Chapter 9
Cell Cycle Checkpoints and Senescence

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Abstract Cellular senescence, an outcome of finite proliferative, limited repair and defence capacity of normal cells, is a widely accepted in vitro model for ageing studies. In a sharp contrast to cancer cells, it is firmly regulated by cell cycle checkpoints that ensure evasion of stressed and genetically modified cells, limiting their expansion and serve as an innate check to carcinogenesis. Tumour suppressors and their regulatory proteins play key roles, both as molecular sensors and regulators in this process. Aim of the present chapter is to sketch a brief understanding on how cellular senescence is regulated by major tumour suppressor and cell cycle checkpoint proteins as well as by some emerging molecules.

Keywords Stress • Cellular senescence • Cell cycle • Tumour suppressors • DNA damage signalling • Checkpoint proteins

9.1 Cellular Senescence

Senescence (derived from Latin: senescere, meaning "to grow old") is a property of all living organisms. It is a process that leads to functional decline, and an increase in vulnerability to a spectrum of diseases eventually leading to death of an organism.

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From evolutionary perspectives, decline in reproductive capacity and increase in mortality rate with age, set up conditions which eradicate exhaustive competition for resources and favour continual survival of a population versus the individuals (Kirkwood 2008). Senescence is considered to be an outcome of limited maintenance and repair capacity of living organisms leading to accumulation of damage that occurs as a consequence of gene activities or functions essential for survival. In other words, it is an indispensable outcome of life; further complicated by the environmental factors without which life cannot exist or function (Kirkwood 2008). A large number of model systems have been used for studying senescence in vitro. It has been established that a cell, the smallest functional unit of life, mimics highly complex organismal ageing phenomenon, and was first demonstrated by its limited capacity to divide despite the availability of sufficient nutrients, growth factors and space (Hayflick 2007; Hayflick and Moorhead 1961). The total number of population doublings (PDs) that normal cell cultures can attain before senescence depends on the cell type and the age of the donor, and not the chronological age of the culture (Hayflick and Moorhead 1961; Maier and Westendorp 2009). The present chapter provides a simple sketch on the current understanding on the role of cell cycle checkpoint proteins in regulation of proliferation in normal and cancer cells.

Senescent cell can be identified in cell culture by virtue of its phenotypic characteristics including increased cell size, flattened and irregular shape, multinucleation and cytoplasmic vacuolation (Mitsui and Schneider 1976; Robbins et al. 1970; Sikora et al. 2011). Young fibroblasts have an organized fusiform appearance in culture. On the other hand, senescent fibroblasts appear flattened, disorganized and are randomly oriented in culture dish. They show fragmented and distorted subcellular structures including nucleus, mitochondria and endoplasmic reticulum, and have a high rate of autophagy that is associated with an increase in lysosomal mass (Gerland et al. 2003; Goligorsky et al. 2009; Kurz et al. 2000). Young cultures are heterogeneous and contain a mixture of dividing, growth-arrested and senescent cells. The proportion of senescent cells increases progressively until the whole culture has entered senescence, a state in which they can remain metabolically active for long periods of time (Cristofalo and Sharf 1973; Smith et al. 1980; Wadhwa et al. 1991). Senescent culture has a lower cell density at confluence than a confluent young culture suggesting that the senescent cells are more sensitive to cell-cell contact inhibition. Although these phenotypes of senescent cells are firmly established, the underlying molecular mechanism(s) remain obscure. A few molecular events have been allied to senescent cell morphology and replicative senescence. Caveolin-1, an integral membrane protein and the principal component of caveolae, was shown to play an important role in senescence-associated morphological changes by regulating focal adhesion kinase activity and actin stress fibre formation in the senescent cells (Cho et al. 2004). Reduction in the levels of caveolin-1 caused resumption of DNA synthesis in senescent cells (Cho et al. 2003) suggesting that it is required for maintaining the state of replicative senescence. Senescence-associated β-galactosidase (SA β-gal) activity was associated with the unusual behaviour of the enzyme β-galactosidase, a lysosomal hydrolase. β-galactosidase is normally active at pH 4, but in senescent cells it becomes active at pH 6. Cells positive for SA β-gal

increase with cell culture passage and age, both in vitro and in vivo, respectively (Bandyopadhyay et al. 2005; Carnero 2013; Dimri and Campisi 1994; Dimri et al. 1995). Early studies showed that the lysosomes increase in number and size in senescent cells. SA β-gal appeared to be a result of increased lysosomal activity at a suboptimal pH, which becomes detectable in senescent cells due to an increase in lysosomal content (Kurz et al. 2000).

Functionally, senescent cells can be distinguished from pre-senescent cells by their increased resistance to apoptotic death, refractoriness to various growth factors and mitogens and increased sensitivity to toxins, antibiotics, irradiation, oxidation and heat shock (Aggarwal et al. 1995; Blake et al. 1991; Fargnoli et al. 1990). Several studies have demonstrated that the rate of protein, DNA and RNA synthesis is reduced in senescent cells and is accompanied by substantial alterations in gene expression involved in processes including cell cycle control, stress response, signal transduction and synthesis of extracellular matrix, mitochondrial, and cytoskeletal proteins (Carnero 2013; Cristofalo et al. 1998; Duncan and Reddel 1997; Falandry et al. 2013; Goldstein 1990; Holliday 1990; Kuilman et al. 2010; Rattan 1996). Some of the most commonly used senescent cell specific biomarkers are: osteonectin, fibronectin, apolipoprotein J, smooth muscle cells 22 (SM22) protein, and type II (1)-pro-collagen (Gonos et al. 1998; Kumazaki et al. 1991). Senescent cells also display an increased activity of metalloproteinases, which degrade the extracellular matrix (Campisi 2000). Such senescent cell specific markers provide hints for elucidating the underlying molecular mechanism(s).

A large number of studies have confirmed that the replicatively senescent cells make a permanent exit from cell cycle and arrest at the G1/S or G2/M boundary representing exhaustion of their division capacity (Campisi 2000; Cheung et al. 2010; Goldstein 1990; Herbig et al. 2004; Marcotte and Wang 2002; Pignolo et al. 1998; Rayess et al. 2012; Stein and Dulic 1995; Vargas et al. 2012). They have been detected in a variety of tissues and in a number of different organisms including mouse, primate and human (Dimri et al. 1995; Jeyapalan et al. 2007; Krishnamurthy et al. 2004; Krtolica and Campisi 2002; Michaloglou et al. 2005; Molofsky et al. 2006; Prieur and Peeper 2008; Satyanarayana et al. 2004; Campisi and Robert 2014; Demaria et al. 2015). In old baboons, over 15 % of dermal fibroblasts showed a senescent phenotype as determined by damaged telomeres, increased $p16^{INK4A}$ expression and an activation of ATM kinase (Jeyapalan et al. 2007). Michaloglou and colleagues showed that the melanocytic naevi (benign skin moles, that may be precursors of malignant melanoma) have increased levels of senescent markers and do not seem to proliferate, yet can persist for many years (Michaloglou et al. 2005). However, evidence regarding the role of senescent cells in vivo ageing and pathologies of old age is only limited. Apparently, in contrast to replicative senescence of cells in vitro, tissue and organismal ageing is multifactorial and is more complex because of the existence of heterogeneous populations of cell types that function and senesce in different ways influenced by individual genetic and environmental factors. Knockout mouse models have been generated to recapitulate human genetic diseases associated with premature ageing and cancer predisposition. Individuals with premature ageing disorders such as

Werner Syndrome, Down Syndrome and Hutchison-Gilford Progeria have a shorter in vitro life span supporting the use of replicative senescence as a valuable model to understand the ageing in vivo (Chun et al. 2011; Mackenzie and MacRae 2011; Raghu et al. 2001; Thweatt and Goldstein 1993; Vaziri et al. 1993).

9.2 Triggers of Senescence

Over the last three decades, there have been several studies to understand the mechanisms responsible for the limited replicative potential of normal human fibroblasts. The most consistent view probably is that the normal biological functions required for life are important triggers of senescence. Although distinguishing between the causes and consequences of senescence may be difficult, some of the most consistent manifestations of cellular senescence appear to be (i) upregulation of specific tumour suppressor activities (Atadja et al. 1995; Brown et al. 1997; Gire 2005; Gire and Wynford-Thomas 1998; Liu et al. 2015), (ii) accumulation of DNA damage response proteins at telomeres due to telomere shortening (d'Adda di Fagagna et al. 2003; Kaul et al. 2012; Mengual Gomez et al. 2014), (iii) an increase in inadequately repaired single stranded (ss) or double stranded (ds) DNA breaks (Campisi and Robert 2014; Klement and Goodarzi 2014; Wang et al. 2015) (iv) increase in intrinsic stress including mitochondrial dysfunction and accumulation of reactive oxygen species (ROS) (Passos and Von Zglinicki 2006; Passos et al. 2006; Burhans and Heintz 2009; Vurusaner et al. 2012; Yan et al. 2014), and (v) increase in secreted growth factors, matrix remodelling enzymes, and inflammatory cytokines that contribute to age-related pathologies including cancers (Campisi 2005a, b; Krtolica et al. 2001; Tchkonia et al. 2013; Velarde et al. 2013).

9.3 Stress Induced Premature Senescence (SIPS)

Any form of sub-cytotoxic stress that can accelerate the appearance of the senescent phenotype in cells is regarded as a trigger of SIPS. Although replicative- and stress-induced senescence initiate from different origins, both processes demonstrate strong similarities with regard to activation of DNA damage response, upregulation of tumour suppressor functions and permanent growth arrest at G1/S or G2/M checkpoints (Halazonetis et al. 2008; Horn and Vousden 2007), and hence both have been used as convenient model systems for understanding the molecular basis of senescence. Sub-lethal stresses such as oxidative stress or gamma irradiation, chromatin remodelling, oncogenic stress, DNA damage and strong mitogenic responses have been shown to result in SIPS (Campisi and Robert 2014; Di Leonardo et al. 1994; Martien and Abbadie 2007; Passos et al. 2006; Saretzki et al. 1998; Suzuki and Boothman 2008; Toussaint et al. 2000). Nuclear and mitochondrial DNA damage (mtDNA) induced by physiological levels of ROS has been shown to

have a significant impact on cellular senescence. Many studies have shown that the telomere shortening is stress dependent and mtDNA damage is closely related to ROS production (Passos et al. 2007). Improvement in mitochondrial function resulted in less telomeric damage and slower telomere shortening. Moreover, telomerase, an enzyme complex that re-elongates shortened telomeres, was shown to protect against oxidative stress, suggesting a strong link between mitochondrial and telomeric DNA damage leading to cellular senescence (Ale-Agha et al. 2014). Examples of oxidative stress causing senescence include treatment with sub-lethal levels of hydrogen peroxide (Chen 2000), UV (Ma et al. 2002; Wlaschek et al. 2003) and interferon-γ (Weyand et al. 2003) that were shown to induce reactive oxygen species and DNA damage (Ale-Agha et al. 2014; Klement and Goodarzi 2014; Passos et al. 2007; Vurusaner et al. 2012). Whereas lifespan of primary human fibroblasts cultured in 3 % O_2 was extended by 20 PDs (Chen et al. 1995; Gelvan et al. 1995) as compared to the ones cultures in 20 % O_2 by Hayflick and Moorhead (1961), the cells cultured in >20 % O_2 displayed reduced growth rate and underwent fewer PDs (Horikoshi et al. 1986; Indran et al. 2010; Passos et al. 2006; Przybylska and Mosieniak 2014; von Zglinicki et al. 1995).

Oncogene-induced premature senescence (OIPS) is a subset of SIPS that occurs in response to excessive mitogenic signals. Inappropriate mitogenic signaling such as inhibition of phosphatidylinositol 3-kinase or constitutive MAP kinase signaling via overexpression of oncogenic Ras, Raf or MEK induces premature senescence in human diploid fibroblasts (Lin et al. 1998; Tresini et al. 1998; Zhu et al. 1998). It was shown that the oncogenic Ras-induced SIPS was mediated by an increase in ROS (Lee et al. 1999; Wei and Sedivy 1999; Sikora et al. 2011). It was shown that the ROS acts as both an upstream signal that triggers p53 activation as well as a downstream effector that mediate apoptosis. Low levels of p53 induce expression of antioxidant enzymes, and its high levels promote the expression of genes that contribute to ROS formation (Liu et al. 2008). Upon continuous exposure to over-expression of the oncogene, normal cells stop proliferating long before their telomeres become short. The RAS proto-oncogenes encode small GTPase proteins that are involved in cellular signal transduction pathways including induction of cell growth and survival, as well as differentiation. A mutation in codon 12, 13 or 61 in any of the three RAS genes (N-RAS, K-RAS and H-RAS) transforms them into active oncogenes but, paradoxically, overexpression of mutant RAS oncogenes in normal cells causes senescence rather than malignant transformation. Studies described in 1997 provided a partial explanation on this finding by showing that the activated H-RAS triggered an initial wave of proliferation in vitro, followed by an irreversible growth arrest and a concomitant accumulation of p53 and $p16^{INK4A}$ proteins (Serrano et al. 1997). Several reports have now demonstrated that the oncogene-induced senescence occurs in vivo in mouse tumor models and in human tumors. A mouse strain containing a knocked-in conditional oncogenic mutant H-RAS allele developed lung adenomas that were characterized by a low proliferative index and increased SA β-gal activity and other senescence markers including senescence-associated heterochromatin foci and elevated levels of $p16^{INK4a}$ and $p15^{INK4a}$ (Collado et al. 2007). Eμ-N-RAS transgenic mice harboring targeted

heterozygous lesions in the gene encoding Suv39h (histone methyltransferase) developed mouse T cell lymphomas that entered senescence after drug therapy. Intriguingly, these studies show that tumor cells are still capable of activating the senescence program when triggered by exogenous stimuli such as DNA-damaging anticancer drugs (Braig et al. 2005). Oncogene-induced senescence has been studied largely in vitro, which has evoked debate whether this type of senescence has physiological relevance. Studies have shown that the stress and oncogene-induced senescence occur in vivo in response to mutations in Ras (Serrano et al. 1997), Raf (Dankort et al. 2007; Michaloglou et al. 2005), NF1 (Courtois-Cox et al. 2006) and PTEN (Chen et al. 2005; Courtois-Cox et al. 2006) genes in mouse as well as human tumors. Sarkisian and colleagues have postulated a triple step model for dose-dependent oncogene induced senescence in vivo (Sarkisian et al. 2007). They generated doxycycline-inducible transgenic mice that allowed titrated Ras activation. Initial activation of *Ras* mutation resulted in low levels of oncogene activation that stimulated proliferation; high level of ras activation activated tumor suppressor pathways and caused induction of senescence; inactivation of the latter was indispensible for tumor progression (Sarkisian et al. 2007). Feng and colleagues demonstrated the relationship between oxidative stress and senescence in vivo using mouse oxidative stress model (Feng et al. 2001). Several tissues from the ozone inhalated mice showed decline in anti-oxidative capacity, increased production of ROS and senescence-related alterations in physiological and physical strength parameters.

9.4 Cellular Senescence and Cancer

Cellular senescence is largely accepted as an in-built anticancer mechanism. Whereas cancer cells show a state that has escaped senescence, reimposition of senescence is considered to be a promising anticancer strategy. Pereira-Smith and Smith illustrated that the hybrids obtained from fusion of normal human diploid fibroblasts with immortal human cell lines exhibited limited division potential suggesting that the cellular senescence is dominant over immortalization (Pereira-Smith and Smith 1983). The latter has been widely accepted as an early prerequisite towards tumorigenesis (Campisi 2013; Duncan and Reddel 1997; Reddel 1998; Shay and Wright 2005; Wadhwa et al. 1994, 2000a, 2002b) and is achieved by overriding multiple proliferative checkpoints by events mediated by genetic, epigenetic, intracellular and extracellular environmental factors (Campisi and Robert 2014; Serrano and Blasco 2007; Shay and Roninson 2004; Moore et al. 2003; Vallejo et al. 2004). It was shown that the introduction of transforming genes of DNA tumour virus, such as SV40, papilloma and adenovirus, prevents cells from entering senescence and confers a finite extension of proliferative lifespan, of approximately 20–60 PD, which eventually ends in culture crisis (Girardi et al. 1965). Only a small number of cells (at a frequency of 10^{-5} to $10^{-9)}$ were able to escape from crisis (Girardi et al. 1965; Huschtscha and Holliday 1983; Shay and Wright 1989) by inactivation of cell cycle checkpoints and activation of a telomere maintenance

mechanisms (Campisi 2002, 2005b, 2008; Duncan and Reddel 1997; Reddel 1998; Smith and Pereira-Smith 1996). Reversal of these signalings has been shown to trigger cells to senescence and has been considered to be a promising anticancer strategy. Indeed several anticancer drugs have been shown to induce premature age-related pathologies like visual deterioration, musculoskeletal decline, osteoporosis, skin changes, chronic fatigue, sexual dysfunction and cardiovascular complications (Lee and Lee 2014; Maccormick 2006; Meinardi et al. 2000; Ventura et al. 2007).

9.5 Cell Cycle Checkpoints and Senescence

The cell cycle checkpoints provide important regulatory machinery for ensuring integrity of genetic information in normal cells. In response to genotoxic stress, they block cell cycle progression, thus allowing DNA repair systems to correct replication errors. Whereas upon correction of the DNA errors, checkpoint signals are attenuated resulting in cell cycle renewal, failure of repair triggers senescence/apoptosis, failure of the latter results in carcinogenesis. Recent studies have shown that the checkpoints-mediated DNA damage response (DDR) signalling acts as "a double-edged sword" in cancer prevention and cancer therapy (Tian et al. 2015). On one hand, it safeguards genomic stability and prevent from tumorigenesis, and on the other, it contributes to the resistance of cancer cells to chemo- and radiotherapy.

The DNA damage checkpoint control is constituted of sensors (MRN complex and RPA), transducers (Ataxia telangiectasia mutated, ATM; Ataxia telangiectasia and Rad3-related, ATR and DNA-PK) and their effector proteins (Chk1, Chk2, p53, Cdc25A, Cdk1, Cdk2 and several others) (Broustas and Lieberman 2014; Elias et al. 2014; Stracker et al. 2013; Zannini et al. 2014; Zhang and Hunter 2014). The ATM/ATR-Chk1/Chk2-p53-p21 axis is a primary regulator of DNA damage response (Stracker et al. 2013). Each of these proteins plays a specific role in regulation of cell cycle and DNA damage response. ATM and DNA-PK respond to double strand DNA breaks, ATR is involved in single-strand DNA breaks. In response to genotoxic, oncogenic and environmental stresses, it is activated by phosphorylation on specific Ser or Thr residues and causes G1/S and G2/M cell cycle arrest. Furthermore, they exhibit stress specific activation. Whereas ATM and DNA-PK respond mainly to DSBs, ATR is activated by single-strand DNA and stalled DNA replication forks (Sperka et al. 2012). Chk1, Chk2, p53 and its downstream regulators execute cell cycle arrest and are most frequently inactivated in cancer cells. On the other hand, they are activated in DDR and OIPS. Furthermore, whereas precancerous cells possess active DDR and OIS, aggressive and advanced cancers show their inactivation suggesting that DDR and checkpoint barriers are overridden during the process of carcinogenesis (Broustas and Lieberman 2014; Sperka et al. 2012; Wang et al. 2015). p53 is activated in response to a variety of stresses and inactivated in large majority of cancers (Blagosklonny 2002; Wynford-Thomas 1996; Xue et al. 2007). Although, mechanisms of functional inactivation

of this axis at various checkpoints are not well understood, recent data show that many checkpoint recovery proteins are overexpressed in various cancer tissues suggesting that they function not only in the cell cycle control, but also in the process of cancer development. On the other hand, several proteins, such as Wee 1, Claspin, Plk1, Wip1, Gwl, FoxM1, Cdh1/APC, and PP2A have been shown to inactivate checkpoint regulators by ubiquitin mediated degradation or other mechanisms and promote cell transformation (Wang et al. 2015; Zannini et al. 2014; Zhang and Hunter 2014). The following discussion is limited to the major cell cycle checkpoints involved in senescence.

9.6 p53 Checkpoint

Involvement of multifunctional p53 tumour suppressor protein in senescence has been firmly established in last two to three decades. The two main activities of p53, DNA binding and transcriptional activation, have been shown to increase as cells approach senescence (Atadja et al. 1995; Bond et al. 1996; Kulju and Lehman 1995) or undergo SIPS in response to oncogenic or environmental stimuli (Chen et al. 1998). It activates transcription of a large variety of genes including $p21^{WAF1}$, GADD45, MDM2, Bax, thrombospondin 1, cyclin G, IGF-BP3, TGFα, 14-3-3 s and MDM2 (el-Deiry 1998; Elias et al. 2014; Fang et al. 1999; Liu et al. 2015; Menon and Povirk 2014; Mirzayans et al. 2012; Zhang et al. 2014) and in turn regulated by HDM2, predominantly, by proteasome mediated degradation (Courtois-Cox et al. 2006). Several studies have shown that p53 must be transcriptionally active in order to induce senescence through its downstream effector cyclin dependent kinase inhibitor $p21^{WAF1}$ that causes arrest at G1/S or G2/M stage of cell cycle. Whereas overexpression of $p21^{WAF1}$ in p53 compromised cells resulted in their senescence (Wang et al. 1999; Fang et al. 1999), $p21^{WAF1}$ compromised cells were refractory to this effect (Brown et al. 1997). Microinjection of anti-p53 antibodies rescued cells from senescence and this effect was accompanied by a decrease in $p21^{WAF1}$ expression (Bond et al. 1994; Gire and Wynford-Thomas 1998; Shay et al. 1993). p21 expression is upregulated in a p53-dependent manner as cells approach senescence (Alcorta et al. 1996; Dulic et al. 1994; Harper et al. 1993; Noda et al. 1994; Stein et al. 1999; Vaziri et al. 1993). Exogenous expression of $p21^{WAF1}$ induced senescence in early passage human diploid fibroblasts (Fang et al. 1999; McConnell et al. 1998; Vogt et al. 1998). Disruption of both p21 alleles conferred an extended lifespan (Brown et al. 1997). However, a high level of p21 expression was neither maintained in human senescent cells nor was necessary for acquisition of senescence in mouse cells (Medcalf et al. 1996; Pantoja and Serrano 1999). Thus it was proposed that $p21^{WAF1}$ may initiate senescence but may not be involved in its maintenance. On the other hand, p53 is inactivated in about 60 % of human cancers (Sharpless and DePinho 2002) by mechanisms involving (i) mutations, (ii) inactivation by either DNA tumour virus oncoproteins or cellular partners/antagonists. Cells from individuals with Li-Fraumeni syndrome (an inherited mutation in one TP53

allele) were shown to exhibit lifespan extension when their wtTP53 was inactivated spontaneously (Maclean et al. 1994; Rogan et al. 1995). And, introduction of wtp53 into immortalized cells resulted in their growth arrest or apoptosis.

For its role as a guardian of the genome, p53 has been shown to induce a variety of genes that promote cell death or apoptosis in response to stress (DNA damage, hyperoxia, hypoxia, activated oncogenes, heat shock, cytokines and growth factors) and evade expansion of cells with genomic anomalies. These include BAX, APAF1, PUMA, p53AIP1, NOXA, Wip1 and Gadd45 (Elias et al. 2014; Liu et al. 2014, 2015; Menon and Povirk 2014). During apoptosis, several of these proteins are found in mitochondria and involved in triggering the caspase cascade. It is still unclear what regulates p53 activities to induce either cell cycle arrest or apoptosis.

Site-specific phosphorylation of p53 in response to DNA damage and other stresses has been used as a reliable indicator of stressed state of cells. For example, ionising radiation induces phosphorylation of p53 at serine 15, and this requires Checkpoint kinase 2 (Chk2) and ATM kinase (Banin et al. 1998; Canman and Lim 1998; Knippschild et al. 1996). UV induces phosphorylation at serines 15 and 37, which is dependent on Chk1 and ATR kinase (Chehab et al. 1999; Hirao et al. 2000) and Chk1 also specifically phosphorylates p53 at serine 20 (Chehab et al. 1999; Shieh et al. 2000). UV has also been found to induce phosphorylation at five other N-terminal serines and two threonine residues and, in the C-terminus, at serine 392 (Appella 2001; Appella and Anderson 2001). Phosphorylation of p53 has been shown to prevent its binding to antagonist HDM2, resulting in its stability and upregulation in stressed cells.

Due to critical role of p53 in cell cycle arrest, and its frequent inactivation in human cancers, it has been established as a key regulator of ageing and carcinogenesis. Mouse models with enforced increase in p53 activity have provided contrasting results due to differences in the transgenic p53 allele (Garcia-Cao et al. 2002; Lavigueur et al. 1989; Maier et al. 2004; Tyner et al. 2002). These studies showed that (i) mice with truncated or mutated p53 allele have accelerated ageing and shorter lifespan, and (ii) the mice with multiple copies of the entire p53 locus (super p53) exhibit decreased cancer incidence. Furthermore, the transgenic animals with supernumerary copies of both p53 and its associated regulator $p19^{ARF}$ (super $p19^{ARF}$/p53) exhibited a high degree of tumour resistance and delay in ageing (Matheu et al. 2007). These studies have highlighted the importance of regulation of p53 activity and not just its expression (Liu et al. 2015; Papazoglu and Mills 2007). Furthermore, It has recently been shown to regulate microRNAs that mediate its spectrum of activities in cell cycle arrest, apoptosis and metabolic regulation (Zhang et al. 2014) (Liao et al. 2014; Musilova and Mraz 2015; Penna et al. 2015).

Just as p53 regulates a large number of proteins, it is affected and regulated by a enormous number of factors. One of its strong upstream regulators of p53 is ARF (Alternate Reading Frame) protein coded by INK4a locus on human chromosome 9p21 that also encodes $p16^{INK4A}$, an upstream regulator of pRB (Quelle et al. 1995). Both these proteins have been shown to act as key regulators of replicative senescence, SIPS, OIPS and immortalization of human cells (Kamijo et al. 1997; Quelle et al. 1995; Serrano et al. 1996). ARF was shown to inactivate ubiquitin

ligase HDM2, responsible for degradation of p53 and pRB, resulting in increased level of expression and activation of these proteins. It also functions independent of HDM2 and involves several other interacting proteins including E2F family members, spinophilin, topoisomerase I, Pex19p, cyclin G1, p120 (E4F), WRN helicase c-myc and CARF (Menendez et al. 2003; Martelli et al. 2001; Vivo et al. 2001; Karayan et al. 2001; Sugihara et al. 2001; Zhao et al. 2003; Rizos et al. 2003; Woods et al. 2004; Hasan et al. 2002, 2004, 2008; Qi et al. 2004). It has been established that compared to human cells, mouse cells possess milder tumour suppressor mechanisms and hence undergo spontaneous immortalization in culture. Explanation to such difference in the activity of mouse and human p53 was provided by the study that isolated Pex19p as an ARF interacting partner in mouse cells. It was shown that Pex19p interacts with mouse ARF ($p19^{ARF}$), but not human ARF ($p14^{ARF}$), and inactivate its p53-activating function accounting for weaker p53 activity in mouse cells (Wadhwa et al. 2002b). ARF was also found to interact with a novel 61-kDa serine-rich ubiquitous unique protein coded by human chromosome 4q35. It was named CARF (collaborator of ARF) due to its interaction and collaboration with ARF for activation of p53 function. Targeted siRNA mediated knockdown of CARF resulted in downregulation of ARF expression and its activity that was also translated to downregulation of p53 and $p21^{WAF1}$ expression and activities (Hasan et al. 2002, 2004). The data suggested that CARF is required for ARF function. Furthermore, CARF interacted with p53 causing its stability and activation (Hasan et al. 2004), and HDM2 (Hasan et al. 2008) resulting in its degradation. In a feedback regulation, CARF acts as a transcriptional suppressor of HDM2 and protects itself from HDM2-mediated proteasomal degradation (Cheung et al. 2010; Hasan et al. 2008). It was shown that CARF regulates senescence and carcinogenesis by its dose dependent two-way regulation of DNA damage response. Whereas high level of CARF activated DNA damage response and p53 pathway, its super high levels were shown to inactivate these pathways and lead to malignant transformation of cells (Cheung et al. 2014). Knockdown of CARF on the other hand caused apoptosis depicting that it is an essential protein for cell survival (Cheung et al. 2011, 2014).

p53 has been shown to be regulated by stress chaperone mortalin that is enriched in cancer cells (Deocaris et al. 2013; Wadhwa et al. 2006). Amino-terminus region of mortalin binds to the carboxy-terminus region of p53 (Kaul et al. 2001, 2005; Wadhwa et al. 1998). The small molecules and peptides that bind to mortalin were able to act as binding antagonists resulting in translocation and reactivation of wild type p53 (Deocaris et al. 2007; Grover et al. 2012; Kaul et al. 2005; Wadhwa et al. 2000b, 2002a). Furthermore, an activation of p53 was observed in cells compromised for mortalin expression (Wadhwa et al. 2003; Yoo et al. 2010). It included not only the activation of transcriptional activation function but also control of centrosomal duplication (Kanai et al. 2007; Ma et al. 2006) and apoptotic functions (Lu et al. 2011a, b). Based on these findings, a model on stress-regulation of mortalin-p53 interaction was proposed. Unstressed normal, immortalized and non-malignant cancer cells possess low level of p53 expression and does not interact with mortalin. Genotoxic or environmental stress induces mortalin-p53 interaction leading to inhibition of the apoptotic ability of p53. Physiologically stressed and

malignant cancer cells accumulate p53 (mutant) that is highly phosphorylated and have mortalin-p53 interaction (Lu et al. 2011a, b).

An allelic form of mouse mortalin (mot-1) that differs by two amino acids, M618V and G624R, in the carboxy-terminus substrate-binding domain was earlier shown to induce senescence in mouse immortal cells. By genome sequencing of human mortalin (hmot-2) from Parkinson disease (PD) patients two missense mutants, R126W and P509S, were identified. In comparative functional analysis mouse mot-1 and human PD mutants, R126W and P509S, it was shown that these lack mot-2 functions involved in carcinogenesis. These included p53 inactivation, hTERT/hnRNP-K activation. Of note, mot-1 and PD mutants caused increased level of endogenous oxidative stress, and resulted in decreased tolerance of cells to exogenous oxidative stress. Growth characteristics of hmot-2 and PD mutant revealed that whereas hmot-2 promotes cell cycle progression, PD mots caused cell cycle retardation (Wadhwa et al. 2015). By functional and biochemical assays on protein-protein interactions, it was found that they possess differential chaperoning activities and binding to proteins including RPL-7 and EF-1α proteins. These factors were predicted to mediate the transformation of longevity/pro-proliferative function of hmot-2 to the premature aging/anti-proliferative effect of PD mutants, that operates through their impact on cell cycle checkpoints involved in regulation of cellular senescence and carcinogenesis.

9.7 pRB Checkpoint

Retinoblastoma protein (pRb) is a negative regulator of cell cycle. In its un-phosphorylated form, it binds to E2F family of transcription factors and inactivate their function for cell cycle progression. Phosphorylation of pRB abrogates its interaction with E2F proteins and activate cell cycle progression through G1 to S phase (Nevins 1992; Benevolenskaya and Frolov 2015; Dyson 1994). Several studies have shown that pRB is under-phosphorylated in senescent cells causing them to arrest at the G1 stage of cell cycle (Futreal and Barrett 1991; Stein et al. 1990). Similar to p53, pRB is a target of DNA tumour virus transforming proteins (Ludlow et al. 1989) and is inactivated in large majority of tumours (Shay et al. 1993; Cipressa and Cenci 2013; Jarrard et al. 1999). Introduction of pRB gene into p53/pRB deficient immortal tumour cells induced senescence (Xu et al. 1997). Downstream target of the pRB, the family of E2F transcription factors, is the key regulator of cell cycle progression, apoptosis and a number of other biologic processes. Most recently, it is implicated in regulation of mitochondria-associated genes (Benevolenskaya and Frolov 2015).

Phosphorylation of pRB is regulated by $p16^{INK4a}$ (an inhibitor of the cyclin D-dependent kinase) coded by the CDKN2A locus on chromosome 9p21 that also encodes ARF (Kamijo et al. 1998; Stott et al. 1998). It has been shown to maintain hypo-phosphorylated pRB in senescent human cells. Unlike $p21^{WAF1}$, $p16^{INK4A}$ remains high in late senescent cells (Alcorta et al. 1996; Hara et al. 1991; Reznikoff

et al. 1996; Jarrard et al. 1999; Stein et al. 1990). Introduction of exogenous $p16^{INK4A}$ into normal or immortal human cells resulted in their growth arrest, and induction of premature senescence by ectopic expression of activated Ras or Raf was mediated by $p16^{INK4A}$ (Kato et al. 1998; Serrano et al. 1997; Lin et al. 1998; Zhu et al. 1998). Whereas elevated level of $p16^{INK4A}$ is also responsible for maintenance of a senescent-like state in cells treated with DNA damaging agents (Robles and Adami 1998), its spontaneous loss was associated with lifespan extension in mammary epithelial cells (Brenner et al. 1998; Huschtscha et al. 1998). In Li-Fraumeni syndrome fibroblasts, loss of wild type p53 or $p16^{INK4A}$ caused lifespan extension and, the effects of losing both the p53 and the pRb/$p16^{INK4A}$ pathways were additive (Huschtscha and Reddel 1999). Consistent with the role of p53 and pRb in cellular senescence in vitro, mice with mutations in p53, and pRb or $p16^{INK4A}$ were prone to tumour formation (Donehower et al. 1992; Sharpless et al. 2001), suggesting the role of these proteins in organismal ageing. The level of $p16^{INK4A}$/ARF was elevated when cells were accelerated to age with the exogenous stress (Halvorsen et al. 2000; Krishnamurthy et al. 2004). On the other hand, caloric restriction, known to retard ageing, caused marked reduction (2–16-fold) in age-induced $p16^{INK4A}$/ARF (Krishnamurthy et al. 2004). Induction of ARF expression has been shown to stabilize and increase the activity of p53, resulting in upregulation of $p21^{WAF1}$, which in turn inhibits CDKs and pRB phosphorylation. ARF and $p16^{INK4A}$ are proposed as biomarkers of ageing through their tumour suppression and senescence-inducing functions. Krishnamurthy and colleagues demonstrated a significant increase in expression of the $p16^{INK4A}$ and ARF in most of the tissues in aged mice and rats (Krishnamurthy et al. 2004). They were associated with upregulation of SA-β-gal activity in several tissues. In calorie-restricted animals, increase in lifespan and reduction in age-associated pathologies was correlated with decrease in both $p19^{ARF}$ and $p16^{INK4A}$ expression (Krishnamurthy et al. 2004). Sharpless and colleagues showed that the animals deficient in $p16^{INK4a}$ and/or p53 are developmentally normal, but showed increased frequency of cancer; notably, $p16^{INK4a}$ and p53 double knockout mice have severely shortened lifespan (Sharpless 2004). In contrast, super Ink4A/ARF mice, carrying its extra locus in addition to the endogenous alleles, are more resistant to the development of a variety of chemically-induced tumorigenesis, and have a lower incidence of spontaneous tumours without affecting normal viability or ageing (Matheu et al. 2004, 2007).

Stress induced senescence in human cells is associated with increase in the expression of $p16^{INK4A}$ (Toussaint et al. 2000; Suzuki and Boothman 2008; Serrano and Blasco 2007; Mirzayans et al. 2012). On the other hand, it was shown that ARF is not directly induced by acute DNA damage (Zindy et al. 2003). It mediates the DNA damage response through its effects on HDM2, p53, ATM and ATR (Pauklin et al. 2005). It may interact directly with ATM and/or ATR kinases or may regulate them through TIP60 (Kim and Sharpless 2006). Although the two major checkpoint pathways, p53 and pRB, show overlapping activities to trigger and maintain senescence through activities of $p21^{WAF1}$ and $p16^{INK4A}$, they also work independent to each other and have a vital role in senescence related checkpoint controls, and their loss during cancer development and progression.

Research in last two to three decades has resolved several questions on functional intricacies of in-built cell cycle checkpoints and tumour suppressor mechanisms that regulate limited proliferative capacity of cells and safeguard them against cancer. Further research on feed-back and feed-forward regulation of these cell cycle checkpoints in normal and stressed physiological conditions, their crosstalk with intra- and extra-cellular regulators will be helpful in designing novel strategies for extending functional lifespan of normal, and therapy of cancer cells.

Conflict of Interest The authors declare that they have no competing interests to disclose.

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