptides, 289R and 243R, whereas E1B transcript specifies two overlapping open reading frames

which encode E1B 19 kDa and 55 kDa proteins. The E1A proteins bind to Rb and inhibit its function in regulating cell cycle progression and also appear to affect p53 functions (Steegenga et al., 1996). The E1B 19 kDa protein functions as an inhibitor of apoptosis (Roulston et al., 1999). The E1B 55 kDa protein suppresses p53 transactivation activity and also p53-mediated apoptosis (Yew and Berk, 1992). Both E1B proteins are required to fully transform cells in cooperation with E1A (Barker and Berk, 1987). Adenovirus E1B 55 kDa oncoprotein has also been shown to inhibit p53 acetylation by PCAF thereby preventing its activation (Liu et al., 2000). This study may prove insightful in functionally linking ING1 with p53 activity since our laboratory and others have found that ING1 and PCAF form an active acetyltransferase complex and that ING1 induces apoptosis in both a p53-dependent and p53-independent manner (Feng et al., 2002; Vieyra et al., 2002a).

Combined with the wealth of knowledge gained from the recent published literature and the evidence presented in this doctoral dissertation, a paradigm is developing whereby a multi-factorial hierarchy of molecular events leads to a unique growth arrested state in senescent cells. Clearly it is essential to consider both genetic and epigenetic modifications in order to elucidate the differential gene expression patterns and altered DNA metabolism that accompanies cellular senescence and oncogenic transformation. These factors, working in concert, ultimately contribute to the senescence-mediated growth arrested state and can be influenced by environmental factors throughout the normal life course. Not surprisingly, some of these seminal findings using *in vitro* cellular senescence

models including the upregulation of the INK4a gene locus are now being shown to occur *in vivo* (Krishnamurthy et al., 2004). This further supports the idea that there is a cellular component to organismal aging and that cellular senescence is a valid model in the understanding of whole organism aging.

From a purely reductionist perspective, one of the major factors underlying the replicative arrest in senescent cells is the differential expression of growth regulatory genes by genetic and epigenetic mechanisms. This differential activity can serve to actively inhibit cell growth and proliferation by targeting key cell cycle regulators for inactivation/down-regulation or hyperactivation/up-regulation depending on their role in cell cycle progression. There are several examples of this, some of which include the loss of serum response factor (SRF) activity resulting in reduced c-fos expression (Atadja et al., 1994), down regulation of PCNA resulting in inability of cells to progress to the DNA synthesis phase of the cell cycle (Paunesku et al., 2001) and down regulation of Bmi1 leading to loss of telomerase expression/activity in adult progenitor cells and p16^{INK4a} de-repression (activation) resulting in inhibition of cyclin/cdk kinase activity (Dimri et al., 2002; Park et al., 2004). Additionally, the up-regulation of cyclin D1 (Atadja et al., 1995b) and the activation of tumour suppressor pathways (Atadja et al., 1995a; Garkavtsev and Riabowol, 1997) results in cell cycle checkpoint activation and halted cell cycle progression. This differential gene activity/expression may also serve to mediate a feedback loop that perpetuates the changes that lead to altered chromatin structure and expression of genes in

senescent cells by their interactions with target promoters and recruitment of histone acetyltransferase and deacetylase enzymes (Figure 35). In summary, a complex hierarchy of gene regulation is being established in which both epigenetic and genetic factors play equally important roles in determining the biochemical signatures of various growth states.

The study of cellular senescence pathways will therefore provide a clearer understanding of the molecular processes that contribute to cellular aging and aging *in vivo* and determine how these processes contribute to the molecular etiology of several age-associated diseases. It will also enable us to elucidate mechanisms by which normal cells limit cell growth and by extension molecular pathways that cancer cells abrogate in order to become immortalized. This would provide better detection and treatment strategies since the inhibition of the senescence program is common to most malignancies. Finally, the development and understanding of cellular senescence models using fibroblasts can now be extended to other cell types and tissues to elucidate the molecular circuitry of progenitor cell self-renewal, immunosenescence, terminal differentiation and the like. Since these are all processes that involve growth arrest and replicative potential loss, the understanding of them will surely shed light on the decline associated with organismal aging.

Chapter 5
SIGNIFICANCE

Chapter 5: Significance of the research and future directions

5.1 Significance

The broad study presented in this dissertation focused on both the genetic and epigenetic mechanisms by which genes are differentially expressed during cellular replicative senescence. This is based on the rationale that an intrinsic genetic program contributes to the process of *in vitro* cellular aging, but also has an epigenetic component that is strongly influenced by stochastic environmental effects. Addressing the genetic program first, we further characterize the promoters of a subset of growth regulatory genes that are differentially expressed in young versus senescent fibroblasts. With the initial focus on cyclin D1, we contradict the dogma that cyclins always function to promote cell cycle progression and growth and corroborate a growing body of evidence suggesting that cyclin D1 has a growth suppressive function (Lucibello et al., 1993; Fukami et al., 1995; Morisaki et al., 1999). In fact, we and others support the notion that the cyclins show high levels of functional redundancy and that they may have dual roles in growth promotion or growth arrest depending on their absolute abundance in various cell types (Lucibello et al., 1993; Sandhu et al., 2000). This is likely a consequence of stoichiometric changes in various cell cycle dependent protein levels during different growth states. Thus we speculate that high levels of cyclin D1 elicit a negative effect on cell growth in one context because it sequesters various binding partners such as cdk2 in inactive complexes during arrested states such as senescence. Since cyclin D1 as part of a multi-protein

complex plays important roles in cell proliferation and senescence, the cis-acting factors that regulate its transcription may be fundamental to these processes.

The identification of common gene regulatory elements in other genes that are differentially regulated during cellular replicative senescence raises the intriguing possibility that there may be coordinate regulation of subsets of genes affected by cellular aging. Precedence for the coordinate regulation of functionally linked genes are now coming to light; however these studies have not focused on the senescence function *per se* (FitzGerald et al., 2004; Xie et al., 2005). Instrumental in further elucidating this hypothesis would be the identification of the DIE-binding protein. As discussed, this protein may act in a similar fashion to the polycomb group of protein insulators which establish higher order domains of chromatin organization thereby altering transcription factor accessibility to target promoters (Gerasimova and Corces, 1998). This is a plausible hypothesis since the DIE element is normally found in gene promoters as tandem repeats, which may act to bind proteins and limit promoter accessibility. Testing this would be very informative in determining regulatory proteins capable of regulating cyclin D1 expression. This can be achieved by performing additional EMSAs using fibroblast nuclear protein extracts and antibodies designed against various polycomb group proteins. Proteins suspected of binding the DIE, based on sequence similarity to other genes that are regulated in this manner, can be included in EMSA analysis to determine if complexes will 'super-shift.' Other approaches can include overexpressing various insulator

proteins and determining the effect on other gene promoters, such as the genes know to be transcriptionally activated during cellular senescence.

The epigenetic changes associated with cellular replicative senescence and aging also provide another layer of complexity to the current paradigm when trying to understand how stochastic environmental effects play a role in organismal aging by affecting DNA packaging. This also acts to corroborate our belief that there is a strong cellular component to organismal aging since environmental stressors can induce phenotypic changes in the absence of DNA sequence changes (Waterland and Jirtle, 2003; Brelloch et al., 2004; Hochedlinger et al., 2004a; Hochedlinger et al., 2004b). This can be interpreted to be much like what is seen in higher organisms such as humans where differing lifestyles affect the incidence of age-associated diseases and aging itself. Since the 'rate' of aging or senescence can be largely influenced both at the cellular and organismal level by environmental factors in the absence of any genetic changes, this lends further weight to the hypothesis that cellular and organismal aging occur as a consequence of concerted genetic and epigenetic influences through the passage of time. Therefore, the latter portion of this dissertation focused primarily on how ING1 could modulate chromatin structure in a stress and cell growth dependent manner to contribute to the differential gene expression patterns seen during these states. This reinforces data from other groups showing that senescent cells accumulate senescence associated heterochromatic foci (SAHF) and silence the expression of key cell cycle promoting genes.

The understanding of how gene expression patterns seen in various cell types change as a consequence of replication potential and/or telomere erosion will surely help elucidate the molecular etiology of various age-associated disease states such as cancer and cardiovascular disease and possibly aging itself. Once we delineate how these changes take place and to what extent they affect cell microenvironments and tissue homeostasis, we can begin to identify novel therapeutic targets to treat these diseases in a preventative fashion. Since there is accumulating evidence suggesting that there is a large cellular component to organismal aging, we must now systematically define how gene expression changes occur, and how the outcome of biochemical changes on cells through the life course affect disease and lifespan.

5.2 Future Directions

Data generated during the course of this dissertation has helped to elucidate various levels of gene regulatory changes during cellular senescence. It has identified a novel gene regulatory element that acts in a senescence specific manner and has contributed to the functional characterization of the ING1 protein isoforms in the context of various cell stress responses and growth states. Even though initial attempts at isolating the DIE-binding protein were unsuccessful, further work on this project is warranted and will shed new light on genetic regulation of senescence associated genes. This could be done using large volumes of homogenous whole cell lysates as opposed to the mosaic of cell types and connective tissues isolated from placentas. Furthermore, efforts can be made to understand how these senescence associated gene regulatory changes affect cell function and tissue maintenance. One major shortcoming of the work outlined in this thesis is that it does not address how these gene regulatory changes affect cell function in the context of tissues. Linking this data to parallel reports of a senescence specific secretory phenotype would further elucidate how differential gene expression is regulated at the genetic and epigenetic levels and does not only affect cell cycle progression, but also cell function and overall tissue homeostasis. This could be addressed by performing functional assays using *in vitro* co-cultures or co-microinjection of cells in mouse models aimed at understanding how cellular senescence affects the interplay between two different cell types.

Addressing gene regulatory mechanisms by transcription factor activation and epigenetic modification can also be extended to adult pluripotent progenitor cells, since these cells are known to maintain many tissues during the lifetime of an animal. Consequently, stem cell senescence must be prevented to maintain an organism's vital tissues throughout life. Understanding stem cell self-renewal and further elucidating the role of telomerase activation and gene transcription would surely elucidate how these progenitor cells functionally decline with age and possibly shed light on potential therapeutic targets in order to maintain the self-renewal capacity of these cell types. Moreover, understanding how epigenetic modifications and transcription factor mediated gene expression change as these cells either differentiate down their various lineages or retain their pluripotent capacity (thereby repressing genes involved in the senescence pathway) is surely going to have tremendous influence on maintaining health in old age.

5.3 Publications produced during the the course of this dissertation

5.3.1 Manuscripts (Published/Submitted/In preparation)

Berardi, P., M. Meyyappan, and K.T. Riabowol. 2003. A novel transcriptional inhibitory element differentially regulates the cyclin D1 gene in senescent cells. *Journal of Biological Chemistry* **278**: 7510-19.

Berardi, P., M. Russell, A. El-Osta, and K. Riabowol. 2004. Functional links between transcription, DNA repair and apoptosis. *Cellular and Molecular Life Sciences* **61**: 2173-80.

Feki, A., C. Jefford, **P. Berardi**, J. Wu, L. Cartier, K. Krause and I. Irminger-Finger. 2005. BARD1 induces apoptosis by catalyzing phosphorylation of p53 by DNA-damage response kinase. *Oncogene*, Electronic Publication Mar. 2005.

Berardi, P., S. Pastryeva, P. Bonnefin, A. Colina, D. Young and K. Riabowol. 2005. The ING1 PHD protein alters chromatin structure and is activated in senescent fibroblasts. *Journal of Cell Biology*. Under Revision.

Russell, M., **P. Berardi** and K. Riabowol. 2005. Stress-ING out: Roles for ING1 in lipid signaling and the response to stress. *EMBO Reports*. In Preparation.

Berardi, P., P. Bose, P. Bonnefin and K. Riabowol. 2005. ING1 binds PCNA during DNA synthesis and repair integrating synthesis and repair pathways to chromatin modification. *Molecular Cancer Research*. In Preparation.

5.3.2 Successful Grant Applications

Berardi, P. and K. Riabowol. Evaluating the Effects of ING1 and Bard1 on Telomere Homeostasis. *The University of Calgary Office of the President International Fellowship*. Fellowship Grant (01/2004 – 05/2004) \$6000.00.

Riabowol, K. (Grant application co-authored by **P. Berardi**). A Novel Regulator of Cancer-Linked Genes: The Cyclin D1 Inhibitory Element. *The Cancer Research Society Inc.* Laboratory Operating Grant (09/2003 – 08/2005) \$118 000.00.

5.3.3 Published Abstracts

Gene Structural Changes that Regulate Aging

Berardi, P., Bonnefin P, Pastryeva S, Suzuki K, Colina A, Young D and Riabowol K. Canadian Association of Gerontology: The Canadian Journal on Aging (Oct. 2004) *Victoria, Canada*

The Effects of ING1-Induced Chromatin Modulation on Cell Growth and Senescence

Berardi P, Bonnefin P, Pastyryeva S, Suzuki K, Colina A, Young D and Riabowol K.
The 4th Geneva Aging Workshop: The International Journal of Biochemistry and Cell Biology (Oct. 2004) *Geneva, Switzerland*

Involvement of the ING1 PHD Protein in the Processes of Aging and Development

Berardi, P., Quarrie, J.K., Loken, S., Bain, G., Scott, M., Pastyryeva, S., Demetrick, D., Rancourt, D. and Riabowol, K.
CSHL Meeting: Molecular Genetics of Aging (Oct. 2004) *Cold Spring Harbour Lab, USA*

Ageing and Tumour Suppressor Protein Activity in Epithelial Cells

Berardi P, Feki A., Jefford C.E., Riabowol K., Irminger-Finger I.
Functional Genomics of Ageing: Mechanisms of Ageing and Development (April 2004) *Crete, Greece*

Chromatin Remodeling During Cell Cycle Arrest and Senescence

Berardi, P, Bonnefin P, Pastyryeva S, Colina A, Young D and Riabowol K.
ASCB Abstract: American Society for Cell Biology Annual Meeting (Dec. 2003) *San Francisco, USA*

Genetics of Aging: The Next Generation of Biomedical Discovery

Berardi, P and Riabowol K.
Canadian Association of Gerontology: The Canadian Journal on Aging (Nov. 2003) *Toronto, Canada*

A Novel Transcriptional Regulatory Element Involved in Differential Gene Expression During Cellular Ageing.

Berardi P, Meyyappan M and Riabowol K.
Functional Genomics of Ageing: Mechanisms of Ageing and Development (April 2002) *Seville, Spain*

Increased Association of p33^{ING1b} with PCNA in Senescent Human Diploid Fibroblasts.

Pastyryeva S, Berardi P, Hara Y and Riabowol K.
IABG Abstract: 9th International Congress of Biomedical Gerontology (June 2001) *Vancouver, Canada*

Senescence-specific Regulation of Growth Regulatory Genes by a Novel Transcriptional Inhibitory Element.

Berardi P, Meyyappan M and Riabowol K.
CSBMCB Abstract: From the Genome to Structure and Function (May 2001) *Alliston, Canada*

Senescence Induced Regulation of Cyclin D1: Identification of a Candidate Repressor Element.

Berardi P, Meyyappan M, Chebib I, Schade K and Riabowol K.

CSHL Meeting: Cancer Genetics & Tumor Suppressor Genes (Aug. 2000) *Cold Spring Harbour Lab, USA*

5.3.4 Local Presentations

Berardi P. The Effects of ING1 on Chromatin Structure. Oral presentation at the 2004 Alberta Cancer Board Annual Research Meeting. (Nov. 2004) *Banff, Canada*

Berardi P. Chromatin Remodeling during Cell Cycle Arrest and Senescence. Poster presentation at the 2003 Alberta Cancer Board Annual Research Meeting. (Nov. 2003) *Banff, Canada*

Berardi P. Mechanisms of Transcriptional Regulation during Senescence of Human Diploid Fibroblasts. Poster Presentation at the Cancer Biology Research Group Biennial (May 2002) *Kananaskis, Canada*

Berardi P. Differential Gene Expression during Human Diploid Fibroblast Senescence. Poster presentation at the 2000 Alberta Cancer Board Annual Research Meeting. (Nov. 2000) *Banff, Canada*

5.3.5 Invited Presentations

Berardi P. The Role of Cell Replicative Senescence in Human Biological Aging. Oral presentation at the 2004 Canadian Association on Gerontology Meeting. (Oct. 2004) *Victoria, Canada*

Berardi P. A Novel Transcriptional Regulatory Element Involved in Differential Gene Expression During Cellular Ageing. Poster presentation at the First Annual Functional Genomics of Ageing Meeting. (April 2002) *Seville, Spain*

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APPENDIX I

ING1 Nucleotide and Amino Acid Sequences

p33ING1b

GCGAGTTGCGGTAGTTGCTGTGTACCATGGTCTCGGAGGTTTCTGTCCC GCGGCCCGTTA
GGTCTGCTCGGGTTTTTCAGCGAAGCAGGCCGCTCCCCTGCGTTTCCCAGCGGGCGTGCT
GTGCCGCCCAACAGGCTCTGCCTCCAAGTGCCAAAACTCCTAGTAAAGTTTGCGCCTCG
CCCGCCGTCCACACCCAGCGGCCCTGACGCTGTCCCCCTCCGCGACCCCTCGCCTCTGGAA
AAAGTGACAGGCAAGGCCACGCCCCCGCGAGGGCCGGCCTGGAGCCCGCAGCCCCAGGG
CCTGGGACGGTGAGGGGCGTGAATGCGGCGGGGGCGGGGCCGTTGCCGGGGGAGGGGGC
CGGGGCGCATGCGCGCTGCGCAGCGGGGCTGAATGTTTTCCAAGTGTTTGAAACTGGTAT
TTGGGTTTTTCCACGTTGGACAAGTGC GGCTCGGCGGCCAGCGGAGCGCGCCCTTCCCCG
TGCCCGCTCCGCTCCTCTCTTCTACCCAGCCAGTGGGCGAGTGGGCAGCGGCGGCCGCG
GCGCTGGGCCCTCTCCCGCCGGTGTGTGCGCGCTCGTACGCGCGGCCCCCGGCGCCAGCC
CCGCCGCTGAGAGGGGGCTGCGCCGCGGCCGGGGCGTGCGCCGGGAGCCACCGCCA
CCGCGGCCCGCGCCCTCAGGCGCTGGGGTCCCCGCGGACCCGGAGGCGGCGGACGGGCTC
GGCAGATGTAGCCGCGGGCCGAAGCAGGAGCCGGCGGGGGGGCGCCGGGAGAGCGAGGG
CTTTGCATTTTGCAGTGCTATTTTTT GAGGGGGCGGGGGTGGAGGAAGCGAAAGCCG
CGCCGAGTCGCCGGGACCTCCGGGGTGAACCATGTTGAGTCTGCCAACGGGGAGCAGC
TCCACCTGGTGAACATATGTGGAGGACTACCTGGACTCCATCGAGTCCCTGCCTTTTGCACT
L--H--L--V--N--Y--V--E--D--Y--L--D--S--I--E--S--L--P--F--D--
TGCAGAGAAATGTCTCGCTGATGCGGGAGATCGACGCGAAATACCAAGAGATCCTGAAGG
L--Q--R--N--V--S--L--M--R--E--I--D--A--K--Y--Q--E--I--L--K--
AGCTAGACGAGTGCTACGAGCGCTTCAGTCGCGAGACAGACGGGGCGCAGAAGCGGGCGGA
E--L--D--E--C--Y--E--R--F--S--R--E--T--D--G--A--Q--K--R--R--
TGCTGCACGTGTGTCAGCGCGCTGATCCGCGAGCCAGGAGCTGGGCGACGAGAAGATCC
M--L--H--C--V--Q--R--A--L--I--R--S--Q--E--L--G--D--E--K--I--
AGATCGTGAGCCAGATGGTGGAGCTGGTGGAGAACCGCACGCGGCGAGGTGGACAGCCACG
Q--I--V--S--Q--M--V--E--L--V--E--N--R--T--R--Q--V--D--
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V--E--L--F--E--A--Q--Q--E--L--G--D--T--A--G--N--S--G--K--A--
GCGCGGACAGGCCCAAAGGCGAGGCGGCAGCGCAGGCTGACAAGCCCAACAGCAAGCGCT
G--A--D--R--P--K--G--E--A--A--A--Q--A--D--K--P--N--S--K--R--
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S--R--R--Q--R--N--N--E--N--R--E--N--A--S--S--N--H--D--H--D--
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D--G--A--S--G--T--P--K--E--K--K--A--K--T--S--K--K--K--K--R--
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K--W--Y--C--P--K--C--R--G--E--N--E--K--T--M--D--K--A--L--E--
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p47ING1a

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M--S--F--V--E--C--
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 -M--D--K--A--L--E--K--S--K--K--E--R--A--Y--N--R--*--.....
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APPENDIX II

Publications Produced During the Course of this Dissertation

A Novel Transcriptional Inhibitory Element Differentially Regulates the Cyclin D1 Gene in Senescent Cells*

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Senescent human diploid fibroblasts are unable to initiate DNA synthesis following mitogenic stimulation and adopt a unique gene expression profile distinct from young or quiescent cells. In this study, a novel transcriptional regulatory element was identified in the 5'-untranslated region of the cyclin D1 gene. We show that this element differentially suppresses cyclin D1 expression in young versus senescent fibroblasts. Electrophoretic mobility shift assays revealed abundant complexes forming with young cell nuclear extracts compared with senescent cell nuclear extracts. Binding was maintained in young quiescent cells, showing that loss of this activity was specific to senescent cells and not an effect of cell cycle arrest. Site-directed mutagenesis within this cyclin D1 inhibitory element (DIE) abolished binding activity and selectively increased cyclin D1 promoter activity in young but not in senescent cells. Sequences with homology to the DIE were found in the 5'-untranslated regions of other genes known to be up-regulated during cellular aging, suggesting that protein(s) that bind the DIE might be responsible for the coordinate increase in transcription of many genes during cellular aging. This study provides evidence that loss of transcriptional repressor activity contributes to the up-regulation of cyclin D1, and possibly additional age-regulated genes, during cellular senescence.

Normal human diploid fibroblasts (HDFs)¹ are widely used as a model system to study the process of replicative or cellular senescence (1, 2). These cells have a finite proliferative life span, at the end of which they are unable to enter the S phase in response to mitogenic stimuli, but they remain metabolically active for long periods of time (3). They have prominent and

active Golgi apparatus, a large, flat morphology, invaginated and lobed nuclei, large lysosomal bodies, and an increase in cytoplasmic microfibrils compared with young cells (reviewed in Ref. 4). They also show a senescence-associated β -galactosidase (β -gal) activity, staining blue under acidic conditions (pH 6.0), whereas young cells do not (5). Some of the biochemical hallmarks that accompany cellular senescence include up-regulation of cyclin D1 (6–8), p21^{Waf1/Cip1/Sdi1} (9), p16^{INK4a} (10, 11), and insulin-like growth factor binding protein-3 (12). Furthermore, senescent cells arrest with a DNA content characteristic of the G₀ and G₁ phases of the cell cycle; yet the expression and activity of many cell cycle regulatory proteins during G₀, G₁, and senescence are distinct, implying that senescent cells exist in a unique, nonproliferative state that we have termed G_S (13).

Cyclin D1 is a member of the D-type family of cyclins that associates with cyclin-dependent kinases 4 and 6 (14, 15). Cyclin D1-cyclin-dependent kinase 4 promotes the G₁ to S phase transition of the cell cycle by cooperating with cyclin E-cyclin-dependent kinase 2 to sequentially phosphorylate the retinoblastoma tumor suppressor protein (16). However, cyclin D1 knockouts are rescued by cyclin E expression, whereas the reverse does not hold, suggesting that cyclin D1 does not play a central role in regulating the cell cycle, which is further suggested by the phenotype of cyclin D1 knockout mice (17–19). Normal human diploid fibroblasts that have reached the end of their *in vitro* life span (senescent cells) express 3-fold higher levels of cyclin D1 protein than low passage cells. Individual cells in mass culture that fail to initiate DNA synthesis in response to serum addition have severalfold higher levels of this cyclin than proliferation competent cells (7). It has been shown that cyclin D1 overexpression may inhibit this entry into S phase through binding to proliferating cell nuclear antigen and cyclin-dependent kinase 2 (7, 20). Cyclin D1 dysregulation and gene amplification have been implicated in a variety of cancers (21), suggesting that deregulated expression of cyclin D1 contributes to abnormal cell proliferation.

Ectopic overexpression of cyclin D1 in normal HDFs, the mammary epithelial cell line MCF-7, Dami megakaryotic cells, and rat embryo fibroblasts inhibits DNA synthesis and cell growth (7, 20, 22–25). Cyclin D1 has also been shown to be up-regulated in numerous nonproliferative differentiated cell types (26–29) and during apoptosis (30), lending further mass to the idea that it may also provide growth suppressive functions. In senescent fibroblasts, the cyclin D1 mRNA and protein levels are constitutively up-regulated by ~3–5-fold compared with serum-stimulated young cells (6–8, 31).

The 5'-regulatory region of the cyclin D1 gene has been well characterized, and the regions responsible for serum-inducible transcription have been identified (32, 33). However, the regulation of cyclin D1 gene expression is not well understood under conditions of senescence-associated growth arrest where

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¹ The abbreviations used are: HDF, human diploid fibroblast; β -gal, β -galactosidase; DIE, cyclin D1 inhibitory element; UTR, untranslated region; MPD, mean population doubling; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; CRE, cyclic AMP response element.

induction is at least as great as seen in response to serum. The increased expression of cyclin D1 is specific for senescence-associated growth inhibition in HDFs and is not apparent in contact-inhibited or serum-deprived cells arrested at a similar place in the cell cycle (24). This is in contrast to the closely related cyclin D2 where expression is increased in senescent, quiescent and contact-inhibited cells (24). Thus, unique transcriptional mechanism(s) may strongly contribute to cyclin D1 expression during senescence. In this report, we examine potential 5'-regulatory regions and mechanisms that may be responsible for the up-regulation of cyclin D1 seen in aging HDFs. Analysis of the cyclin D1 promoter via transient transfections of nested promoter deletions into young and old fibroblasts has identified a 15-bp cyclin D1 inhibitory element (DIE). This element is located in the 5'-UTR of cyclin D1 and has been shown to bind a low molecular mass protein more avidly in young cells compared with senescent cells, suggesting that loss of binding to this element in senescent cells contributes to the increased expression of cyclin D1 seen during cellular senescence. Consistent with a role in cell aging, it was noted that several other genes that are up-regulated during cell aging were found to have DIE-like elements in their 5'-UTRs, strongly supporting the idea that this element may bind a common regulatory protein that contributes to senescence-specific gene expression.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—HDFs (Hs68: ATCC CRL 1635 from newborn foreskin, reaches 85 mean population doublings (MPDs) in culture; WI-38: CCL 75, from embryonic lung, reaches 56 MPDs under our culture conditions) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin. In all experiments, low passage (young) cells were between 28–32 MPDs and high passage (senescent) cells were used at 82–86 MPDs (Hs68) or 53–56 MPDs (WI-38). Senescence was defined as a state in which less than 10% (typically 3–5%) of cells enter DNA synthesis in response to mitogens in a 36-h period. Senescent cells used in this study did not reach confluence within 2 weeks after subculturing at a 1:2 split ratio, demonstrated a characteristic large, flattened senescent cell morphology, and were positive for senescence-associated β -gal activity.

Senescence-associated β -Gal Staining—*In vitro* senescence-associated β -gal activity was detected as described (5, 34) with minor modifications. The cells were washed with phosphate-buffered saline (PBS; pH 7.2), fixed with 0.5% glutaraldehyde in PBS for 3–5 min at room temperature, washed several times with PBS (pH 6.0), and stained for 16–24 h at 37 °C with 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -galactosidase (X-gal) in PBS (pH 6.0) containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mM $MgCl_2$.

Nested Deletion Constructs—The 1.3-kb human cyclin D1 promoter in pUC118 vector was a generous gift from Yue Xiong (University of North Carolina, Chapel Hill). The vector was digested with *SacI/PvuII* to release the 1291-bp promoter containing 138 bp of the 5'-UTR. The ends of the fragment were filled in with Klenow polymerase (Invitrogen), and the fragment was subcloned into the *SmaI* site of pBluescript II KS. Nested deletions were performed with a double-stranded nested deletion kit (Pharmacia Corp.). For 5' nested deletions, the cyclin D1 promoter in pBluescript was digested with *HindIII*, filled in with thionucleotides, and redigested with *EcoRV* at the 5' end. The deletions were made by exonuclease III digestion for various time points followed by S1 nuclease treatment. The deleted constructs were analyzed by agarose gel electrophoresis, ligated, and transformed to obtain clones, which were screened and sequenced for the desired deletions. Deleted clones in pBluescript were digested with *SalI/BamHI* and subcloned into the *SalI/BamHI* site of the pBLCAT3 vector. The clones were designated as -1154 (full length), -858, -580, -459, -425, -198, -85, -23, and +26 relative to the transcriptional start site.

For 3' deletions of the 5'-UTR, the -23 clone in pBluescript was cut with *SpeI*, filled in with thionucleotides, and redigested with *BamHI* at the 3' end. After exonuclease III/S1 nuclease treatment, the deleted clones were ligated, screened, and sequenced. Clones with 3' deletions were digested with *SalI/XbaI* and subcloned into the *SalI/XbaI* sites of the pBLCAT3 vector. Two clones with the 3' end deleted were designated as -23 to +74 and -23 to +24.

Transient Transfections

Electroporation— 5×10^5 log phase young and senescent Hs68 fibroblasts were seeded in 150-mm plates and harvested when ~80% confluent. The cells were trypsinized, suspended in 400 μ l of serum-free Dulbecco's modified Eagle's medium, and transferred to 4-mm gap cuvettes (BTX Inc., San Diego, CA). Cyclin D1 promoter-chloramphenicol acetyltransferase (CAT) reporter plasmid (30 μ g) was added to 5 μ g of a cytomegalovirus-driven β -galactosidase expression construct and 15 μ g of salmon sperm DNA to make a total DNA content of 50 μ g/cuvette. Electroporations were done using a Bio-Rad gene pulser at 250 V and 960 microfarad, and following transfection, the samples were transferred to 10-cm plates. The cells were harvested 48 h post-transfection and assayed for CAT activity.

LipofectAMINE 2000— 5×10^3 log phase (unsynchronized) young and senescent (arrested) Hs68 fibroblasts were seeded in 6-well plates and treated when ~95% confluent. The cells were washed in PBS (pH 7.2), and the medium was replaced with OPTI-MEM (Invitrogen). LipofectAMINE 2000/OPTI-MEM (Invitrogen) solution (250 μ l) was incubated for 20 min with DNA/OPTI-MEM solution (250 μ l). Immediately after incubation 500 μ l was added to each well of the 6-well plate. Approximately 6 h later, the medium was replaced with L-Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. The cells were harvested 48 h later using passive lysis buffer (Invitrogen) and assayed for luciferase expression using an EG & G Berthold luminometer.

β -Galactosidase and CAT Assays—Electroporated cells were washed twice with ice-cold PBS, harvested, and centrifuged. The cell pellet was washed with PBS, resuspended in 100 μ l of lysis buffer (0.25 M Tris-Cl, pH 7.8), and subjected to three freeze-thaw cycles in liquid nitrogen with rigorous vortexing between each step. The cell lysates were centrifuged at 10,000 rpm for 5 min, and the supernatants containing the cell extract were used for β -galactosidase assays as previously described (5). Briefly, 10 μ l of cell extract was added to a tube containing 90 μ l of lysis buffer, 350 μ l of LacZ buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , pH 7, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β -mercaptoethanol) and 50 μ l of chlorophenolred- β -D-galactopyranoside (30 μ g/ μ l) (Roche Molecular Biochemicals). The mixture was incubated for 1 h at 37 °C, and the absorbance at 574 nm was determined. Activity resulting from the co-transfected cytomegalovirus- β -galactosidase plasmid was determined by subtraction of endogenous basal activity values obtained from mock-transfected young and old fibroblasts. Extracts corresponding to equal units of β -galactosidase activity were used for CAT assays as described previously (35). The proportion of acetylated product was determined by liquid scintillation counting of excised thin layer chromatography spots.

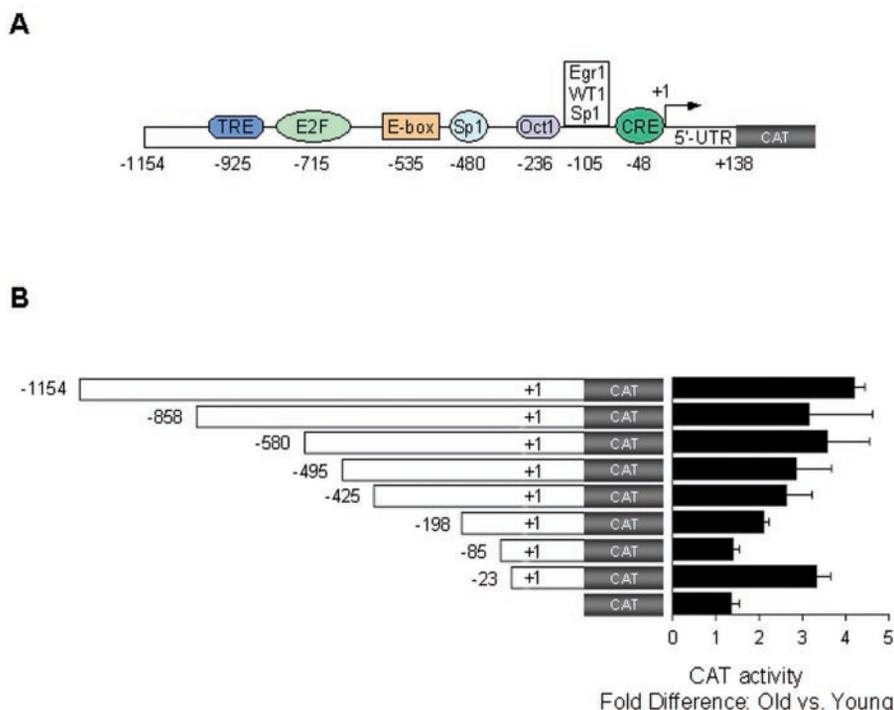
Electrophoretic Mobility Shift Assays—Nuclear extracts from young and old Hs68 HDFs were prepared as described previously (36, 37). A double-stranded 64-bp oligonucleotide spanning nucleotides +75 to +138 and the overlapping oligonucleotides (both wild type and mutant) were synthesized with 5' overhangs, end-labeled with Klenow enzyme (0.5 unit; Invitrogen) and 50 μ Ci of [α - ^{32}P]dCTP (3000 Ci/mmol; Amersham Biosciences), and gel-purified. 20,000 cpm of labeled probe (0.1–0.5 ng) was incubated with 5 μ g of nuclear extracts from young or old cells in binding reactions that contained 20 mM Hepes (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM dithiothreitol, 0.1 mM EDTA, and 2 μ g of poly(dI-dC) as described previously (36). The reactions were incubated for 30 min at room temperature, electrophoresed through 5% nondenaturing polyacrylamide gels at 150 V at room temperature, dried, and visualized by autoradiography.

Oligonucleotides corresponding to the cyclic AMP response element (CRE) and binding conditions for this element have been described previously (36). Briefly, 20,000 cpm (0.1–0.5 ng) of gel-purified [α - ^{32}P]dCTP-labeled probe was incubated with 5 μ g of nuclear protein extract in a buffer that contained 20 mM Tris (pH 7.6), 4% Ficoll, 50 mM KCl, 1 mM EDTA, 0.2 mM dithiothreitol, 10 μ M $ZnCl_2$, 1 μ g of partially denatured salmon sperm DNA, and 30 μ g of bovine serum albumin in a final volume of 20 μ l for 30 min at room temperature. Unlabeled wild type and mutant competitor DNA was added at a 100-fold excess and incubated for 10 min at room temperature before the addition of hot probe.

Site-directed Mutagenesis—A total of 8 bp between +117 and +131 of the 5'-UTR were mutated using a QuikChange site-directed mutagenesis kit (Stratagene). A mutagenic primer (46-mer) was synthesized and annealed to the double-stranded pBLCAT3 construct containing the full-length cyclin D1 promoter. *Pfu* DNA polymerase was used to synthesize the mutagenic promoters followed by digestion of the parental plasmid by *DpnI* as per the manufacturer's instructions. The mutated

FIG. 1. 5'-nested deletion analysis of the cyclin D1 promoter.

A, schematic representation of several potential transcription factor binding sites in the cyclin D1 promoter. *TRE*, TPA response element; *Egr1*, immediate-early growth response gene; *WT1*, Wilms' tumor suppressor gene product; *E2F*, transcription factor E2F-binding site; *Sp1*, promoter-specific transcription factor; *Oct1*, octamer-like transcription factor binding site; *E-box*, insulin-responsive region. **B**, a series of 5'-nested deletions of the cyclin D1 promoter in pBluescript vector was generated by exonuclease III digestion, and the resulting deletions were subcloned into the pBLCAT3 vector for transient transfections into young and old fibroblasts (left panel). The bar graphs in the right panel represent the fold difference in CAT activity in old versus young cells after normalizing for transfection efficiencies by measuring activity from co-transfected β -galactosidase constructs. The results represent CAT activity from three independent transfections with the standard error indicated.



plasmid was transformed into XL1-Blue competent cells, and the resulting plasmid was isolated and sequenced to confirm the mutations.

UV Cross-linking of DNA-Protein Complexes—To estimate the relative molecular mass of the DNA-binding proteins, binding reactions using various oligonucleotides as described for the band shift assays were performed. Twice the amount of labeled probe (40,000 cpm) and nuclear extract (10 μ g) were used. After 30 min of incubation at room temperature, the binding reaction was subjected to UV light, and unprotected DNA was digested. The samples were irradiated by a 305-nm inverted UV transilluminator at 7 mW/cm² for 5 min. The cross-linked reactions were electrophoresed through 15% SDS-polyacrylamide gels, dried, and visualized by autoradiography.

Sequence Analysis—All of the sequence analyses were performed using the Wisconsin Package™ (version 9.1) from the Genetics Computer Group available through the Canadian Bioinformatics Resources website. Promoter sequences in the GenBank™ DNA sequence data base were obtained from the NCBI. The accession numbers identifying particular sequences are listed under "Results." The sequences were searched for the 15-bp DIE using the FINDPATTERNS program, allowing for 33.3% (5 of 15) random mismatch. Further DIE-like element comparison was done using the PRETTY program to find those elements with highest sequence similarity to the DIE.

Dual Luciferase Reporter Assays—Constructs containing wild type and mutant DIE elements were made using pGL3 Control reporter plasmids (Promega). The 15-bp high pressure liquid chromatography-purified oligonucleotides were cloned into the *Hind*III site found between the SV40 promoter and the luc⁺ reporter gene. This cloning site is found outside of the pGL3 control multi-cloning site and was chosen because of its proximity to the functional gene (i.e. the *in vivo* DIE is located in the 5'-UTR of the cyclin D1 gene). Therefore, the DIE was cloned upstream of the luc⁺ start codon but downstream of the SV40 promoter (see Fig. 8A). These constructs were then co-transfected into young and old HDFs with a *Renilla* luciferase construct (pRL-TK) as a transfection efficiency control. Firefly luciferase expression was measured using an EG & G Berthold luminometer and normalized based on pRL-TK expression.

RESULTS

Removal of a 64-bp Region in the 5'-UTR of Cyclin D1 Abolishes Increased Promoter Activity in Old Cells—To determine the basal activity of the cyclin D1 promoter in young and old cells, a 1.3-kb cyclin D1 promoter fused to a CAT reporter gene (Fig. 1A) was used in transient transfection studies. Various 5' deleted cyclin D1 promoter-CAT reporter constructs were also generated (Fig. 1B) and transiently co-transfected with a cyto-

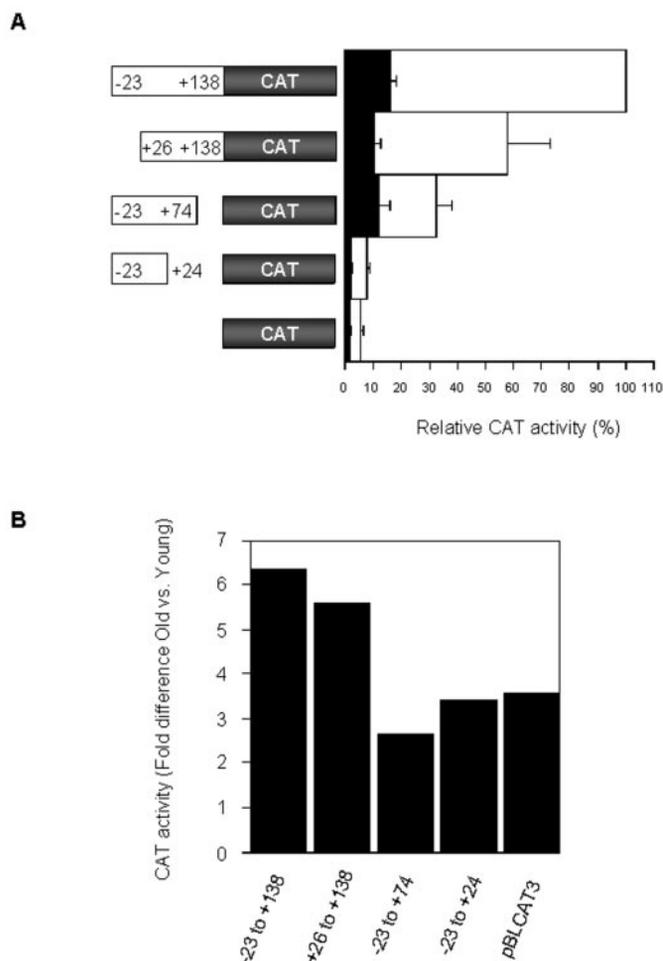
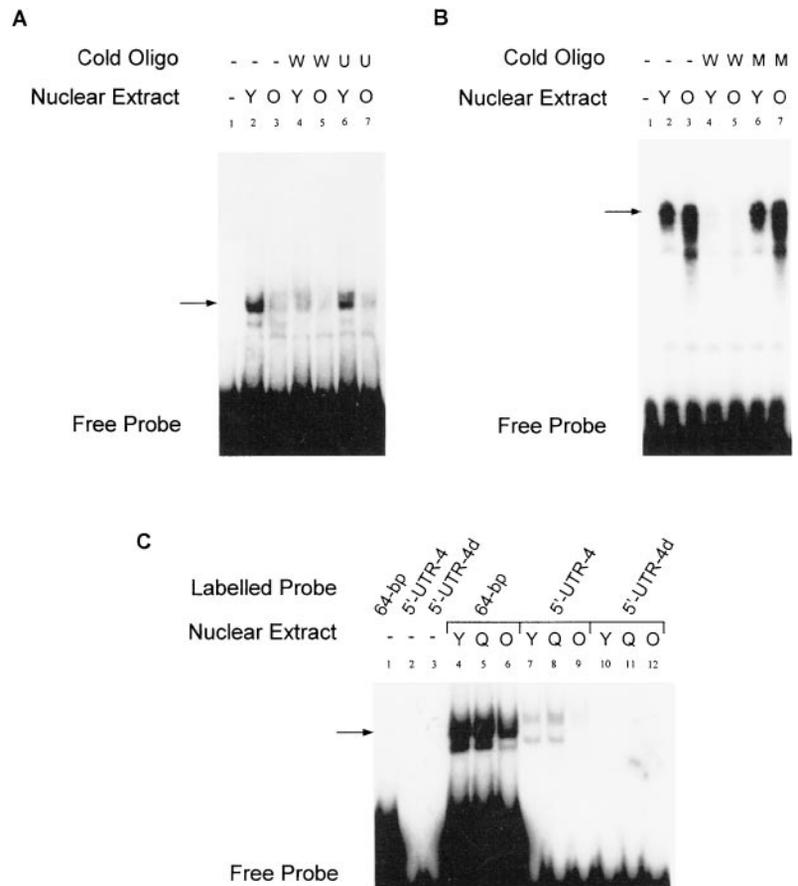


FIG. 2. 5' and 3' nested deletion analysis of the -23 to +138 region of the cyclin D1 promoter. **A**, 5' and 3' deletions of the cyclin D1 promoter (left panel) and the resulting CAT activity in young (black bars) and old (white bars) cells plotted after normalizing for β -galactosidase activity (right panel). **B**, fold difference in CAT activity in old versus young cells from A.

FIG. 3. DNA binding activity of the cyclin D1 5'-UTR. *A*, nuclear extracts from asynchronous young (*Y*) and old (*O*) Hs68 HDFs were incubated with an oligonucleotide corresponding to the 64-bp sequence of the cyclin D1 5'-UTR (lanes 2 and 3). The arrow points to the major specific DNA-protein complex. Lane 1 corresponds to the reaction with the labeled oligonucleotide in the absence of nuclear extracts. Lanes 4 and 5 show the reaction with the 64-bp oligonucleotide in the presence of a 100-fold excess of unlabeled (Cold) wild type (*W*) oligonucleotide or in the presence of a 100-fold excess of cold unrelated (*U*) CRE oligonucleotide (lanes 6 and 7). *B*, control gel shift using a consensus *c-fos* CRE binding site with the same young and old nuclear extracts described in *A*. Lane 1 shows probe alone in the absence of nuclear extract. Lanes 2 and 3 show complexes formed on the CRE when young and old nuclear extracts were added. Lanes 4 and 5 show the reaction in the presence of a 100-fold cold wild type oligonucleotide (*W*) or a 100-fold cold CRE mutant oligonucleotide (*M*) (lanes 6 and 7). *C*, electrophoretic mobility shift assays using independently isolated extracts from young growing cells (*Y*), young quiescent cells (*Q*), and old subconfluent cells (*O*). The first three lanes show oligonucleotide in the absence of extract, and lanes 4–12 show the degree of binding activity of the extracts of the extracts to the 64-bp oligonucleotide, a subdomain (5'-UTR-4), and a mutant subdomain (5'-UTR-4d) of the oligonucleotide as identified in Fig. 4.



megalovirus-driven β -galactosidase construct (as internal control) into young and old cells. CAT activity caused by the full-length cyclin D1 promoter was 4–5-fold greater in old than in young cells (Fig. 1B), consistent with endogenous levels of cyclin D1 expression in those cells (7). Deletion of segments from the 5' end from –1154 to –85 progressively reduced the expression difference between young and old cells to levels observed with the promoterless pBLCAT3 vector (Fig. 1B). However, the smallest deletion construct containing –23 to +138 of the cyclin D1 promoter retained 3–4-fold higher expression levels in old compared with young cells, indicating that this region is sufficient to confer increased expression in old cells.

We then generated further 5' and 3' nested deletions of the –23 to +138 region to further define the region involved in the differential regulation of cyclin D1. As shown in Fig. 2A, removal of the transcription initiation site resulted in approximately equivalent levels of inhibition of CAT activity in young and old cells. However, when 64 bp (+75 to +138) of the 5'-UTR were deleted, it resulted in a further 44% decrease in old cells, but the CAT activity in young cells remained relatively unchanged, thus reducing the fold difference in CAT activity to levels observed with the control vector (Fig. 2B, compare –23 to +138 with –23 to +74). Further removal of the 5'-UTR did not have any differential effect on CAT activity because it resulted in an approximately 77% decrease in young and an 82% decrease in old cells. These results indicate that together, other regions of the promoter are likely to contribute as strongly to the differential expression of cyclin D1 in young and old cells. The 64-bp sequence between +75 and +138 appeared to be a potential binding site for a transcriptional repressor in young cells and/or a transcriptional activator in old cells.

Decreased DNA Binding Activity in Extracts of Old Cells to

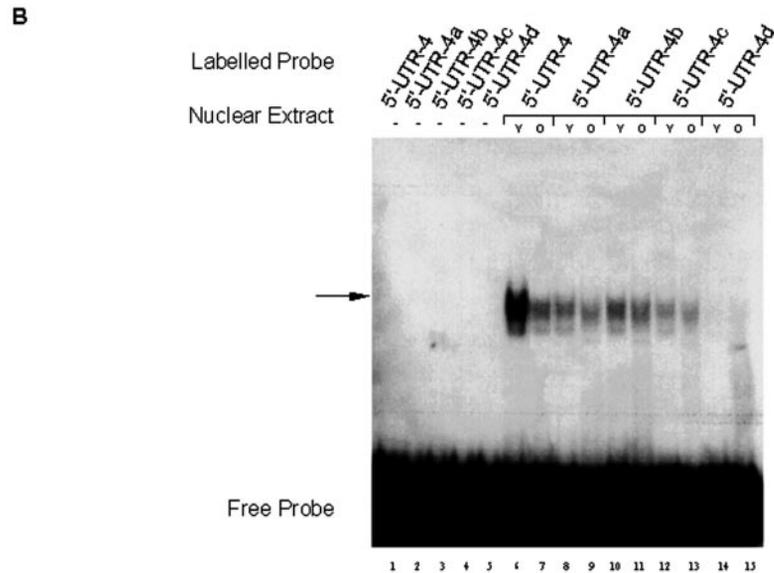
the +75 to +138 Region of the Cyclin D1 Promoter—To determine whether any proteins could be detected binding to this 64-bp region, electrophoretic mobility shift assays with young and old cell nuclear extracts were performed. Specific complexes were formed with young cell extracts, whereas the levels were dramatically reduced with old cell extracts (Fig. 3A, lanes 2 and 3). Specificity of the complexes was confirmed by incubation with a 100-fold excess of unlabeled oligonucleotide, which effectively competed with the labeled probe (Fig. 3A, lanes 4 and 5), whereas a 100-fold excess of unlabeled, unrelated CRE oligonucleotide did not compete efficiently (Fig. 3A, lanes 6 and 7).

To rule out the possibility that the nuclear extracts from old Hs68 fibroblasts were deficient in their ability to bind DNA through some nonspecific mechanism, we performed gel shift studies using the same extracts with an oligonucleotide containing the CRE present in the *c-fos* promoter (36). As shown in Fig. 3B, CRE binding activity was actually about 50% higher in old cell extracts than in young (lanes 2 and 3). As expected, 100-fold competition with unlabeled oligonucleotide bearing the same CRE sequence competed with the complexes formed on the labeled CRE (Fig. 3B, lanes 4 and 5), whereas mutant sequences did not (Fig. 3B, lanes 6 and 7). Given previous reports that CRE binding activity is about equal in young and old cells, specific binding activity of the complex in Fig. 3A may actually be even more dramatically reduced in extracts from old compared with young cells (36).

Although extracts from young cells showed more binding activity than extracts from old cells, it was possible that reduced binding in old cells was due to cells exiting the cell cycle when senescent rather than to a senescence-specific event *per se*. For example, a clear passage-related up-regulation of cyclin D2 is seen in all strains of primary HDFs examined, but increased expression is also seen upon serum withdrawal or con-



FIG. 5. Mutations of oligonucleotides in the 5'-UTR-4 region. *A*, to determine the effect of various base pair changes on protein binding activity, four sets of oligonucleotides (5'-UTR-4a to 5'-UTR-4d) were synthesized and used in mobility shift assays. *B*, electrophoretic mobility shift assays with young and old cell nuclear extracts using the mutant 5'-UTR-4 oligonucleotides shown in *A*. Lanes 1–5 are control lanes in the absence of nuclear extracts. Introduction of 2 or 4-bp changes inhibited binding activity (lanes 8–13), whereas an 8-bp change within the DIE nearly abolished binding activity (lanes 14 and 15) compared with the wild type 5'-UTR-4 oligonucleotide (lanes 6 and 7).



binding, oligonucleotides of 5'-UTR-4 containing various mutations (5'-UTR-4a to 5'-UTR-4d) were generated as shown in Fig. 5A. In each case, purines and pyrimidines were exchanged for noncomplementary pyrimidines and purines, respectively. Electrophoretic mobility shift assays of young and old cell nuclear extracts with these oligonucleotides showed that the mutations in 5'-UTR-4a, -4b, and -4c reduced binding in young cell extracts significantly compared with 5'-UTR-4 (Fig. 5B, compare lanes 8–13 with lanes 6 and 7), and that 5'-UTR-4d, which contains all of the individual mutations, nearly abolished binding in both young and old extracts (lanes 14 and 15). Densitometric scanning of different exposures of the lanes in Fig. 4B indicated that the binding to these oligonucleotides was reduced by greater than 90% in the case of the most mutated (5'-UTR-4d). Relative binding values for the oligonucleotides were 5'-UTR-4 (100%), 5'-UTR-4a (53%), 5'-UTR-4b (42%), 5'-UTR-4c (22%), and 5'-UTR-4d (9%). Therefore, the base changes in the 5'-UTR-4d oligonucleotide were used for site-directed mutagenesis of the DIE in the context of the full-length cyclin D1 promoter.

Mutation of the DIE Increases Cyclin D1 Promoter Activity in Young Cells—An 8-bp change in the full-length cyclin D1 promoter-CAT reporter construct from +117 to +131, corresponding to the mutations in 5'-UTR-4d, was introduced by site-directed mutagenesis. The 1.3-kb full-length cyclin D1 promoter-CAT reporter construct and the mutated construct were transfected into young and old cells, and the CAT activity resulting from each construct was measured after normalizing for β -galactosidase activity. As shown in Fig. 6A, mutation of the DIE in the 5'-UTR resulted in nearly a doubling of CAT activity in young cells relative to old cells, compared with the activity of the wild type construct. In contrast, the CAT activity

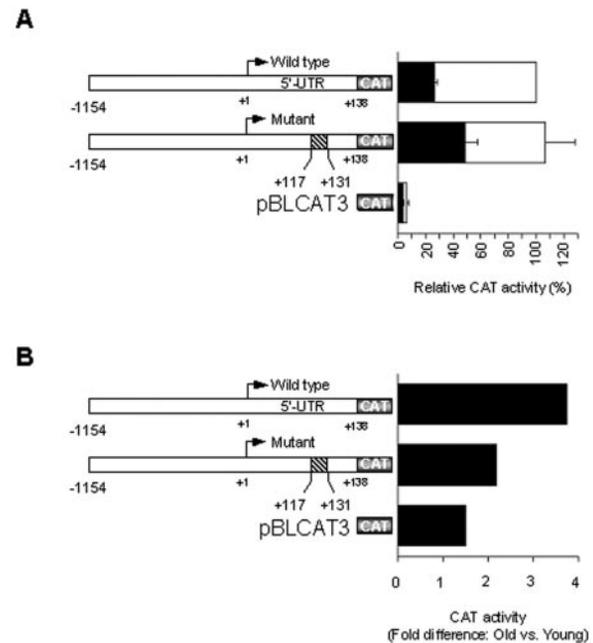
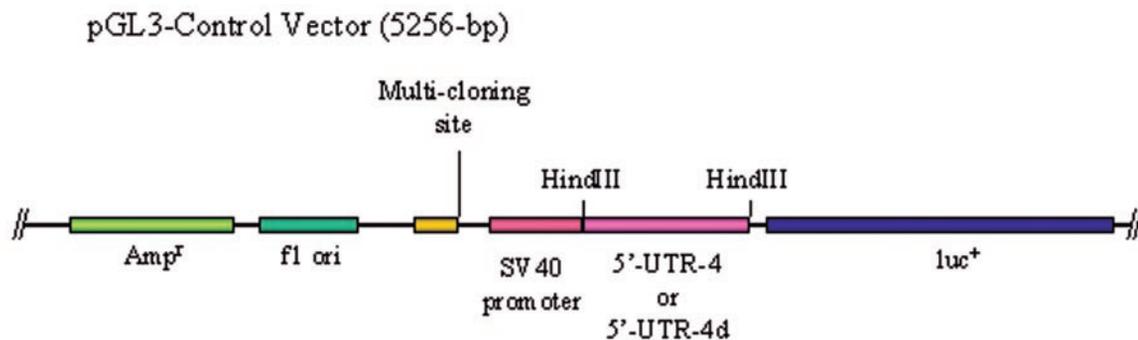


FIG. 6. CAT activity in young versus old cells using a cyclin D1 promoter containing a mutant DIE. The 8-bp change corresponding to 5'-UTR-4d was introduced into the full-length cyclin D1 promoter by site-directed mutagenesis, and the construct was transfected into young and old cells. *A*, the graph represents CAT activity from young (black bars) and old (overlapping white bars) cells after normalizing for transfection efficacy using co-transfected β -galactosidase construct. *B*, results from *A* plotted as fold difference in CAT activity in old versus young cells. An average of three independent transfections were performed with the standard deviations indicated by error bars.

A.



B.

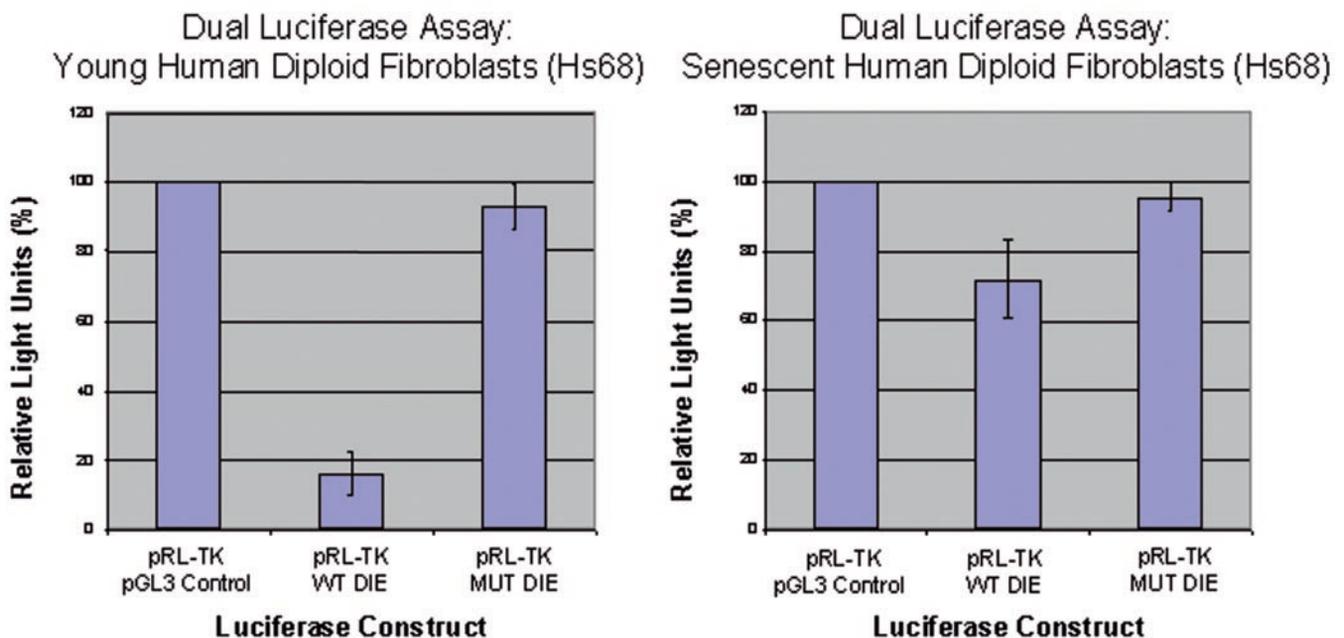


FIG. 8. Dual luciferase reporter assays using wild type and mutant DIE sequences. 26-bp sequences containing wild type or mutant forms of the 15-bp DIE were cloned into pGL3-control firefly luciferase reporter plasmids in the sense orientation. The assays show differential inhibition by the wild type *versus* mutant DIE on luc^+ expression. Young HDFs show more than 80% reporter inhibition, whereas senescent HDFs show less than 30% inhibition. Mutant constructs show little to no inhibition of reporter expression.

although their contribution to gene expression is unknown. Interestingly, the sequence similarity comparison revealed two additional DIE-like sequences within the cyclin D1 5'-UTR (Fig. 9). This included a sequence with limited homology within the 5'-UTR-2 oligonucleotide, with which weak complex formation was observed in mobility shift assays (Fig. 4). Based on our results, it appears that specific interactions of the repressor are stabilized by multiple copies of the DIE such that mutation of one can only partly reverse cyclin D1 gene repression in young cells. This would be consistent with the stronger binding seen using the 64-bp *versus* the shorter 22-bp oligonucleotides.

Although regulation of gene expression via the 5'-UTR has not been widely reported, such mechanisms might act to re-

press transcription more commonly than previously thought. For example, p53 suppresses the expression of *bcl-2* at least partly through a p53 response element located in the 5'-UTR of the *bcl-2* gene (46). Similarly, a suppressor element has been identified in the 5'-UTR of the androgen receptor gene (47). Although the exact mechanisms operating to repress transcription are unknown, the results presented here raise the possibility that other genes that are up-regulated during cellular senescence could be regulated by a mechanism similar to if not identical with the DIE identified here. Indeed, comparison of the DIE with a selected number of genes up-regulated during cellular aging revealed the presence of DIE-like sequences in their 5'-UTRs and the absence of these elements in control

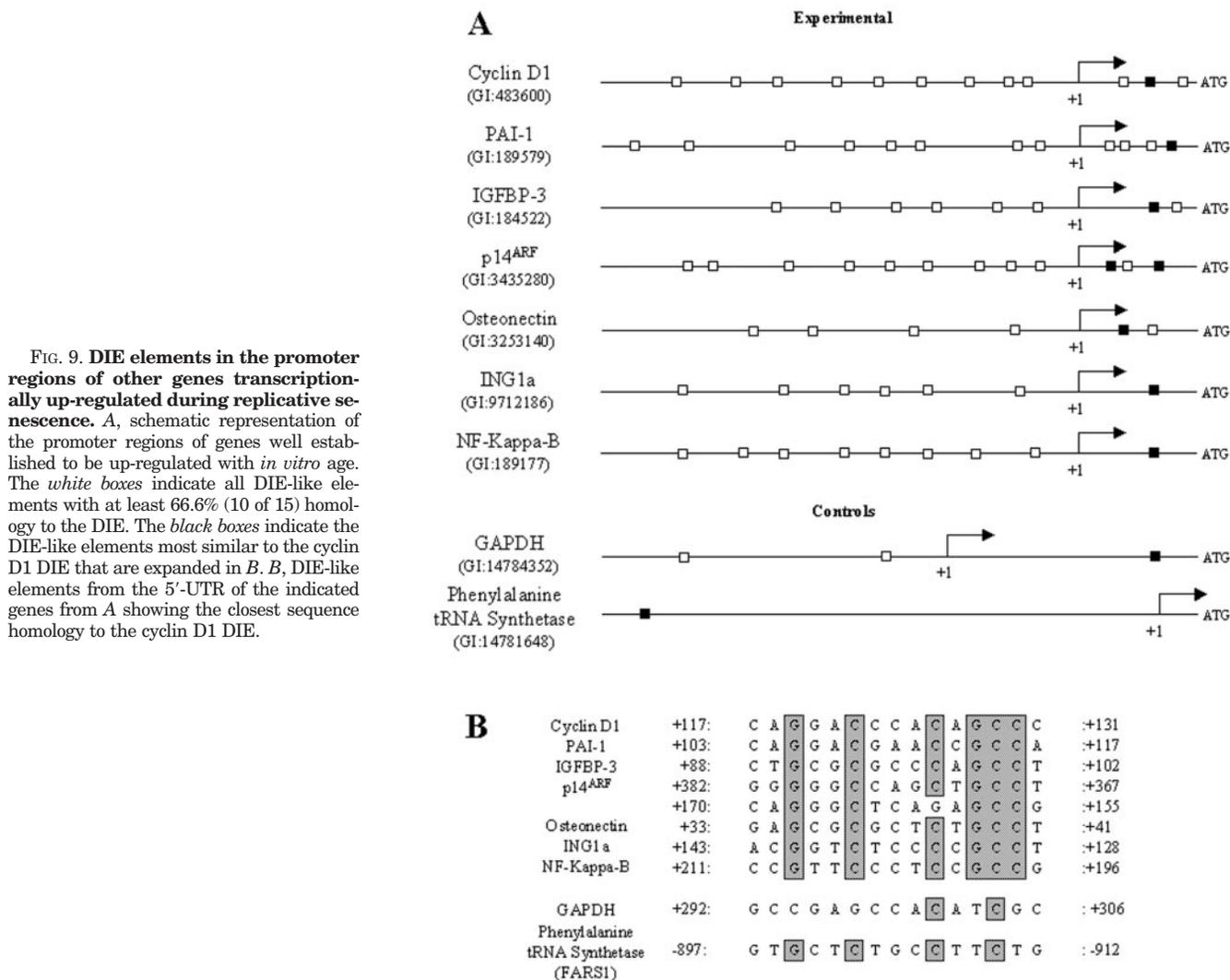


FIG. 9. DIE elements in the promoter regions of other genes transcriptionally up-regulated during replicative senescence. A, schematic representation of the promoter regions of genes well established to be up-regulated with *in vitro* age. The white boxes indicate all DIE-like elements with at least 66.6% (10 of 15) homology to the DIE. The black boxes indicate the DIE-like elements most similar to the cyclin D1 DIE that are expanded in *B. B.* DIE-like elements from the 5'-UTR of the indicated genes from A showing the closest sequence homology to the cyclin D1 DIE.

genes. This raises the possibility that the expression of a group of age-related genes is coordinately regulated by one principal mechanism and that it may be possible to influence the expression of many genes by targeting a single repressor element or protein.

Taken together, these data indicate that a specific transcriptional mechanism contributes to the increased expression of cyclin D1 during cellular senescence. Searches of transcription factor data bases using the DIE revealed no homologies to known human transcription factor binding sites, suggesting that the protein(s) binding the DIE are novel. Experiments to purify the putative repressor protein(s) by DNA affinity chromatography are underway and should reveal important insights regarding the regulation and identification of additional genes containing DIE elements and their role(s) in cellular aging.

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Functional links between transcription, DNA repair and apoptosis

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Abstract. DNA damage initiates damage response pathways, cell cycle arrest and apoptosis. These processes act in a concerted fashion and remain functionally linked through mechanisms not completely understood. Programmed cell death, referred to as apoptosis, is a tightly regulated phenomenon ensuring that cells that accumulate irreversible DNA damage do not replicate. Interestingly, hyperacetylation of histone proteins, which alters transcription patterns and appears linked to DNA repair, also induces apoptosis, suggesting that aspects of chro-

matin modification link these very distinct processes. Modulating chromatin structure in the absence of any DNA lesions also activates key DNA damage-signalling proteins, further supporting the role of higher-order chromatin structure in mediating stress responses. This review will present an overview of the epigenetic control of eukaryotic genomes by chromatin remodelling as it pertains to DNA damage and highlight the potential role of the ING PHD proteins in linking apoptosis and DNA repair to gene transcription.

Key words. Histone acetylation; HAT/HDAC; ING1; p53; PCNA; transcription; DNA damage/repair; apoptosis/senescence.

Introduction

A variety of exogenous and endogenous genotoxic agents are capable of inducing DNA damage and eliciting DNA repair, stress-induced premature senescence (SIPS) that resembles a severe cell cycle checkpoint, or apoptotic responses. It is believed that the cell's initial response is to attempt to repair damage, but if the lesion is too extensive or compromises DNA metabolism, a signalling cascade triggers alternative mechanisms so as to inhibit cellular transformation and immortalization. Therefore, it is crucial that cells are capable of recognizing the severity of damage and simultaneously activating the appropriate checkpoint responses, otherwise mutations will be introduced and faulty genomes will be propagated. Several key DNA damage sensors that bind specifically to DNA lesions such as the Mre11-Rad50-Nbs1 (MRN) complex and the BRCA-1-associated surveillance complex

(BASC), are believed to activate kinases that are presumed to transduce signals generated by various types of genomic insults. However, it has recently been shown that these kinase transducers can be activated in the absence of DNA damage [1]. These stress-induced kinase proteins harbour significant sequence homology to the catalytic domains of the phosphoinositide (3) kinase [PI(3) kinase] and are therefore referred to as phosphoinositide (3) kinase-related kinases (PIKKs). Indeed, it is now clear that different forms of DNA damage and some other types of cellular stress prompt a protein phosphorylation cascade that ultimately impinges upon the transcription, replication and cell cycle machineries. Mutations that are known to increase the cell's vulnerability to specific DNA-damaging agents have helped to elucidate the mechanisms by which these proteins are activated and how their downstream targets mediate the cell's response to genomic insult (table 1). Furthermore, since genomic integrity is a vital component of all life forms, studies in a wide range of model organisms have enabled us to identify and char-

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acterize evolutionarily conserved repair protein orthologues.

The packaging of genetic material into chromatin has pronounced effects on transcriptional regulation and plays a pivotal role in DNA repair, senescence and apoptotic pathways. This type of genomic regulation is often referred to as epigenetics (literally translated to 'on genes') since it involves modifications of DNA and its associated proteins, resulting in altered gene expression without altering the DNA sequence per se. Chromatin structure can be modified by both internal stressors such as lesions to DNA and external stressors such as a non-hospitable growth environment [2, 3]. Therefore, it is likely that there is a high degree of downstream overlap in

a cell's response to intracellular DNA damage as well as to extracellular stress (fig. 1).

Supporting this hypothesis is the yeast target of rapamycin (TOR) protein kinase, whose homologue in mammalian cells (mTOR) is a member of the PIKK family that responds to DNA damage [4]. In yeast, TOR has recently been shown to modulate chromatin in response to nutrient availability [2]. This finding is especially intriguing since nutrient deprivation, like DNA damage, can influence chromatin structure and initiate a signalling cascade in response to the stress. Therefore, it seems that several types of cellular stress could alter chromatin equilibrium, thereby activating similar downstream stress responses (fig. 2).

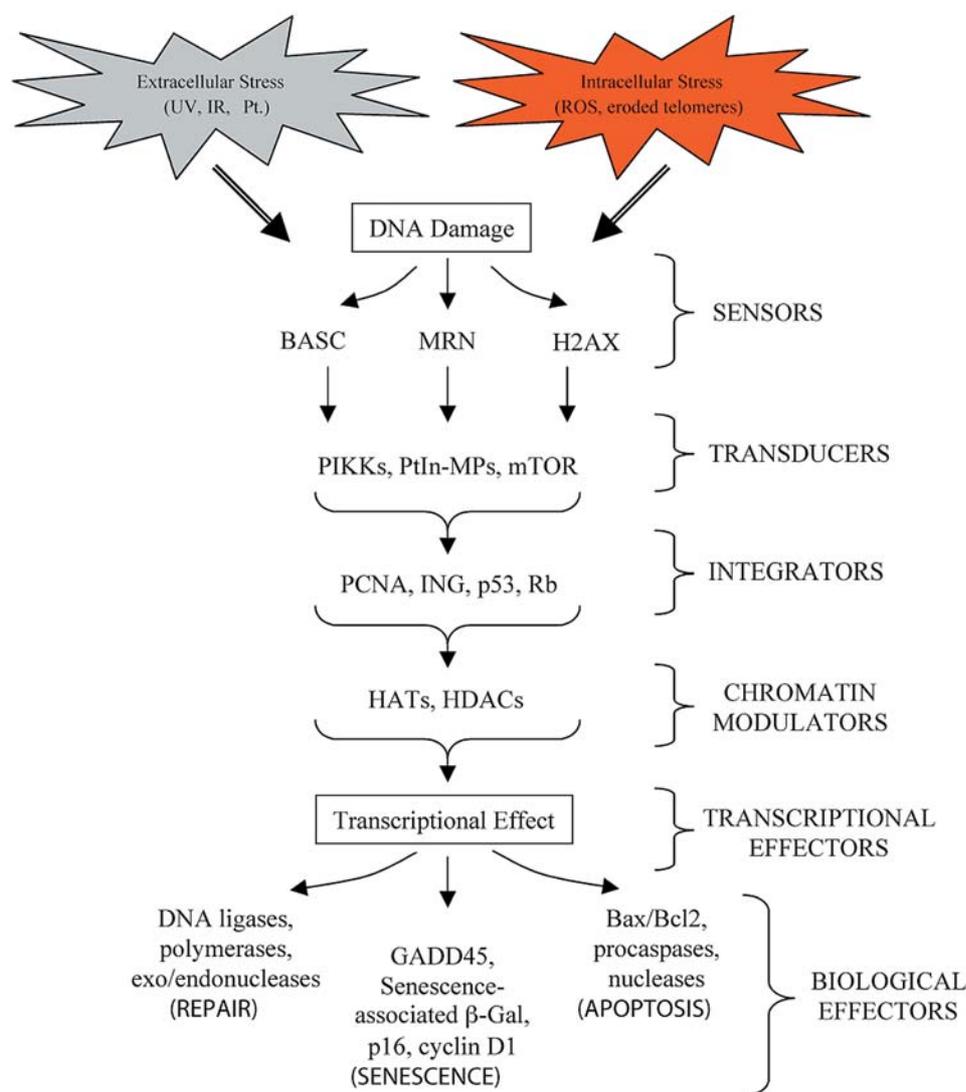


Figure 1. Cellular responses to DNA damage, telomere erosion or extracellular stress all impinge upon common regulators of transcription. Both external stresses such as UV or chemical toxins and internal stresses such as reactive oxygen species activate PIKKs. This pathway is common to all DNA damage-signalling models in that it involves sensor, transducer and effector molecules. Here we propose the addition of integrators that target chromatin-modifying proteins to particular regions of chromatin to activate or repress particular genes. The activation of ING2, and possibly other ING1s by phosphatidylinositol monophosphates (PtIn-MPs) has recently been observed in response to DNA damage.

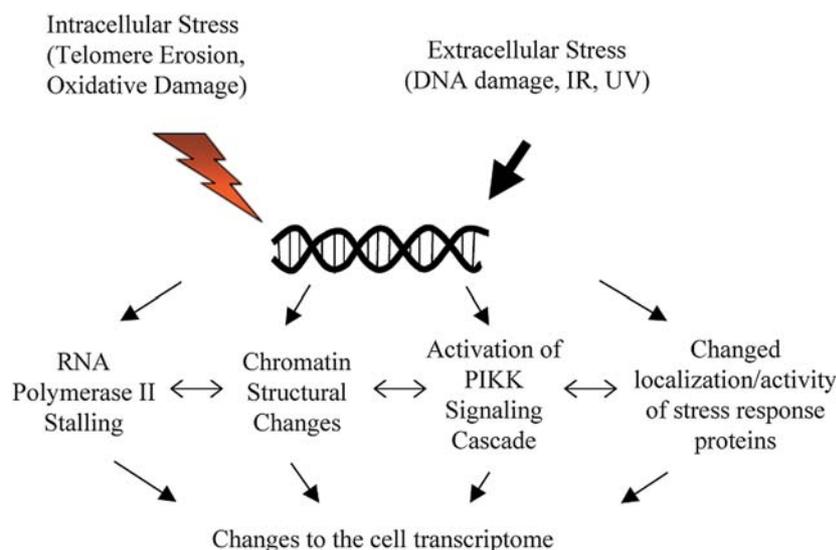


Figure 2. Several types of cellular stress could alter chromatin equilibrium, thereby activating similar downstream stress responses. Cellular stress responses impinge upon several common downstream targets that ultimately have effects on gene transcription. These targets also show a bidirectional activation possibly to reinforce the stress response pathways.

Table 1. Genomic vulnerability and accelerated aging syndromes.

Syndrome	Defective gene(s) or gene product(s)	DNA repair defect
Ataxia telangiectasia	ATM	Unable to activate cell cycle checkpoints in response to DNA double-strand breaks
Nijmegen breakage syndrome Ataxia-telangiectasia-like disease	NBS MRE11	part of MRE11-RAD50-NBS1 (MRN) complex involved in initial processing of DNA double-strand breaks
Werner's syndrome	WRN helicase	WRN DNA helicase
Bloom's syndrome	BLM helicase	ablated homologous recombination repair and repair of damage at stalled replication forks
Xeroderma pigmentosum	XPA, XPB, XPC, XPD, XPF	ablated nucleotide excision repair (NER)
Trichothiodystrophy	XPB, XPD in transcription	abrogated function of DNA helicases involved
Cockayne's syndrome	CSA, CSB	unable to recognize stalled polymerase (TCR)
Fanconi's anemia	multiple 'FA genes'	abrogation of DNA damage response system
Rothmund-Thompson syndrome	RECQL4	RecQ-like DNA helicase

Chromatin structure and transcription

Chromatin in interphase cells is made up of 146 bp of DNA coiled around an octamer of histones (H2A, H2B, H3 and H4), forming a 10-nm nucleosome fiber [5, 6]. The 10-nm string of polynucleosomes can be obtained under conditions of low ionic strength; however, a 30-nm fiber consisting of an underlying coiled structure also forms under some conditions. The 30-nm fiber is the basic constituent of both interphase chromatin and mitotic chromosomes (fig. 3) [7, 8]. Chromatin structure is very dynamic and is affected by multiple modifications of chromatin-associated proteins, including, but not limited to, histones and remodelling cofactors within particular

chromatic regions. Indeed, chromosomal DNA and its associated proteins undergo dramatic alterations in structure during normal cellular processes such as DNA synthesis, transcription and repair [9, 10]. Conversely, it is known that DNA damage leads to changes in gene expression [11–13], and it is now clear that mechanisms that impinge directly upon higher-order chromatin structure regulate cellular metabolic processes such as transcription, DNA replication and DNA repair. This bidirectional interaction is outlined in figure 2.

Chromatin structure is increasingly being attributed to modification of the subunits of nucleosomes, the basic histones. Histones are positively charged, low-molecular-weight DNA scaffolding proteins that are subject to numerous post-translational modifications including acety-

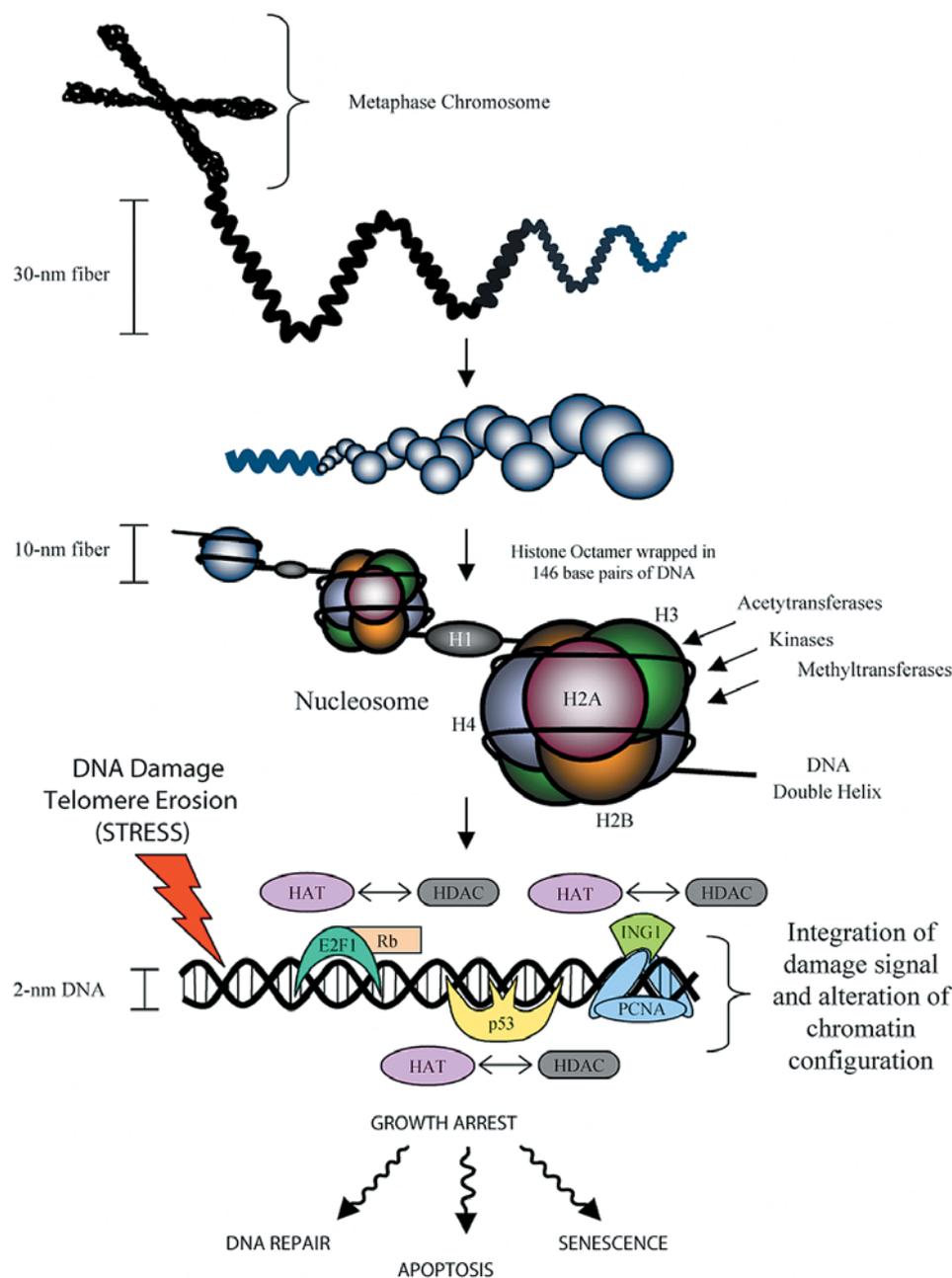


Figure 3. Chromatin modulation can result from both intracellular and extracellular stressors, ultimately inducing a common downstream signalling cascade. Recent studies have highlighted the importance of epigenetic control of the human genome. Here we propose that structural changes to chromatin such as those seen at sites of DNA damage as well as those induced by extracellular stressors can activate common key transducers of cellular stress. This signalling cascade impinges upon diverse mediators of the stress response and results in changes to the transcriptome. Ultimately, the mammalian cell's fate is dictated by how severe the stress is and culminates in either survival when the stress is repaired or removed, apoptosis or senescence when the stress is too severe, or transformation when the damage remains unchecked.

lation, methylation, phosphorylation, poly(ADP-ribosylation) and ubiquitination [14]. These modifications play diverse roles in modulating chromatin structure and have been linked to the regulation of gene transcription [15]. Histone acetylation neutralizes the charge of basic (positively charged) lysine residues within histone proteins. Consequently, there is destabilization of the binding of

histones to the negatively charged DNA so that other enzymes/protein complexes are capable of unwinding the chromatin, accessing the DNA at selective sites and transcribing target genes. In other words, the dynamic modification of histones through the enzymatic actions of histone acetyltransferase (HAT) and histone deacetylase (HDAC) protein complexes modifies nucleosome struc-

ture, altering the degree of DNA relaxation and subsequently modifying the accessibility of regions of DNA to transcription factors [14].

Not surprisingly, HAT and HDAC protein complex activity must be tightly regulated in order to maintain the appropriate level of histone acetylation in a given cellular environment. To date several HAT/HDAC coactivators and corepressors have been identified. The INhibitor of Growth (ING) family of proteins are involved in chromatin remodeling, and bind to and affect the activity of both HAT and HDAC protein complexes. The first member of the ING gene family, ING1, induces histone acetylation, promotes DNA repair and interacts with proliferating cell nuclear antigen (PCNA). The ING proteins have a highly conserved plant homeodomain (PHD) zinc finger, a nuclear localization sequence (NLS) and nucleolar targeting sequences (NTS) that target the proteins to nuclear domains under certain conditions [16–18]. ING1 also has a PCNA-interacting protein (PIP) domain through which it binds PCNA in a DNA damage-inducible manner [19]. Since PCNA is an essential factor for DNA replication and repair, ING1 may act to couple these processes to chromatin remodeling. Therefore, the ING family of PHD proteins may act to help functionally link transcription to DNA repair and apoptosis.

Chromatin modifications other than acetylation also play clear roles in DNA damage repair and apoptosis. For example, phosphorylation of the histone variant H2AX on its canonical SQE motif acts to localize DNA damage repair proteins to the site of DNA damage [20, 21]. Interestingly, this has also been reported to occur in replicative senescence initiated by telomere erosion [22], suggesting that senescence may share some of the same pathways involved in the DNA damage signalling cascade initiated by external damaging agents such as ionizing radiation (IR), ultraviolet (UV) and oxygen radicals, among others. Transduction of internal and external stress signals by common pathways is illustrated in figure 1. Histone and DNA methylation also play important roles in chromatin

Table 2. Types of DNA damage.

DNA damage	Repair mechanisms*
Double-strand breaks (DSBs)	NHEJ, HR
Single-strand breaks (SSBs)	NHEJ, HR, nucleotide excision repair (NER), base excision repair (BER)
Thymidine dimers	NER
Mismatch	mismatch repair (MMR)
C-U deamination	BER
Covalent cross linking	NER, BER
Apurinic/aprimidinic (AP) site (missing bases)	NER, BER

*Repair mechanisms are not an exhaustive list and do tend to show considerable overlap based on additional characteristics of DNA damage.

homeostasis and gene activity [23, 24]; however, for the sake of brevity we will focus on the role of histone acetylation in these processes.

Upon induction of a cellular stress such as DNA damage, it is apparent that particular genes are activated or repressed and different types of DNA damage trigger different repair responses depending on the severity and type of lesion (summarized in table 2). Recently, a model by which DNA double-stranded breaks (DSBs) activated the ataxia telangiectasia-mutated (ATM) protein (a PIKK kinase) through intermolecular autophosphorylation [1] was proposed, where DNA damage induces inactive multimeric ATM to autophosphorylate, dissociate and become catalytically active. Surprisingly, chromatin structural changes in the absence of any DNA damage also appeared to activate ATM [1], supporting the idea that changes to local chromatin architecture can elicit a DNA damage repair response via a mechanism that remains to be identified. Furthermore, treatment of cells with low doses of ionizing radiation (IR) increased the amount of catalytically active monomeric ATM in the nucleus, sug-

Table 3. Key DNA damage protein motifs.

Motif*	DNA damage proteins	Role in damage	Modifications
BRCT	BRCA1, BARD1, MDC1, 53BP1, Rad9	localization of host protein	phosphorylation
PHD	ING1-5, CBP, TIP5/NoRC	chromatin modulation	phosphorylation
SQE	p53, H2AX, E2F1, SMC1, Nbs1, TRF1	activation of host protein targeted by ATM	phosphorylation
PIP	ING1, p21, GADD45, FEN1, XPG	loading dock near site of lesion	competitive binding of proteins
FAT/FATC PI(3)K	ATM, ATR, SMG1, DNA-PK _{cs} , mTOR (FRAP), TRRAP	protein binding, structural scaffold catalytic site of active protein kinases	none reported phosphorylation

*BRCT, BRCA1 C-terminus; PHD, plant homeodomain; SQE, serine, glutamine, glutamic acid; PIP, PCNA interacting protein; FAT, FRAP, ATM and TRRAP; FATC, FRAP, ATM and TRRAP extreme C terminus; PI(3)K, phosphoinositide 3-kinase catalytic domain.

gesting that ATM was not activated by DNA lesions themselves via MRN or BASC complexes, but rather by modifications to the higher-order structure of chromatin.

DNA damage and the decision between senescence and apoptosis

The processes necessary to initiate and mediate DNA damage repair versus apoptotic signalling cascades are tightly regulated, and making an incorrect decision would have dire consequences to cells and organisms. Recently, it was shown that in addition to the repair-versus-apoptosis decision, low levels of DNA damage can alternatively induce a senescent-like phenotype referred to as stress-induced premature senescence (SIPS) [25–27]. Further complexity exists when we consider that different types of DNA lesions result in aberrant DNA structures and recruit various repair mechanisms, as outlined in table 2. We now know that certain proteins such as the tumour suppressors p53 and retinoblastoma (Rb) are germane to these complex cellular processes, but we still do not fully understand the context in which these and other stress response proteins act. Members of the PIKK family such as ATM and ATR (ataxia-telangiectasia mutated and Rad3 related) are believed to act as transducers of the DNA damage signal by phosphorylating downstream effector molecules, resulting in their activation [28]. It has also been shown that in response to DNA damage, the HAT and transcriptional coactivator p300, as well as ING1, which activates HATs, binds PCNA and participates in chromatin remodelling at DNA lesion sites [19, 29, 30]. This indicates that the ING1:p300 HAT protein complex associates with dynamic DNA structures, again linking DNA damage repair to chromatin modification via histone acetylation. It remains unclear whether PIKK activation and HAT complex localization to sites of DNA damage are functionally linked, but recent evidence suggests this [1].

PCNA is an essential processivity factor for DNA replication and repair. It is highly conserved evolutionarily, essential for cell survival and forms a sliding homotrimeric platform encircling DNA that can mediate the local interaction of proteins with DNA [19, 29–31]. Many proteins bind to PCNA through a small region containing a conserved motif; these include proteins involved in cell cycle regulation as well as those involved in DNA processing and chromatin modulation (table 3) [31, 32]. We and others speculate that PCNA associates with protein complexes in a DNA damage and cell growth-dependent manner, suggesting that its role in the context of DNA association is as a ‘liaison’ for the integration of various chromatin- and DNA-modifying proteins [30]. For instance, HAT protein complexes such as p300/CBP which interact with PCNA also interact with proteins such as

BRCA1, which is involved with DNA repair [33], and with the ING1 protein, which binds PCNA directly and plays a role in chromatin remodelling and apoptosis [17, 19, 30] through interactions with HATs and HDACs. It is therefore possible that a DNA repair pathway, an apoptotic pathway or a senescence pathway could be triggered based on the specific multiprotein complex tethered to PCNA at any given time. This complex of proteins, therefore, could play the role of a molecular integrator of signals, as outlined in figure 1. Protein motifs that are common on proteins functioning in DNA repair pathways and that may promote complex formation are listed in table 3.

Transcription-coupled repair and RNA processing

It has been known for some time that transcriptionally active genes are repaired significantly faster than non-transcribed genes [34, 35]. This is consistent with the role of PCNA-binding partners, which include chromatin-modifying complexes, in DNA transcription, synthesis and repair. Although it is evident that chromatin structure needs to be modulated to allow transcription, DNA replication or DNA repair, the role of modular protein complexes in these processes was initially unexpected.

The transcriptional response to DNA damage may help to elucidate how chromatin modulation is linked to DNA repair and apoptosis (fig. 2). Normally, DNA damage interrupts RNA synthesis via RNA polymerase II (RNAP-II) stalling, allowing DNA damage to be repaired before transcription resumes [36, 37]. In fact, several lines of evidence indicate that a stalled polymerase is necessary to elicit transcription-coupled repair (TCR). Moreover, it ensures that DNA damage is not propagated, since the cell is temporarily put on ‘hold’ in order to accurately assess the degree of damage. It has been reported that prolonged RNAP-II stalling results in RNAP-II cleavage and apoptosis [38], suggesting a mechanism by which the cell can establish if the DNA damage is repairable or whether the insult is too severe and cell suicide must ensue. A number of models for the mechanism of transcriptional downregulation in response to DNA damage have been proposed [39]. These include RNAP-II phosphorylation, TATA-binding protein depletion and transcription/repair factor TFIIH depletion [40]. However, inactivation of RNAP-II by phosphorylation seems to be the most plausible in light of the fact that several kinase proteins that can phosphorylate RNAP-II are activated upon induction of DNA damage [41, 42].

In addition to altering transcription in response to DNA damage, recent studies suggest that preexisting messenger RNA (mRNA) is also regulated. Since genes transcribed just prior to DNA damage can potentially promote inappropriate cell growth, it is critical that this message be intercepted. This occurs by interruption of

pre-mRNA processing. Briefly, DNA repair is linked to mRNA 3' processing [43] by virtue of the mRNA polyadenylation factor CstF (cleavage stimulation factor) interacting with the BRCA1-associated protein BARD1. The BARD1-CstF interaction represses the nuclear mRNA polyadenylation machinery, thereby inhibiting the export of newly transcribed genes. The interaction between the N-termini of BRCA1 and BARD1 mediates the effect of CstF on mRNA. Consistent with this mechanism, mouse embryonic stem cells that are BRCA1 null are defective in TCR of oxidative DNA damage [44] possibly due to an inability to process mRNA. Therefore, it appears that cells have developed mechanisms not only to abrogate de novo RNA synthesis, but also to stop the processing and export of newly transcribed genes.

Can ING proteins link transcription, DNA repair and apoptosis?

Many recent studies of DNA damage signalling have highlighted the importance of key, evolutionarily conserved protein motifs [12, 19, 28, 31, 45], some of which are listed in table 3. Many of these motifs are modified to change catalytic activity, localization or association with transcriptional coactivators or -repressors. For example, the activity of the PIKK kinases in response to DNA damage and the constellation of proteins that bind to PCNA changes markedly in response to UV and other DNA-damaging agents.

The ING family of proteins, with their evolutionarily conserved PHD domain as well as a PIP motif found in the p33^{ING1b}-splicing isoform of ING1, represent a family of proteins that utilize different functional domains to link different cellular processes that arise from internally generated and/or external stresses. For example, PCNA forms a homotrimeric clamp around DNA as diagrammed in figure 3 and is an essential processivity factor for DNA polymerase δ and ϵ , functioning in both DNA replication and nucleotide excision repair. Since PIP motifs are found in growth regulators such as p21^{WAF1} and the growth arrest and DNA damage-inducible gene GADD45 and with DNA replication and repair proteins, it suggests that PCNA acts to integrate the intensity of the DNA damage signal by binding particular proteins with PIP motifs and translating the signal into distinct responses such as cell cycle arrest with subsequent DNA repair, stress-induced premature senescence, or apoptosis. Therefore, ING proteins could serve a central role in this decision-making process since they could recruit different chromatin-remodelling (HAT and HDAC) complexes to sites of repair where PCNA is bound.

Conclusion

Clearly, the links between how cellular stresses such as DNA damage, oxidative damage, heat shock and particular metabolic poisons alter DNA transcription and initiate repair, apoptosis or senescence are too numerous to mention in the context of this review. However, the aim of this report is to highlight the expanding roles of multifunctional proteins such as PCNA and ING1 in the regulation of the biological responses to DNA damage through their ability to coordinate DNA repair with higher-order chromatin structure, which subsequently impinges upon transcription regulation. The numerous functions of these proteins in the context of DNA damage and repair, apoptosis or senescence can be a consequence of protein modification and/or localization within the cell. One common feature of most DNA damage response models is that of sequential signalling of DNA damage from initial sensors of DNA damage, such as the MRN and BASC complexes, to the transducers of this signal, such as the PIKK family of protein kinases, and finally to the mediators or effectors of this signalling cascade that are ultimately responsible for generating a physiological response. As diagrammed in figures 1 and 3, we propose the addition of a signal integrator that initially blocks cell growth and, through the regulation of local chromatin structure, determines the subset of genes that are transcriptionally activated or repressed. This integrator may include tumour suppressors such as p53, Rb and ING1 that can target HAT and HDAC complexes to particular domains of chromatin. Subsequently, the genes that are regulated determine whether cells repair DNA and resume cell cycling, enter a stress-induced senescent state or undergo apoptosis.

Much like the naïve belief that individual genes were regulated entirely by cis-acting promoter elements, we are now recognizing the importance of epigenetic mechanisms in DNA damage responses and beginning to appreciate the complexity of the molecular circuitry that enables cells to shut down general transcription in response to genomic insult while concomitantly transactivating specific subsets of damage-inducible genes.

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ORIGINAL PAPER

BARD1 induces apoptosis by catalysing phosphorylation of p53 by DNA-damage response kinaseAnis Feki^{1,2}, Charles Edward Jefford¹, Philip Berardi^{1,3}, Jian-Yu Wu¹, Laetitia Cartier¹, Karl-Heinz Krause¹ and Irmgard Irminger-Finger^{*1}¹Biology of Aging Laboratory, Department of Geriatrics, University of Geneva, Chemin de Petit Bel Air 2, CH-1225 Geneva/Chêne-Bourg, Switzerland; ²Department of Gynecology and Obstetrics, University Hospital of Geneva, Geneva, Switzerland; ³Biochemistry and Molecular Biology and Oncology, University of Calgary, Calgary, Canada

The BRCA1-associated RING domain protein BARD1 acts with BRCA1 in double-strand break repair and ubiquitination. BARD1 plays a role as mediator of apoptosis by binding to and stabilizing p53, and BARD1-repressed cells are resistant to apoptosis. We therefore investigated the mechanism by which BARD1 induces p53 stability and apoptosis. The apoptotic activity of p53 is regulated by phosphorylation. We demonstrate that BARD1 binds to unphosphorylated and serine-15 phosphorylated forms of p53 in several cell types and that the region required for binding comprises the region sufficient for apoptosis induction. In addition, BARD1 binds to Ku-70, the regulatory subunit of DNA-PK, suggesting that the mechanism of p53-induced apoptosis requires BARD1 for the phosphorylation of p53. Upregulation of BARD1 alone is sufficient for stabilization of p53 and phosphorylation on serine-15, as shown in nonmalignant epithelial cells and ovarian cancer cells, NuTu-19, which are defective in apoptosis induction and express aberrant splice variants of BARD1. Stabilization and phosphorylation of p53 in NuTu-19 cells, as well as apoptosis, can be induced by the exogenous expression of wild-type BARD1, suggesting that BARD1, by binding to the kinase and its substrate, catalyses p53 phosphorylation.

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Keywords: apoptosis; BARD1; DNA-PK; Ku-70; p53; phosphorylation

Introduction

Apoptosis is an important means of eliminating dangerously damaged cells. Cancer cells have developed strategies to escape apoptosis and consequently become resistant to apoptosis inducing treatments. Along this

line, the tumor suppressor p53, a key element in the apoptosis response pathway, is mutated in more than 50% of tumors. Several signaling pathways converge on the activation of p53, which leads either to cell cycle arrest and DNA repair or senescence, or apoptosis (Levine, 1997). P53 activation and stabilization depend on its phosphorylation at multiple sites, by a number of kinases (Meek, 1994; Milczarek *et al.*, 1997). Phosphorylation at serines 6 and 9 by casein kinase 1-delta and casein kinase 1-epsilon occurs both *in vitro* and *in vivo* (Knippschild *et al.*, 1997; Kohn, 1999), the checkpoint kinases Chk1 and Chk2 phosphorylation at serine 20 enhances its tetramerization, stability, and activity (Shieh *et al.*, 1999; Hirao *et al.*, 2000). Phosphorylation of p53 at serine-392 is observed *in vitro* by CDK-activating kinase (CAK) (Lu *et al.*, 1997) and *in vivo* (Hao *et al.*, 1996; Lu *et al.*, 1997), but is altered in human tumors. Serine-392 phosphorylation has been reported to influence the growth suppressor function, DNA binding, and transcription activation of p53 (Hao *et al.*, 1996; Lohrum and Scheidtmann, 1996; Lu *et al.*, 1997; Kohn, 1999). Phosphorylation of p53 at serine-46 is important for regulating its ability to induce apoptosis (Oda *et al.*, 2000). However, the rapid response to DNA damage is induced by phosphorylation of p53 at serines 15, 20, and 37 and leads to reduced interaction of p53 with its negative regulator, oncoprotein MDM2 (Shieh *et al.*, 1997).

Known kinases for the DNA damage response are ATM, ATR, and DNA-PK, which can phosphorylate p53 at serines 15 and 37 (Shieh *et al.*, 1997; Tibbetts *et al.*, 1999). These phosphorylation events impair the ability of MDM2 to bind p53, promoting both the accumulation and functional activation of p53 in response to DNA damage (Shieh *et al.*, 1997; Tibbetts *et al.*, 1999). MDM2 acts as inhibitor of p53 function by targeting it for ubiquitination and proteasomal degradation (Honda *et al.*, 1997; Chehab *et al.*, 1999).

The tumor suppressor BRCA1 Associated Ring Domain protein (BARD1) can act as a mediator between genotoxic stress and apoptosis by binding to and stabilizing p53 (Irminger-Finger *et al.*, 2001; Irminger-Finger and Leung, 2002). BARD1 is the major protein-binding partner of BRCA1 (Wu *et al.*, 1996)

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and the BARD1/BRCA1 heterodimer has functions in DNA repair (Jin *et al.*, 1997; Scully *et al.*, 1997) and ubiquitination (Baer and Ludwig, 2002). Both proteins are homologous within their RING domains and two BRCT domains. The BRCT domains of BRCA1 have recently been characterized as modules for kinase and target interaction (Manke *et al.*, 2003; Yu *et al.*, 2003) and a role of BARD1–BRCA1 complexes in signaling from DNA damage to p53 phosphorylation has been described recently (Fabbro *et al.*, 2004).

The repression of BARD1 expression *in vitro* results in genomic instability and cellular changes suggestive of a premalignant phenotype (Irminger-Finger *et al.*, 1998) and resistance to apoptosis (Irminger-Finger *et al.*, 2001). Similarly, BARD1, as well as BRCA1, knockout cells exhibit high levels of genomic instability (Joukov *et al.*, 2001; Ludwig *et al.*, 2001; McCarthy *et al.*, 2003). However, excess of BARD1 over BRCA1 results in apoptosis independently of BRCA1 (Irminger-Finger *et al.*, 2001). Interestingly, the expression of BARD1, but not BRCA1, is hormonally controlled in uterine tissue (Irminger-Finger *et al.*, 1998), suggesting a role in tissue homeostasis. Further evidence for a role of BARD1 in tumor suppression is presented by BARD1 mutations found in breast, ovarian, and most intriguingly in endometrial tumors (Thai *et al.*, 1998; Hashizume *et al.*, 2001; Ghimenti *et al.*, 2002; Ishitobi *et al.*, 2003). In contrast, BRCA1 mutations have not been found in uterine cancers, supporting a BRCA1-independent role of BARD1 in tumor suppression by apoptosis induction (Irminger-Finger and Leung, 2002).

To further elucidate the mechanism by which BARD1 induces apoptosis, we investigated its possible function in the regulation of p53 phosphorylation required for apoptosis (Lakin and Jackson, 1999).

Here we report that BARD1 is involved in p53^{ser15} phosphorylation and BARD1 overexpression is sufficient for this phosphorylation. We identified a cancer cell line which is resistant to apoptosis and deficient in p53^{ser15} phosphorylation in response to genotoxic stress. Indeed, BARD1 is mutated and deleted in this cell line and exogenous expression can restore p53^{ser15} phosphorylation and apoptosis.

Results

Given the role of BARD1 in p53 stabilization, we investigated its expression in relation to p53 in various cell types. In nonmalignant mammary gland cells, TAC-2, treatment with the apoptosis-inducing drug doxorubicin resulted in the upregulation of BARD1 and accumulation of the p53 protein, as shown previously (Irminger-Finger *et al.*, 2001). Simultaneously to the increase of BARD1 and p53 protein levels upon genotoxic stress, the accumulation of phosphorylated p53^{ser15} was observed when phospho-epitope-specific antibodies against phospho-p53^{ser15} were used on Western blots (Figure 1a). This is consistent with the

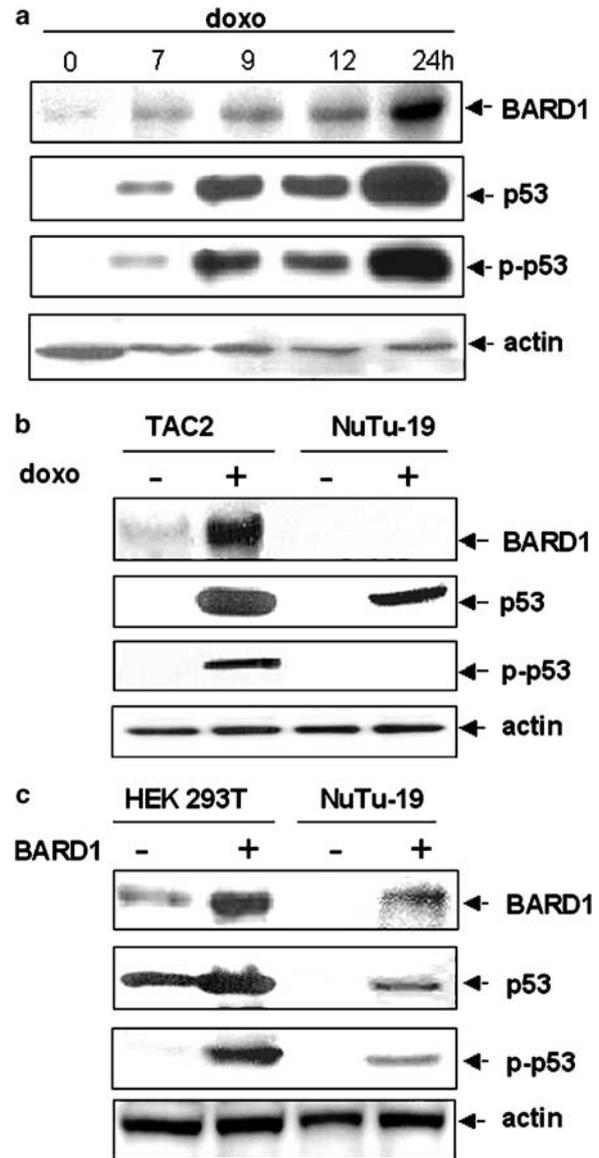


Figure 1 p53 and phospho-p53 expression correlated with BARD1 expression. (a) Doxorubicin treatment of TAC-2 cells induces BARD1, p53 and phospho-p53 (p-p53). Western blots were performed with respective antibodies on cell extracts treated with doxorubicin for various intervals. (b) BARD1 is expressed in TAC-2 but not NuTu-19 cells after treatment with doxorubicin. Correlated expression of p53 and phospho-p53 is shown. (c) Exogenous expression of BARD1 restores p53 expression and phosphorylation. HEK 293T cells with constitutive expression of p53 and NuTu-19 cells were transduced with lentiviral vector expressing BARD1. The membranes were probed with anti-actin as loading control

reported stabilization of p53 by phosphorylation upon stress induction (Shieh *et al.*, 1997). Phosphorylation of serines 15, 20, and 37 is essential for p53 function during apoptosis (Shieh *et al.*, 1997; Tibbetts *et al.*, 1999). Since serine 15, but not serines 20 and 37, is conserved between human, mouse, and rat p53, we determined whether this site was implicated in the resistance to apoptosis in cancer cells.

We first compared the effect of doxorubicin treatment applied to TAC-2 cells and to highly malignant rat ovarian cancer cells, NuTu-19 (Rose *et al.*, 1996). NuTu-19 lack the full-length BARD1 protein and showed only a slight upregulation of p53, as compared to TAC-2 cells (Figure 1b). Most importantly, no trace of phospho-p53 could be detected in doxorubicin-treated NuTu-19 cells, while phospho-p53 was readily detectable in TAC-2 cells.

Since overexpression of BARD1 by itself can lead to p53 stabilization and apoptosis (Irminger-Finger *et al.*, 2001), we tested the effect of BARD1 expression on p53 phosphorylation. HEK 293T cells were used and compared to malignant NuTu-19 cells, since both cell types showed similar transduction efficiency with lentiviral vectors. Transduction of HEK 293T and NuTu-19 cells with wild-type BARD1, under the control of the EF1-alpha promoter, resulted in increased expression of BARD1 in HEK 293T cells and expression of exogenous BARD1 in NuTu-19 cells. An increase of total p53 in HEK 293T cells, and less pronounced in NuTu-19 cells, was observed. Phosphorylation of p53 p53^{ser15} was observed in HEK 293T cells and NuTu-19 cells after exogenous expression of BARD1 (Figure 1c).

These data indicate that upregulation of BARD1 protein is sufficient for p53 upregulation and phosphorylation, suggesting that the function of BARD1 in p53 stabilization involves phosphorylation of p53 at the key residue serine-15 and that increased BARD1 protein levels can induce this post-translational modification.

To investigate whether BARD1 and p53 or phospho-p53 physically interact, we used HEK 293T cells transfected with a cDNA expression vector of a BARD1-EGFP fusion construct expressed from the CMV promoter (Jefford *et al.*, 2004). Immunofluorescence microscopy was performed to visualize BARD1-EGFP, p53, and phospho-p53^{ser15} with specific antibodies. When antibodies recognizing total p53 were used, we observed that all cells expressed p53 independently of BARD1-EGFP expression, whereas some accumulation of p53 in nuclear dots was correlated with BARD1-EGFP expression (Figure 2a). Using p53 antibodies specific for phospho-serine-15, no staining was observed in untransfected HEK 293T cells, while phospho-p53 was expressed in BARD1-EGFP-positive cells, and BARD1-EGFP and phospho-p53^{ser15} co-localized in nuclear dots (Figure 2a). Comparison of p53 and phospho-p53^{ser15} in nontransfected cells and in

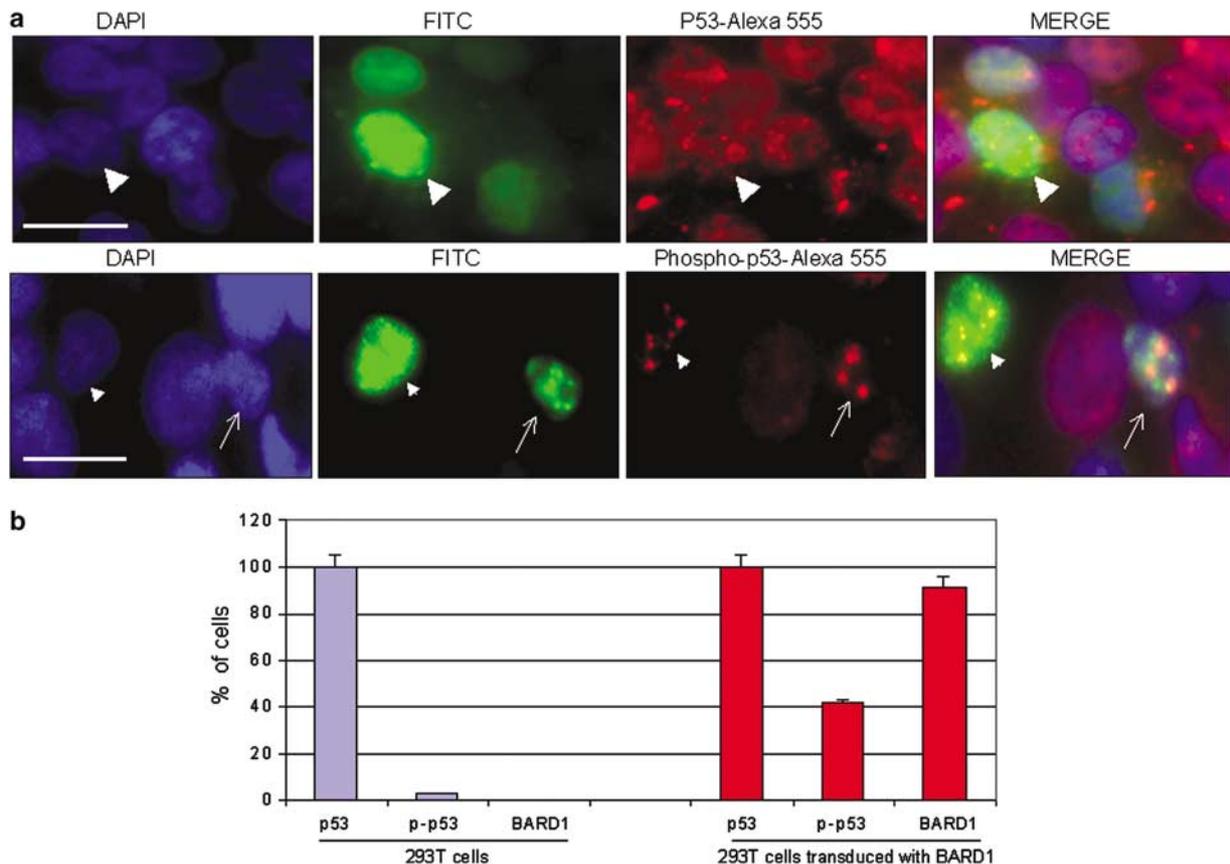


Figure 2 BARD1 co-localizes with phospho-p53. (a) HEK293T cells nontransfected or transfected with BARD1-EGFP expression vector were used for immunofluorescence microscopy. DAPI staining, BARD1-EGFP fluorescence, anti-p53, and anti-phospho-p53 staining are shown. Arrowheads mark cells with high BARD1-EGFP content, arrows mark those with moderated BARD1-EGFP expression. Merge shows colocalization (arrow) of BARD1-EGFP and phospho-p53. (b) The histogram demonstrates BARD1, p53, and phospho-p53 expression in nontransduced and BARD1-transduced cells, derived from three independent transduction experiments

BARD1-EGFP-expressing cells clearly shows that phospho-p53 is only present in BARD1-EGFP expressing cells, and the amount of phospho-p53 labeling is correlated with the level of BARD1-EGFP expression. The quantitative analysis of BARD1-EGFP and phospho-p53^{ser15} expression shows similar percentage of BARD1- and phospho-p53^{ser15}-positive cells (Figure 2b). These results are consistent with the observation that overexpression of BARD1 can induce p53 phosphorylation (Figure 1c).

We have previously shown that BARD1 binds to p53 in co-immunoprecipitation assays (Irminger-Finger *et al.*, 2001). To test BARD1 binding to phospho-p53, immunoprecipitation experiments were performed with cell extracts from HEK 293T, TAC-2, and NuTu-19

cells, using a C-terminal BARD1 antibody (Figure 3a). To obtain increased protein levels of BARD1 and p53, HEK 293T and NuTu-19 cells were transduced with BARD1-expressing lentiviral vector, and TAC-2 cells were treated with doxorubicin. To quantify the transduction efficiency, control transductions were performed using a GFP-expressing lentiviral vector. As shown previously (Irminger-Finger *et al.*, 2001), the increase of BARD1 was associated with the increase of p53 expression as compared to control transduction. Moreover, p53 and phospho p53^{ser15} levels were elevated in doxorubicin-treated TAC-2 cells and in BARD1-transduced HEK 293T and NuTu-19 cells. BARD1 antibodies co-immunoprecipitated p53 and phospho p53^{ser15} in all cell types. Phospho-p53^{ser15} was co-

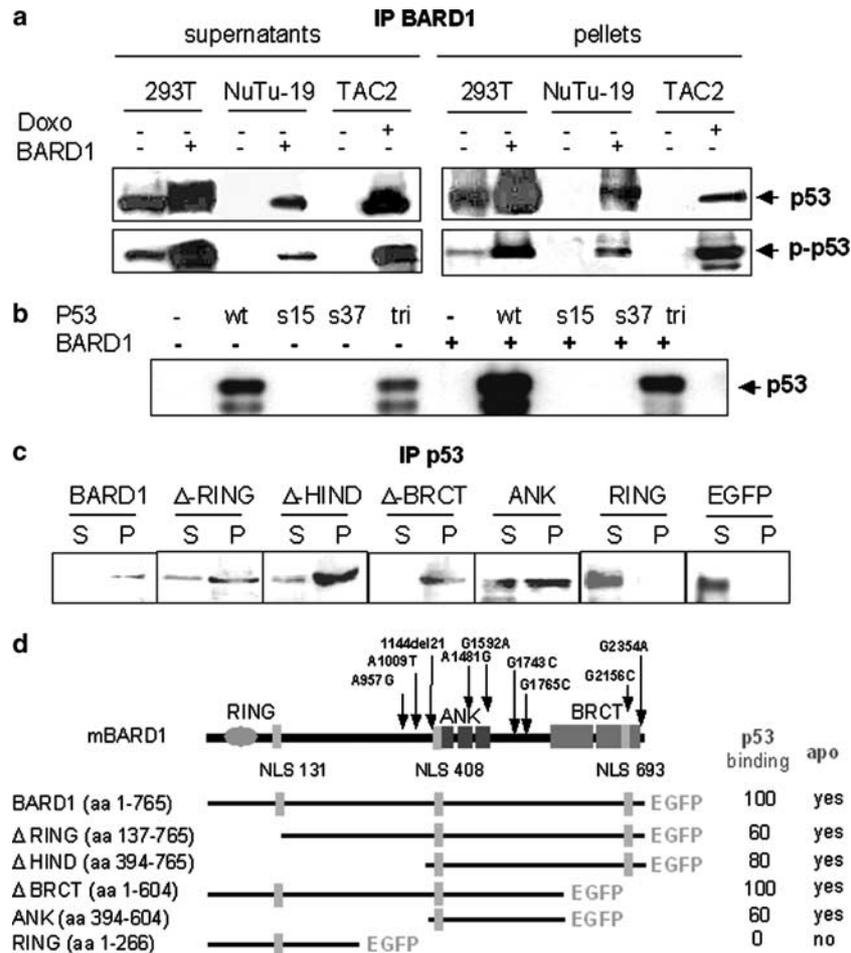


Figure 3 BARD1 interacts with p53 and with phospho-p53. (a) Co-immunoprecipitations were performed with HEK 293T, NuTu-19, and TAC-2 cells, using anti-BARD1 antibody C-20. TAC-2 cells, with or without doxorubicin treatment, and HEK293T and NuTu-19, nontransduced or transduced with BARD1 expression vector, were compared. Western blots were probed with anti-p53 antibodies or anti-phospho epitope serine-15 antibodies. The left panel shows control immunoprecipitation supernatants, the right panel shows pellets. (b) BARD1 stabilizes wild-type (wt) and triple mutant 33-81-315 (tri), but not p53 mutants of ser15 or ser37. Prostate cancer cells PC3, deficient of p53 (Honda *et al.*, 2002), were transfected (+) or not transfected (-) with BARD1 and cotransfected with p53 or p53 mutants. Western blots of cell extracts were probed with anti-p53 antibodies. (c) Region sufficient for p53 binding includes ANK and the region between ANK and BRCT. EGFP-tagged BARD1 and deletion constructs, and EGFP alone, were transiently transfected. Immunoprecipitation was performed with anti-p53 antibodies and Western blots were probed with anti-GFP antibodies. The supernatant (S) and pellet (P) fractions show the fractions of the respective fusion proteins. (d) Schematic diagram of BARD1 protein sequence and deletion-bearing fusion constructs is presented. Percentage of p53 binding, determined by gel intensity scan, and capacity of apoptosis induction reported previously, (Jefford *et al.*, 2004), are indicated. Arrows above the protein scheme indicate relative positions of tumor-associated mutations (Thai *et al.*, 1998; Ghimentu *et al.*, 2002; Karppinen *et al.*, 2004)

immunoprecipitated with BARD1 as efficiently as p53, albeit the amounts of p53 and phospho-p53^{ser-15} were reduced in NuTu-19 as compared to TAC-2 and HEK 293T cells. These results indicate that BARD1 interacts with p53 and with phospho-p53^{ser-15} in different cell types and cells from different species.

To further investigate the specificity of BARD1-dependent p53^{ser15} phosphorylation, we tested the mutated forms of p53. Cotransfection assays were performed in p53-deficient human prostate cancer cells PC3 (Honda *et al.*, 2002). No p53 can be detected in PC3 cells without exogenous p53 expression, also ser15 and ser37 mutants do not express detectable amounts of protein after transfection. Triple mutant 33-81-315, however, is stable (Zacchi *et al.*, 2002). BARD1 cotransfection leads to an increase of wild-type p53 and of the triple mutant, but had no effect on p53 mutated at ser15 and ser37 (Figure 3b).

The interaction of BARD1 and p53 was also tested with BARD1-EGFP fusion constructs, using antibodies against p53 to co-immunoprecipitate BARD1-EGFP, which was assayed with anti-EGFP antibodies on Western blots (Figure 3c). To determine the specific region of BARD1 required for this interaction, BARD1-EGFP deletion constructs were used in transfection assays followed by immunoprecipitations with anti-p53 antibodies. Interestingly, all deletion constructs co-immunoprecipitated with anti-p53 except RING-EGFP, which represents the amino-terminal 89 amino acids of BARD1 (Figure 3c, d). The minimal region required for binding to p53 was deletion construct ANK-EGFP, which was previously identified as the minimal region sufficient for apoptosis induction (Jefford *et al.*, 2004). This finding suggests that the molecular pathway of BARD1 signaling towards apoptosis is based on its role in p53 stabilization by phosphorylation.

Since p53 phosphorylation on serine 15 can be induced by overexpression of BARD1, it was tempting to speculate that BARD1 might act as a mediator between p53 and its specific kinase. Phosphorylation of p53^{ser-15} and p53^{ser-37} is required for p53 stabilization and for p53-dependent response to genotoxic stress (Shieh *et al.*, 1997; Tibbetts *et al.*, 1999; Jack *et al.*, 2004). A role of BARD1-*BRCA1* complexes in phosphorylation of p53^{ser-15} in an ATM/ATR pathway has been described recently (Fabbro *et al.*, 2004). We suspected that DNA-PK kinase might be responsible for p53^{ser-15} phosphorylation in a BARD1-dependent pathway, since DNA-PK can phosphorylate p53^{ser-15} upon genotoxic stress (Woo *et al.*, 1998, 2002) and DNA-PK and Ku-70, the active subunit of DNA-PK, are upregulated in ATM/ATR-dependent stress response (Brown *et al.*, 2000; Shangary *et al.*, 2000). Most recently, it was found that Ku-70 mutation partially suppresses the homologous-repair defects of BARD1 disruption (Stark *et al.*, 2004), suggesting that both act in a common pathway.

We performed co-immunoprecipitation assays with anti-p53 antibodies, anti-Ku-70, the active subunit of DNA-PK, or anti-BARD1 antibodies to investigate the interactions between DNA-PK, p53, and BARD1 (Figure 4a). Immunoprecipitation assays, when per-

formed on lysates from HEK 293T cells, or HEK 293T cells transduced with BARD1-EGFP, revealed that BARD1 and p53 were co-immunoprecipitated with Ku-70. When anti-p53 antibodies were used to precipitate p53 or interacting proteins, p53 immunoprecipitation was nearly 100%, as was BARD1 co-immunoprecipitation with anti-Ku-70 antibodies (Figure 4a).

We were interested whether NuTu-19 cells expressed Ku-70 and immunoprecipitation experiments were performed with untransfected and BARD1-transfected NuTu-19 cells (Figure 4b). Only exogenous BARD1 could accumulate detectable amounts of Ku-70 in immunoprecipitation. No precipitation was observed when BARD1 antibodies were omitted.

As further evidence for the implication of DNA-PK, a member of the phosphoinositide 3-kinase related kinase (PIKK) family, in phosphorylation of p53 and interaction with BARD1, we treated cells with caffeine and doxorubicin. Caffeine is a well-known inhibitor of PIKK kinase activity and as a result can effectively inhibit the phosphorylation of p53^{ser-15}. Doxorubicin on the other hand induces genotoxic stress on cells, consequently resulting in phosphorylation of p53 on the same residues inhibited by caffeine. After treatment of HEK 293T cells with 50mM caffeine alone, we observed a slight elevation in p53 levels as compared to untreated cells; however, there was no change in the phospho-p53 levels (Figure 4c). Doxorubicin treatment induces the expression of both p53 and BARD1, as described previously (Irmingier-Finger *et al.*, 2001; Jefford *et al.*, 2004). Since HEK 293T cells have constitutive p53 expression, no significant changes in p53 levels with or without genotoxic stress were observed. We did not observe substantial increase in phospho-p53 protein levels co-immunoprecipitating with BARD1 when treated with caffeine, but when treated with doxorubicin. The combined treatment with caffeine and doxorubicin resulted in decreased phospho-p53 levels in both the supernatant and pellet fractions of the BARD1 immunocomplexes (Figure 4c). This result provides further evidence that p53 is phosphorylated by a PIKK family member and that this reaction might be catalysed by the presence of BARD1, since BARD1 immunocomplexes contain both phosphorylated and unphosphorylated species of p53. The same immunoprecipitation extracts were probed with anti-ATM antibodies and no binding of ATM to BARD1 could be observed (Figure 4c), thus supporting that DNA-PK rather than ATM is responsible for BARD1-dependent p53^{ser-15} phosphorylation.

While p53 phosphorylation was induced in several cell types by exogenous expression of BARD1, this effect was most striking in malignant ovarian cancer cells NuTu-19. NuTu-19 cells were generated by continuous *in vitro* culture of ovarian epithelial cells leading to spontaneous malignant transformation (Rose *et al.*, 1996). These cells lack full-length BARD1 protein (Figure 1b, c). Cloning and sequencing of the BARD1 mRNAs expressed in NuTu-19 cells revealed that none of the cDNAs encoded a full-length BARD1 gene (Figure 5a, c, d). The cDNA corresponding to the 2.2 kb

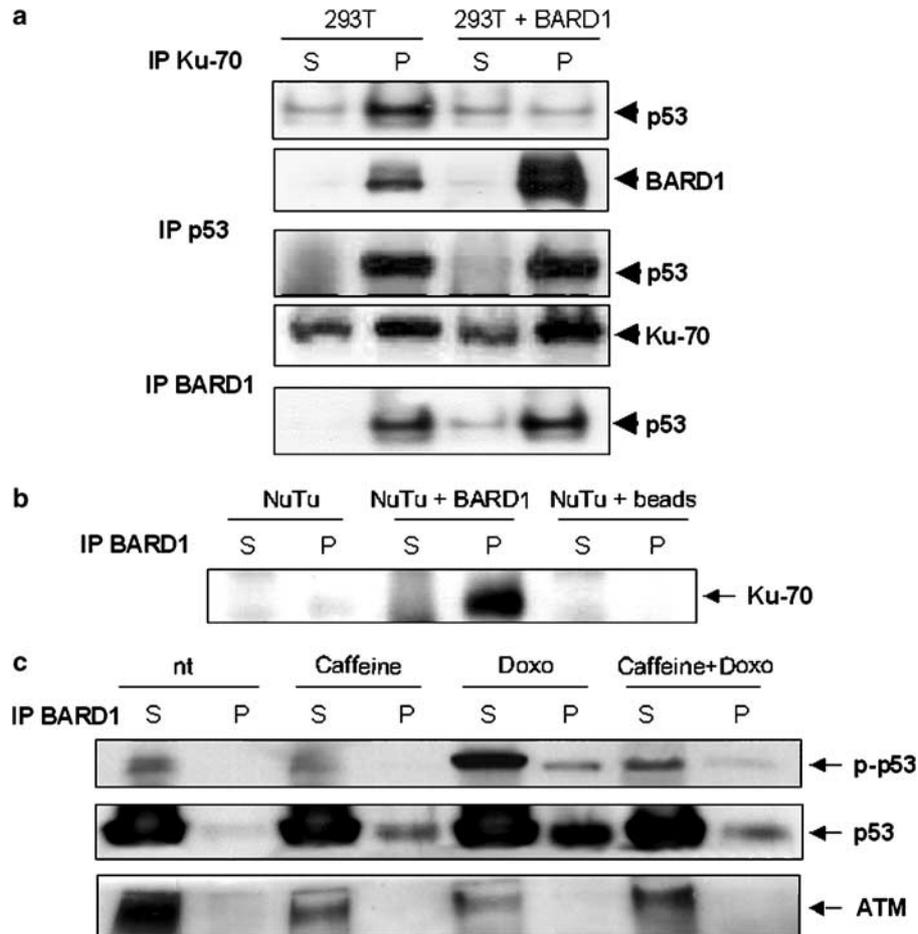


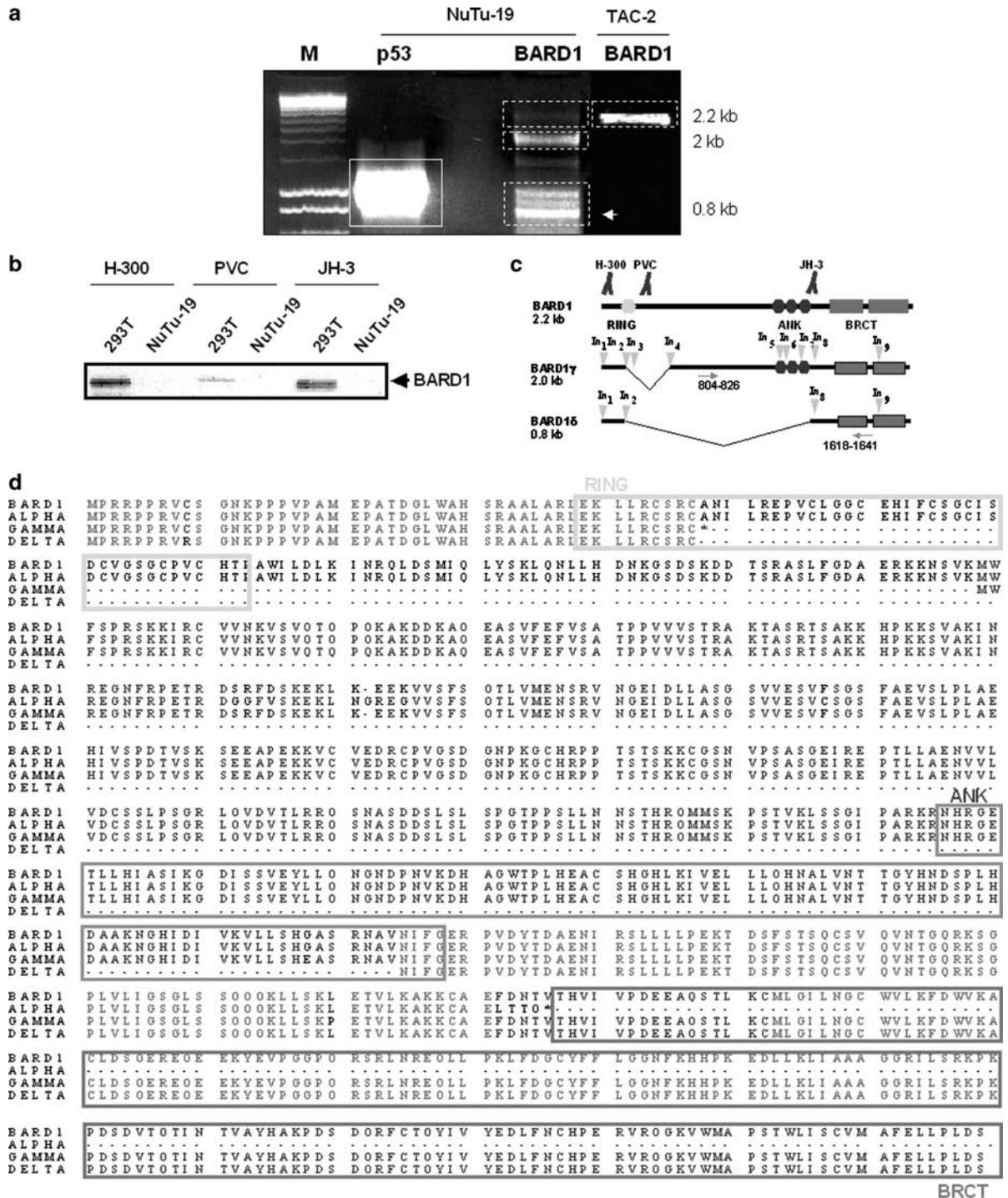
Figure 4 BARD1 interaction with p53 and DNA-PK subunit Ku-70. (a) HEK293T (293T) nontransduced or transduced with BARD1 were used for immunoprecipitation assays with Ku-70, p53, and BARD1 antibodies. Supernatants (S) and pellet (P) fractions are shown. (b) NuTu-19 cells nontransduced or transduced with BARD1 were used to demonstrate BARD1-Ku-70 interaction. Little co-precipitation is observed in the absence of exogenous BARD1. Control experiment without antibodies shows no precipitation (beads). (c) P53 phosphorylation is induced (doxo) or inhibited (caffeine). Immunoprecipitation performed with BARD1 antibody C-20 to show interaction between BARD1, phospho-p53, p53, and ATM. Supernatants (S) and pellet (P) fractions are shown

wild-type BARD1 transcript contained point mutations leading to a premature translation stop at position 1786 between the ankyrin repeats (ANK) and the BRCT domains (Figure 5a). A smaller 2 kb fragment was due to deletion of exons 2 and 3. This mRNA species, herein designated BARD1 γ , was similar to the testis-specific mRNA BARD1 β (Feki *et al.*, 2004). This finding was corroborated by Western blots performed with three different previously characterized anti-BARD1 antibodies (Feki *et al.*, 2004), which showed that full-length BARD1 protein is absent in NuTu-19 cells (Figure 5b). HEK 293T expressed a 94-kDa protein, detectable with N-terminal antibodies H-300 and PVC, directed against the region adjacent to the RING finger, and JH3, directed against the regions between ANK and BRCT. None of the antibodies tested recognized a protein fragment corresponding to the molecular weight of full-length BARD1 in NuTu-19 cell extracts. Consistently, the most abundant transcript of BARD1 in NuTu-19 cells was an 800 bp RT-PCR product, a novel splice variant, hitherto named BARD1 δ . BARD1 δ lacks exons 2–7, which include regions coding for the RING finger

and the ankyrin repeats (Figure 5c, d). Therefore, NuTu-19 cells do not express full-length BARD1 molecules and none of the BARD1 transcripts encode ANK repeats and the region between ANK and BRCT.

Interestingly, nucleotides 1618–1641 represent an inverted repeat of region 804–826, which could act as antisense to abrogate the translation of BARD1, but not BARD1 δ transcripts. Mutations and deletions in the BARD1 coding region were in strong contrast to the status of p53. Cloning and sequencing of the p53 cDNA from NuTu-19 cells revealed two polymorphisms, S66P and L192F, within the less conserved domains of p53, but no mutations that predict structural changes of the encoded protein.

We had evaluated the apoptotic activity of NuTu-19 cells after treatment with UV or doxorubicin. NuTu-19 cells show little response to apoptosis-inducing drugs as measured by FACS and TUNEL assays. Doxorubicin treatment resulted in no significant increase of apoptotic cells as observed after TUNEL or 7AAD staining and flow cytometry analysis (Figure 6a). However, when we overexpressed wild-type BARD1 by



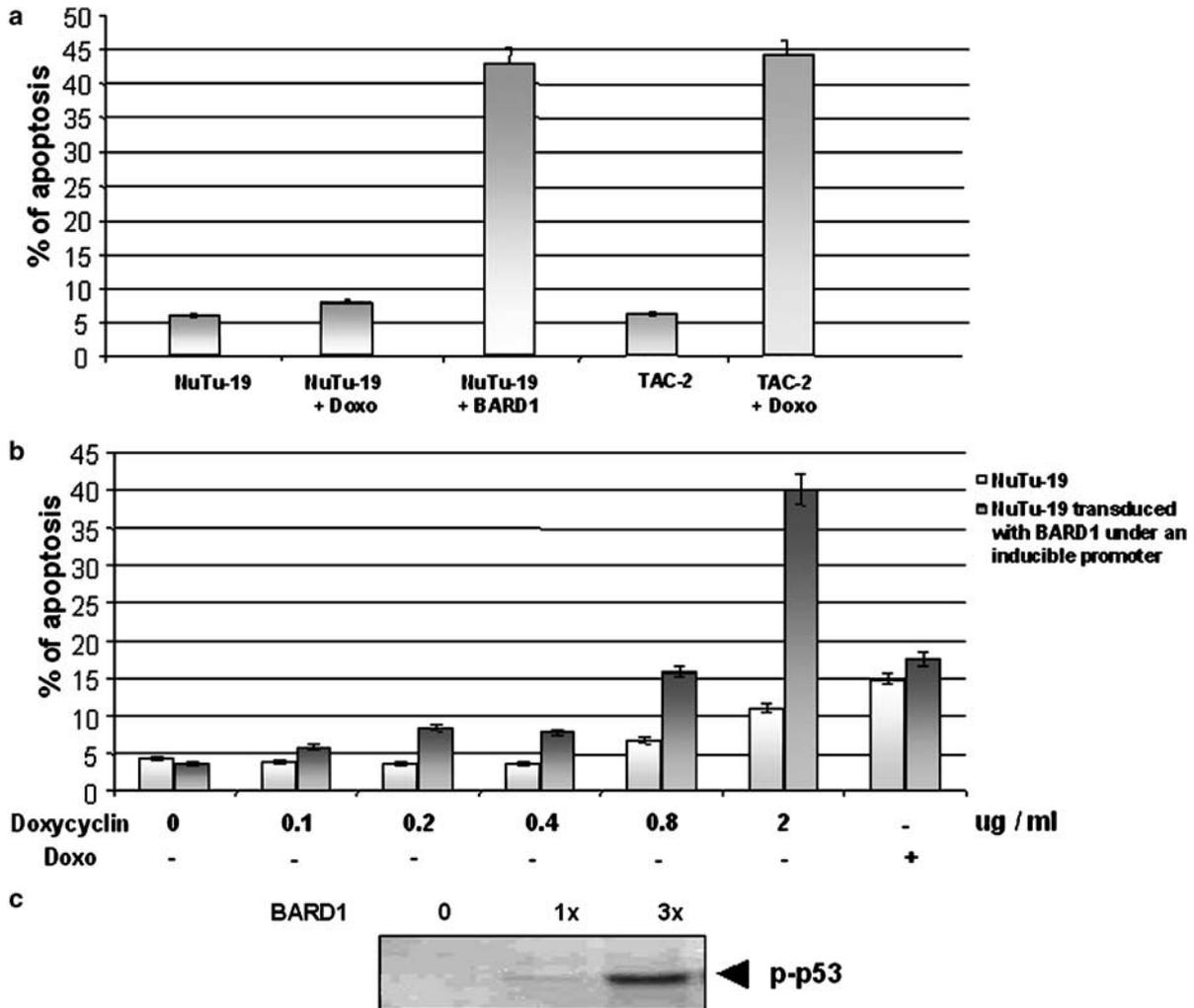


Figure 6 Expression of BARD1 reverses apoptosis resistance in NuTu-19 cells. **(a)** TUNEL assay on NuTu-19 ovarian cancer cells or nonmalignant TAC-2 cells with or without treatment with doxorubicin (doxo) or transfection of BARD1. Percentage of TUNEL-positive cells was quantified by flow cytometry. **(b)** NuTu-19 cells and NuTu-19 transduced with lentiviral vector containing BARD1 were monitored for apoptosis. Cells were incubated with or without doxycyclin to induce BARD1 expression, or with doxorubicin (doxo). **(c)** Dose response of BARD1-induced phosphorylation is observed after transduction of NuTu-19 cells with BARD1. Nontransduced (0), transduced (1 \times), or transduced with threefold (3 \times) concentration of BARD1 expression viral vector, cells were probed on Western blot with anti-phospho-ser15 p53-specific antibody

doxycyclin levels clearly resulted in apoptosis in a dose-dependent manner (Figure 6b). However, BARD1 expression and apoptosis in response to doxorubicin in nontransduced NuTu-19 cells was comparable to background levels of apoptosis induction in BARD1-transduced cells without induction of gene expression by doxycyclin.

Similarly, we tested p53-phosphorylation on p53^{ser15} as dose response to BARD1 expression levels in NuTu-19 cells. Nontransduced cells and cells transduced with increasing amounts of BARD1 expression vectors were compared on Western blots. Phospho-p53^{ser15} increase was observed in a dose-dependent manner on Western blots probed with antibodies against phospho-p53^{ser15} (Figure 6c).

This experiment proved that expression of wild-type BARD1 could reverse apoptosis resistance in cells lacking full-length BARD1. In nonmalignant cells with

endogenous wild-type BARD1, overexpression of exogenous BARD1 is sufficient to stabilize p53 and to induce p53^{ser15} phosphorylation. In BARD1-deficient NuTu-19 cells, phosphorylation of p53 cannot be induced by apoptosis-inducing drugs, but by the exogenous expression of wild BARD1, without other stress signals, suggesting that BARD1 facilitates the phosphorylation of p53 by binding to p53 and Ku-70, the subunit of DNA-PK, and that this is sufficient and required for apoptosis induction.

Discussion

In the present study we demonstrate that BARD1 plays a critical role in phosphorylation of p53, which is required for its apoptotic function. Several kinases

involved in p53 activation have been reported (Lu *et al.*, 1997; Shieh *et al.*, 1997, 1999; Tibbetts *et al.*, 1999; Hirao *et al.*, 2000) and ATM/ATR-dependent phosphorylation of p53^{ser-15}, implicating BRCA1–BARD1 complexes, has been described very recently (Fabbro *et al.*, 2004).

It was shown before that BRCT domains, a protein motif also present in BARD1 and BRCA1, have phospho-epitope-binding capacities (Rodriguez *et al.*, 2003). A phospho-epitope-binding function was reported specifically for BRCT domains of BRCA1. BRCT domains are present in many other repair proteins and seem to facilitate physical interactions among proteins involved in the cellular response to DNA damage check point control and DNA repair (Bork *et al.*, 1997; Callebaut and Moron, 1997). It is speculated that BRCT domains are important modules for tumor suppressor functions, supported by cancer-associated mutations within the BRCT domain of BRCA1 (Williams *et al.*, 2001).

BARD1 contains tandem BRCT motifs, it has tumor suppressor functions (Irminger-Finger and Leung, 2002), and it binds and stabilizes p53 (Irminger-Finger *et al.*, 2001) which is suggestive of a role in a pathway of p53 phosphorylation. Here we show that, indeed, BARD1 binds to p53 and phospho-p53 and, secondly, BARD1 interacts with the DNA-PK subunit, Ku-70, but not ATM. The region sufficient for p53 interaction and apoptosis induction comprises ANK, the region between ANK and BRCT, and part of the BRCT domains. It is possible that the BRCT domains are binding to the kinase and that its target is binding to the adjacent region.

Ku-70 is abundantly expressed in HEK 293T cells, as is p53, as compared to BARD1. Nevertheless, overexpression of BARD1, without an additional stimulus through DNA damage, causes p53 phosphorylation in HEK 293T cells and NuTu-19 cells, suggesting that BARD1 is an essential and limiting partner for the formation of the p53–DNA–PK complex. Consistent with this view, BARD1 co-localizes with phospho-p53 in distinct nuclear dots (Figure 5b). Since overexpression of BARD1 is sufficient for p53^{ser15} phosphorylation, binding of BARD1 to the kinase and its target catalyses p53 phosphorylation.

Supporting this view, several cancer-associated missense mutations (Thai *et al.*, 1998; Ghimenti *et al.*, 2002; Ishitobi *et al.*, 2003), map to the region defined sufficient for p53 binding and apoptosis induction, and it is deleted or mutated in the NuTu-19 cells, described here.

It is likely that BARD1 deficiencies are frequent in cancer cells that have wild-type p53 but are resistant to apoptosis, and significant changes in BARD1 expression have been found associated with malignancies (Pomeroy *et al.*, 2002; Iyengar *et al.*, 2003; Schafer *et al.*, 2003; Zuco *et al.*, 2003). The NuTu-19 ovarian cancer cells, to some extent, are a model of ovarian cancer, which suggests that BARD1 is a key target for mutations leading to carcinogenesis. NuTu-19 cells are derived from a spontaneous transformation of continuously cultured ovarian epithelial cells (Rose *et al.*, 1996),

suggesting that few genetic modifications led to the phenotype of resistance to undergo apoptosis. This process mimics the *in vivo* situation, as it has been hypothesized that proliferation associated with ovarian repair contributes to the risk of ovarian cancer in humans (Fathalla, 1972). We speculated and confirmed here that one of the critical mutations for malignant transformation involves the tumor suppressor BARD1.

Indeed, rat ovarian cancer cells, NuTu-19, have applied several strategies to eliminate the expression of functional BARD1: mutations leading to a truncated form of the protein, differential splicing leading to the deletion of essential parts of the protein, and possibly repression of the partially active forms of the protein by expression of a short inverse repeat sequence that could function as antisense RNA in transcriptional repression of wild-type forms of BARD1. These mechanisms of inhibition of BARD1 function are consistent with the hypothesis that BARD1 is an essential tumor suppressor, critical for apoptosis induction upon damage, by stabilizing p53 (Irminger-Finger *et al.*, 2001). BARD1 binding to p53 could also influence p53 stability by inhibiting its ubiquitination and/or its binding to MDM2 (Meek, 1994; Honda *et al.*, 1997; Milczarek *et al.*, 1997).

Consistently, no phosphorylation of p53 is observed in NuTu-19 cells in the absence of exogenous expression of wild-type BARD1. Co-immunoprecipitation experiments suggest that BARD1 binds both the kinase and its respective target p53 and catalyses its phosphorylation. This could be explained mechanistically by a higher affinity between BARD1 and Ku-70 and between BARD1 and p53 than between p53 and Ku-70. Our data support this view, since co-immunoprecipitations of BARD1 and p53 or BARD1 and Ku-70 are more efficient than co-immunoprecipitations of p53 and Ku-70. In addition, BARD1 protein levels are considerably lower in the cell than either p53 or Ku-70, therefore BARD1 can be regarded as the critical and limiting factor in the formation of the p53/Ku-70 complexes. Furthermore, the overexpression of BARD1 alone without induction of the DNA damage pathway is sufficient for p53 phosphorylation and supports the view that BARD1 catalyses p53 phosphorylation by DNA-PK.

Materials and methods

Cell cultures

The HEK 293T cell line, as well as HeLa cells, were obtained from the ATCC. NuTu-19 cells (Rose *et al.*, 1996) were a generous gift from Dr Attila Major, Geneva, PC3, human prostate cancer cells, were obtained from Dr Timothy MacDonnell, Houston. All cell lines were cultured in 10 cm plastic tissue culture dishes (NUNC, Roskilde, Denmark) and incubated at 37°C in humidified air containing 5% CO₂. Adherent subcultures were detached from culture dishes using a solution of Trypsin/EDTA in HBSS without Ca²⁺ or Mg²⁺. Subconfluent cell cultures were passaged regularly at 1:10 dilution approximately twice a week. All cell culture media, solutions, buffers, and antibiotics were purchased from

GIBCO (Invitrogen AG, Basel, Switzerland). HEK 293T cells were pretreated for 1 h with 50 mM caffeine, after which they were either treated with doxorubicin (50 µg/ml) or left untreated as described previously (Irminger-Finger *et al.*, 1998).

Western blotting

For Western blots, protein extracts from cell cultures were prepared in standard lysis buffer (150 mM NaCl, 0.1% SDS, 50 mM Tris (pH 8.0)). Equal concentrations of all whole cell extracts per gel were loaded onto SDS-PAA gels according to Laemmli's protocol and were transferred to polyvinylidene fluoride (PVDF) membranes. Filters were blocked for 1 h in PBS containing 5% non-fat milk and then incubated at room temperature for 2 h with a polyclonal BARD1 (Irminger-Finger *et al.*, 1998), p53 and P-p53 (Santa Cruz) antibodies. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody and the resulting complexes were visualized by an enhanced chemiluminescence (ECL) system (Boehringer).

Immunoprecipitation

Immunoprecipitation was performed by homogenizing cell samples in 100 µl (100–150 µg total protein) RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% NaDOC, 0.1 SDS, 50 mM Tris (pH 8.0), and 2 mM EDTA (pH 8.0)). Cells extracts were incubated with p53 antibody (FL-393 Santa Cruz), or GFP antibody (Torrey Pines Biolabs, Inc) in a 1:200 dilution overnight, followed by incubation with sepharose-bound protein G for 3 h. Extracts were centrifuged, and pellets were washed twice with RIPA buffer and re-suspended in 100 µl RIPA buffer. In all, 25 µl of supernatant and pellet fractions was analysed by Western blotting. Total protein content in supernatant and pellet fractions was compared by fast green staining after protein transfer and was 90–10% at least. Antibody reactive bands were visualized, scanned, and quantified using Imagequant.

Antibodies

The anti-BARD1 (H300), anti-p53 (FL-393), and anti-phospho p53 ser15 antibodies (Cell Signaling 5284) were purchased from Santa Cruz. Anti-GFP (TP-401) was purchased from Chemokine (Torrey Pines Biolabs, Houston, TX, USA). WFS is a previously described anti-BARD1 antibody (Gautier *et al.*, 2000). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz and were compatible with Cruz Molecular Weight Markers (Santa Cruz, CA, USA).

RT-PCR

Total RNA was purified from NuTu-19 cells using Qiagen reagent. RNA was reverse transcribed using oligo-dT and superscript II (Life Technologies). RT products were subjected

to PCR analysis using specific primers for rat BARD1 cDNA (covering BARD1 coding regions) and rat p53 (covering p53 coding regions). PCR cycles were: 94°C for 15 min, 94°C for 1 min, 56°C for 1 min, 72°C for 2 min 30 s for BARD1 and 1 min 30 s for p53 (35 ×), then 72°C for 10 min. Equal volumes of PCR products were analysed on 1% agarose gel.

Lentiviral vector production and transduction

The packaging constructs used in this study were the pCMVΔR8.92 and the pCMVΔR8.93 plasmids described previously (Dull *et al.*, 1998). The vesicular stomatitis virus G (VSV-G) envelop was the pM2DG plasmid (Zufferey *et al.*, 1997). The BARD1 coding sequence was cloned into the carrier pLOXEW plasmid (Zufferey *et al.*, 1997). The viral particles were produced using the usual transient transfection method (Zufferey *et al.*, 1997). The 293T cells (2.8×10^6 cells in 10-cm tissue culture dishes) were co-transfected with either 10 µg of pLOXEW-BARD1 ires-GFP, 3.75 µg of pCMVΔR8.92, 3.75 µg of pCMVΔR8.93, and 2.5 µg of pM2DG, or 10 µg of pHR'-CMV-BARD1, 3.75 µg of pCMVΔR8.92, 3.75 µg of pCMVΔR8.93, and 2.5 µg of pM2DG.

After an overnight incubation in the presence of the precipitate, the culture medium (10 ml) was changed. Two days later, the supernatant was harvested, filtered through 0.45-µm pore-sized polyethersulfone membrane and subsequently 1 ml of each was added to NuTu-19 target cells in six-well plates (10^4 cells/well). Vector particles were left on the cells for 3 days. Transduction efficiency was monitored using a GFP-expressing vector.

Plasmid constructions and transfection

Different BARD1 mutants fused with GFP, was described previously, were used. Briefly, the full-length mouse BARD1 cDNA (Irminger-Finger *et al.*, 1998) was used to generate the deletion constructs in pcDNA3 behind a CMV promoter. The plasmid pEGFP-N1 (enhanced green fluorescent protein) was purchased from Clontech and was used to generate either the full-length BARD1-EGFP or BARD1 deletion-bearing mutants fused in-frame with the EGFP tag. The deletion-bearing constructs were the same as described (Jefford *et al.*, 2004). HEK293T cells were seeded in 10 cm petri dishes and transfected with 2 µg of DNA using the Effectene reagents from Qiagen. Cells were harvested 48 h post-transfection.

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