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BACHELOR'S THESIS

Replicative stress in cellular senescence and oncogenesis

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Declaration:

Hereby I declare that I wrote this thesis myself with the help of no more than the mentioned literature and auxiliary means.

Prague, April 2009

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Abstract

Cellular senescence is a state of irreversible cell cycle arrest. It represents an active response of the cell to various types of intrinsic and extrinsic stresses. Uncapped telomeres, effects of DNA-damaging agents and activation of certain oncogenes belong among these stresses. This thesis reviews current models of mechanisms which may result in establishment of senescence phenotype. Particular emphasis is being placed on a model of replicative stress, a condition that leads to a halt or a collapse of DNA replication forks. Replicative stress causes DNA damage, preferentially in specific regions of chromosomes, termed fragile sites. DNA damage activates DNA damage response pathways, which are capable of evoking senescence phenotype. Senescent cells with activated DNA damage response signaling have been observed in preneoplastic lesions, supporting a current view that cellular senescence plays a role as an important tumorigenesis barrier. In the end, adverse effects of cellular senescence are briefly discussed including their role in organism aging.

Key words: cellular senescence, replicative stress, fragile sites, DNA damage response, telomeres, oncogenes, cancer, aging

Abstrakt

Buněčná senescence je stavem nevratného zastavení buněčného cyklu. Představuje aktivní odpověď buňky na různé vnější i vnitřní zátěže. Mezi tyto zátěže patří ztráta ochranných konců telomer, účinky DNA poškozující agens a aktivace určitých onkogenů. Tato práce shrnuje současné modely mechanismů, které mohou způsobit ustanovení senescentního fenotypu. Zvláštní pozornost je věnována modelu replikativního stresu, stavu vedoucího k zastavení či kolapsu replikativní vidličky. Replikativní stres způsobuje poškození DNA přednostně ve specifických úsecích chromozomů nazývaných fragilní místa. Poškození DNA aktivuje signální dráhy odpovědi na poškození DNA, které jsou schopné indukovat senescentní fenotyp. Senescentní buňky s aktivovanými drahami odpovědi na poškození DNA byly pozorovány v preneoplastických lézí. Toto podporuje pohled na buněčnou senescenci, jakožto na významnou protinádorovou bariéru. Na závěr jsou krátce zmíněny škodlivé účinky buněčná senescence a jejich role ve stárnutí organismů.

Klíčová slova: buněčná senescence, replikativní stress, fragilní místa, odpověď na poškození DNA, telomery, onkogeny, rakovina, stárnutí

Acronyms and abbreviations

53BP1	53-binding protein 1
ActD	actinomycin D
APC	aphidicolin
AREG	amphiregulin
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
ATRIP	ATR interacting protein
BRCA1	breast cancer 1
BrdU	5-bromo-2′-deoxyuridine
BTG	B-cell translocation gene
C/EBPβ	CCAAT-enhancer-binding proteins β
CDC25A	cell division cycle 25A
CDC25C	cell division cycle 25C
CDC6	cell division cycle 6
CDK(s)	cyclin dependent kinase(s)
CFS	common fragile sites
CUL1	cullin 1
DDR	DNA damage response
dNTP	deoxyribonucleotide triphosphate
DSB(s)	double-strand breaks
dsDNA	double-stranded DNA
E2F1	E2F transcription factor 1
EMF	electromagnetic fields
ERK	extracellular signal-regulated kinases
Ets	erythroblastosis virus E26 oncogene homolog 2
Eya1/3	Eyes Absent 1/3
FGF7	fibroblast growth factor 7
FHIT	fragile histidine triad gene
FMR1	fragile X mental retardation 1
FMR2	fragile X mental retardation 2
FOXO	forkhead box O
FS	fragiles sites
H2AX	H2A histone family, member X
HDF	human diploid fibroblast
HDM2	human double minute 2
HGF	hepatocyte growth factor
HU	hydroxyurea
CHK1	checkpoint kinase 1
CHK2	checkpoint kinase 2
IFN-β	interferon β
IL	interleukin
MDC1	mediator of DNA damage checkpoint protein 1
MDM2	murine double minute 2
MEK	MAP-ERK kinase
MMC	mitomycin C
MMP3	matrix metallopeptidase 3
Mre11	meiotic recombination 11
NBS1	Nijmegen breakage syndrome 1
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NF1	neurofibromin 1
OIS	oncogene-induced senescence
p14^{ARF}	cyclin-dependent kinase inhibitor 2A alternate reading frame
p16	cyclin-dependent kinase inhibitor 2A

p21$^{waf1/cip1}$	cyclin-dependent kinase inhibitor 1A
p53	tumor protein p53
PCNA	proliferating cell nuclear antigen
PIK3	phosphoinositide 3-kinases
Plk3	polo-like kinase 3
PML	promyelocytic leukemia
Pol	polymerase
POT1	protection of telomeres 1
PP5	protein phosphatase 5
PTEN	phosphatase and tensin homolog
RAC1	Ras-related C3 botulinum toxin substrate 1
Rb	retinoblastoma protein
RFC	RAD17-replication factor C
RFC	rare fragile sites
RNF186	ring finger protein 186
RNF8	ring finger protein 8
RNR	ribonucleotide reductase
ROS	reactive oxygen species
RPA	replication protein A
S-phase	synthesis phase
SA-β-GAL	senescence-associated β-galactosidase
SASP	senescence-associated secretory phenotype
SCF	SKP1-CUL1-F-box protein
SIPS	stress-induced premature senescence
SKP1	S-phase kinase-associated protein 1
SMURF2	SMAD specific E3 ubiquitin protein ligase 2
SSB(s)	single-strand breaks
ssDNA	single-stranded DNA
STAT5A	signal transducer and activator of transcription 5A
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor β
Tlk1/2	tousled-like kinase 1/2
TO53BP1	tumor protein p53 binding protein 1
TOPB1	topoisomerase II-binding protein 1
TRF1	telomeric repeat-binding factor 1
TRF2	telomeric repeat-binding factor 2
VEGF	vascular endothelial growth factor
WWOX	WW domain containing oxidoreductase
γH2AX	phosphorylated H2A histone family, member X

Contents

1. Introduction

Cellular senescence, a state of terminal cell-cycle arrest, was first observed more than four decades ago (Hayflick and Moorhead, 1961). Since the time of its discovery a view of cellular senescence made a dramatic development. It has been proved that cellular senescence is not a mere cell culture artifact and that it occurs *in vivo* as well. The hypothesis proposing cellular senescence as a representation of aging on cellular level was also largely reconsidered. Nowadays, cellular senescence is viewed as an active response to various types of intrinsic and extrinsic stresses, such as telomere attrition, effects of DNA-damaging agents and activation of certain oncogenes. In common, these stresses are manifested by DNA damage, particularly by the most severe DNA double-strand breaks. DNA double-strand breaks represent a serious hazard to genome integrity. They may result in chromosomal aberrations, potentially leading to cancer development. Cellular senescence prevents cellular proliferation and thus represents a tumor-suppressive mechanism. The importance of this mechanism was recently evidenced in many *in vivo* studies (Braig *et al.*, 2005; Chen *et al.*, 2005; Collado *et al.*, 2005; Michaloglou *et al.*, 2005).

In this work I attempt to summarize current views on cellular senescence with emphasis on the role of replicative stress in this phenomenon.

2. Replicative stress

All cellular molecules have to constantly face various endogenous and exogenous stresses. They suffer oxidative stress from reactive oxygen species (ROS) generated in mitochondrial respiration, ionizing radiation from environment and numerous others stressors. Deoxyribonucleic acid (DNA) is no exclusion, due to its role as the carrier of genetic information, the damage affecting DNA is even more severe.

Speaking about DNA integrity, S-phase, in which the DNA replication occurs, represents the most vulnerable phase of the cell cycle. DNA is confronted with several difficulties intrinsic or pathological to the nature of the replication event, which can be broadly termed a replication stress. The term replicative stress was originally coined to denote abortion of DNA replication due to the depletion of deoxynucleotide pool. Today this term is generally used to refer to any condition that leads to a halt or a collapse of DNA replication forks. Factors contributing to replicative stress are summarized in Figure 1.

In addition, otherwise relatively rigid double-stranded DNA structure is during replication unwound into single strand regions, which leads to the conversion of single-strand breaks (SSBs) to more harmful double-strand breaks (DSBs). DSBs represent a serious hazard to the structural stability of chromosomes.

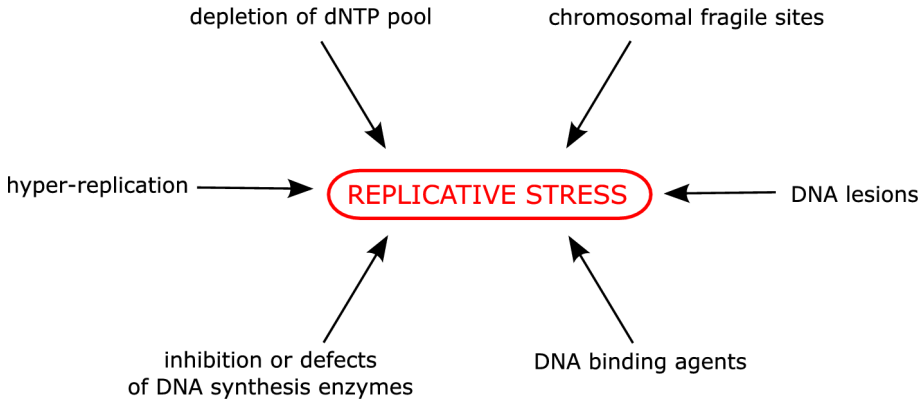

Figure 1 | Factors contributing to replicative stress

- **Depletion of dNTP pool**, caused e.g. by inhibition of ribonucleotide reductase (RNR) by hydroxyurea (Krakoff *et al*., 1968) or folate deficiency (Duthie and Hawdon, 1998).
- **Inhibition of enzymes involved in DNA synthesis** leads to replicative stress. DNA polymerases can be inhibited e.g. by aphidicolin (Tedeschi *et al*., 2004); topoisomerases II by etoposide and doxorubicin (Hande, 1998).
- **DNA binding agents**, like actinomycin D (Hou *et al*., 2002) or distamycin A (Luck *et al*., 1977) interfere with replication fork progression.
- **DNA lesions** result in a collapse of fork.
- **Chromosomal fragile sites** as specific DNA structures represent obstacles for DNA replication and are preferentially prone to induction of DNA damage foci (Durkin *et al*., 2008).
- **Hyper-replication** triggered by activated oncogenes – potent activator of replicative stress, with crucial importance in oncogene-induced senescence (Gorgoulis *et al.,* 2005 and Bartkova *et al*., 2005).

2.1. Fragile sites (FS)

Distribution of DNA lesions caused by replicative stress is not random. Particular chromosomal sites, termed fragile sites (FS), are preferentially prone to induction of DNA damage foci (Magenis *et al*., 1970; Durkin *et al*., 2008). In common, FS contain repetitive sequences – the CGG triplets repeats or AT-rich repeats (Kremer *et al*., 1991; Yu *et al*., 1997). These repeats have been observed to form secondary

structures, such as hairpins, which interfere with replication fork progression and formation of chromatin structures as well (Gacy *et al*., 1995; Zlotorynski *et al*., 2003). The mechanism of FS fragility is still not fully elucidated, effects of secondary structure-forming repeats are plausible candidates for culprits.

Based of the frequency of their occurrence in studied population, FS can be divided into two major groups – the rare fragile sites (RFC), with less than 5% incidence in human population and common fragile sites (CFS), which can be found in every individual. An extensive summary of known fragile sites can be found in Lukusa and Fryns, 2008.

2.1.1. Rare fragile sites (RFC)

RFC can be further classed into folate-sensitive and nonfolate-sensitive groups. The latter are specifically promoted by distamycin A or by distamycin A in cooperation with 5'-bromo-2'-deoxyuridine (BrdU). Folate-induced RFC exhibit CGG-repeats (Kremer *et al*., 1991), whereas folate-independent RFC contain AT-rich repeats (Yu *et al*., 1997).

Particular RFC belonging to folate-sensitive group have been causally linked to certain human disorders. FRAXA site situated in FMR1 gene locus is associated with fragile X syndrome (also known as FRAXA syndrome), which is demonstrated by mental retardation (Verkerk *et al*., 1991). FRAXE site placed in FMR2 gene locus is responsible for milder X-linked non-specific mental retardation (Gu *et al*., 1996).

2.1.2. Common fragile sites (CFS)

CFS occur in every individual and are highly evolutionarily conserved through species. Putting it in perspective with deteriorative effects of CFS breakability, it seems to be a bit self-contradictory. It has been suggested that CFS have not been eliminated by selection, because they play a specific physiological role. Since CFS are replicated very late in S-phase (Le Beau *et al*., 1998), they may act as a sensing element of completed replication (Durkin and Glover, 2007).

The majority of CFS are specifically induced by aphidicolin (APC; Glover *et al*., 1984), the minor and less characterized part by BrdU and 5-azacytidin (Sutherland *et al*., 1985). APC-induced CFS can be induced also by other factors, like hydroxyurea or folate-deficiency, but the treatment with low doses of APC gives the best reproducible results.

2.1.2.1. Role of CFS in cancer

Common fragile sites represent the weak spots of genome integrity. Due to their breakability, they are often involved in chromosomal aberrations, which can lead to cancer development.

The best-characterized examples of fragile sites linked to cancer are FRA3B and FRA16D. These CFS are situated in loci of tumor-suppressor genes. FRA3B lies in the locus of FHIT (Fragile histidine triad) gene. Product of FHIT has been described as the target of the protein kinase Src and it presumably plays a role in triggering apoptosis (Pekarsky *et al*., 2004). FRA16D occurs in the locus of WWOX (WW domain-containing oxidoreductase) gene. Gene product of WWOX interacts with p53, contributing to induction of apoptosis (Chang *et al*., 2005). Deletions that inactivate these genes have been detected in numerous cancers, e.g. in ovarian (Denison *et al*., 2003), pancreatic (Kuroki *et al*., 2004) and lung tumors (Yendamuri *et al*., 2003).

In addition to deletions, CFS are also prone to translocation events, though less frequently. For instance, translocations in FRA6E and FRA6F have been observed in acute lymphoblastic leukemia and acute myeloid leukemia (Sinclair *et al*., 2005). Furthermore, CFS can contribute to genome instability by triggering gene amplification (Coquelle *et al*., 1997).

2.1.2.2. Model of instability of CFS (Figure 2)

APC uncouples polymerases from the helicase/topoisomerase complex. While polymerases are slowed down, helicase/topoisomerase complex still continues in unwinding of dsDNA strand. This results in exhibition of long ssDNA regions (Walter *et al*., 2000). Flexible regions of repetitive sequences can form secondary structures, which consequently cause collapse of polymerases (Zlotorynski *et al*., 2003). Regions of ssDNA also trigger ATR-branch of DNA damage response, which can, among other effects, delay cell-cycle progression to provide enough time to complete replication, and promotes repair of damaged DNA (see below).

Consistently with this model, depletion of ATR leads to the lack in DDR and consequently to increase in DNA lesions associated with CFS. In addition, collapsed fork may lead to induction of DSBs which activate ATM-branch of DDR (Ozeri-Galai *et al*., 2008).

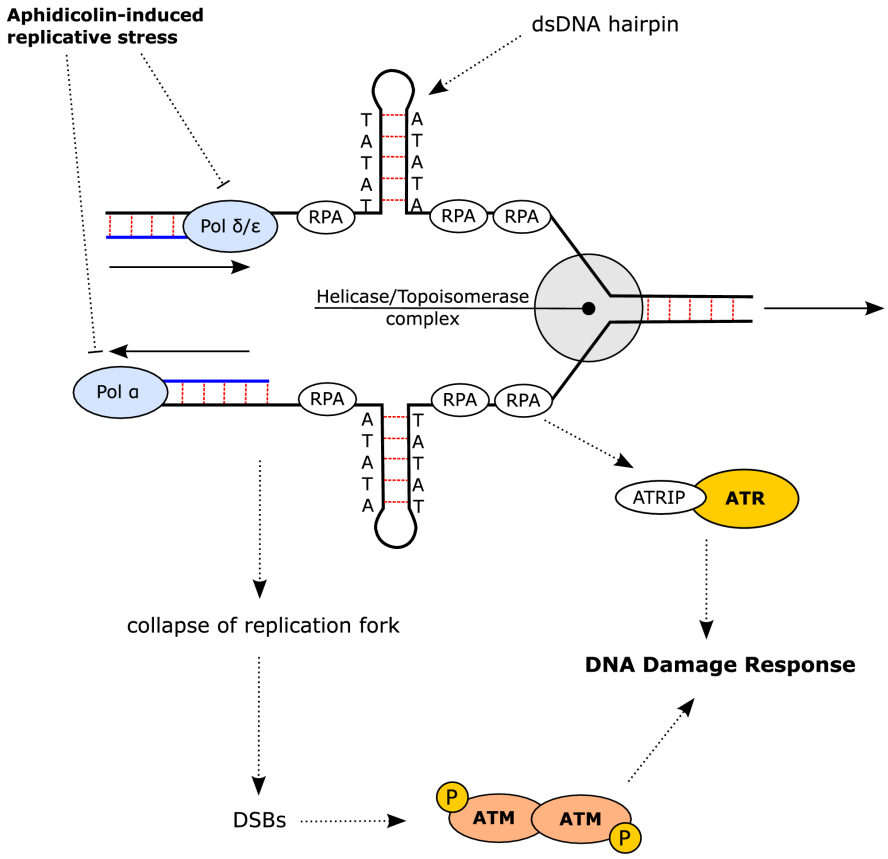

Figure 2 | Model for common fragile site instability proposed by Durkin and Glover - dsDNA secondary structures, established in ssDNA flexible regions of repetitive sequences, interfere with replication fork progression, which leads to triggering of DDR (based on diagram from Durkin and Glover, 2007).

3. The DNA damage response (DDR)

Maintenance of genome integrity is crucial for survival at both the cellular and organism level. Eukaryotic cells have developed specific mechanisms to deal with genotoxic stresses. The DNA damage response pathways represent important guardians of genome stability.

As mentioned above, DNA lesions serve as potent activators of DDR. DSBs activate the response mediated mainly by ATM kinase, while single-strand DNA regions and stalled DNA replication forks trigger ATR kinase. However, it was shown that ATR can be recruited also to DSB damage foci when ATM activates nuclease activity of meiotic recombination 11 (Mre11), which can generate single-stranded DNA regions in DSB foci (Jazayeri *et al.*, 2006).

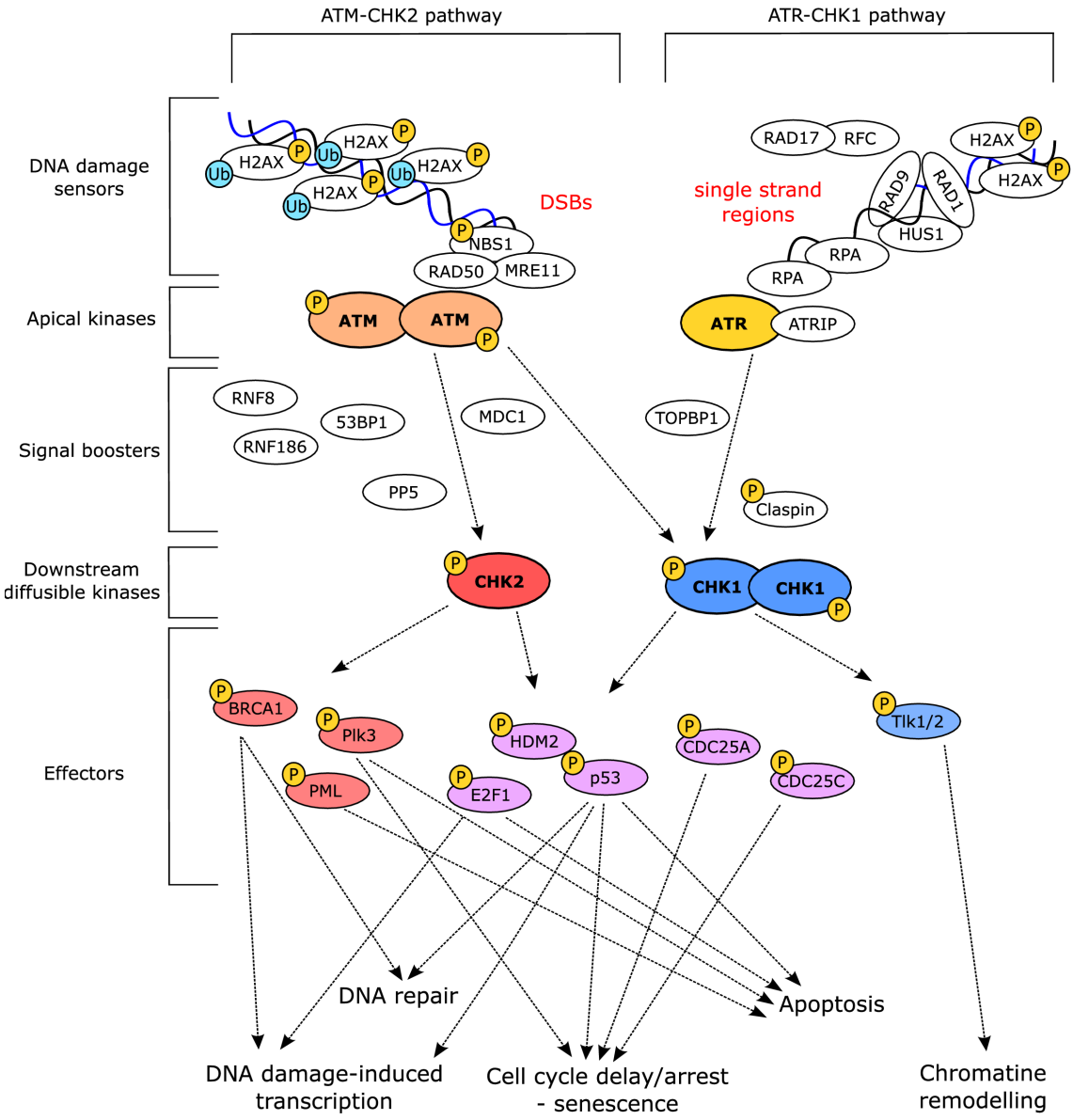

Figure 3 | The DNA-damage response
ATM-CHK2 and ATR-CHK1 damage response pathways and their effectors, with depiction of known involved proteins. Pathways are discussed in detail below. Effectors phosporylated by CHK2 are in red, CHK1 substrates are in blue and substrates phosporylated by both kinases are in pink (based on diagrams from d'Adda di Fagagna, 2008; Campisi and d'Adda di Fagagna, 2007; Bartek and Lukas, 2003).

3.1. ATM-CHK2 branch of DDR

The DSBs are recognized by MRN complex, composed of MRE11 (meiotic recombination 11), RAD50, NBS1 (Nijmegen breakage syndrome 1) proteins (Nelms *et al*., 1998; Grenon *et al*., 2001). MRN complex recruits ATM to sites of DNA damage (Lee and Paull; 2004). Consequently, ATM forms homodimers and is activated through intramolecular phosporylation on serine residues 1981 (Bakkenist and Kastan, 2003).

Recently it was demonstrated that ATM activation is inevitably preceded by its phosphorylation on serine 794 by cyclin-dependent kinase 5 (Cdk5; Tian *et. al*., 2009).

Alternatively, based on the observation that even a few DSBs can rapidly activate a large amount of ATM, Bakkenist and Kastan (2003) suggested that ATM activation is not stimulated via binding to DSBs foci. They proposed that ATM initiating stimulus may be sparked by alteration of chromatin structure, which accompanies the DBSs. However, the exact mechanism by which the chromatin alteration might cause initial activation of ATM has not been clarified.

The role of protein phosphatase 5 (PP5) is essential in ATM activation, as its deficiency results in the lack of ATM-mediated cell cycle arrest (Ali *et al*., 2004; Yong *et al*., 2007). Nevertheless, its direct effect on ATM remains unknown. Speculatively, in response to DSBs, PP5 may remove putative inhibitory phosphates from ATM and thus provide its activation (Ali *et al*., 2004).

Once activated, ATM dimers dissociate into monomers (Bakkenist and Kastan, 2003) which consequentially phosphorylate histone H2AX on serine 139. H2AX can undergo this phosporylation only if it has been earlier dephosphorylated on tyrosine 142 by Eya1/3 phosphatases (Cook *et al*., 2009). H2AX phosporylated on serine 139 (γH2AX) binds MDC1, mediator of DNA damage checkpoint protein 1. MDC1 interacts with NBS1, which leads to the increase of MRN complex concentration at DSBs foci (Melander *et al*., 2008). MRN complexes in turn recruit more ATM, which successively phosphorylates more H2AX. Thus a positive feedback loop is set up.

MDC1 also promotes accumulation of RNF8 ubiquitin ligase at DSBs. RNF8 ubiquitinates histones H2A and γH2AX. (Mailand *et al*., 2007). Ubiquitinated histones H2A attract other ubiquitin ligase, RNF186 that further increases histone ubiquitinylation. In this way modified DSBs-flanking chromatin then serves as a landing platform for downstream DDR mediators, such as tumor protein p53 binding protein 1 (53BP1; Doil *et al*., 2009). 53BP1 promotes ATM activity in a pathway parallel to MDC1-NBS1 pathway. It is suggested that when one of these amplification supporting pathways is set up, the other is inhibited as redundant (Mochan *et al*., 2003).

While the activity of ATM reaches a certain level, it activates CHK2 kinase. Notably, ATM can in a minor manner also activate activate CHK1 (Gatei *et al*., 2003).

3.2. ATR-CHK1 branch of DDR

The ATR branch of DDR consists of different proteins than ATM branch, but in the structure of signal transmission these pathways share many similarities (Figure 3).

Firstly, the ssDNA region is coated by replication protein A (RPA), which recruits the ATRIP-ATR complex (Cortez *et al*., 2001; Zou and Elledge *et al*., 2003). ATRIP role in ATR localization to DNA damage foci via its interaction with ssDNA-RPA complex has been believed to be crucial. Interestingly, a splice variant of ATRIP, which is not able to bind to ssDNA-RPA complex, still supports the ATR activation, implying that binding to RPA–ssDNA is not absolutely required for ATRIP-ATR function (Ball *et al*., 2005).

ATR activity is promoted by the PCNA-related 9-1-1 complex, consisting of RAD9, RAD1, HUS1. 9-1-1 complex is loaded to DNA damage foci by clamp loader RAD17-RFC complex (RAD17-replication factor C) (Zou *et al*., 2002). Additionally, ATR activity can be promoted also by topoisomerase II-binding protein 1, TOPB1 (Kumagai *et al*., 2006) In contrast to ATM, ATR does not undergo phosphorylation during its activation. Boosted ATR then in cooperation with phosphorylated adaptor protein claspin phosphorylates and thus activates CHK1 (Kumagai *et al.*, 2000).

3.3. CHK1 and CHK2 kinases and down-stream signal transduction

Diffusible kinases CHK2 and CHK2 spread the signal through the nucleus to its effectors, Figure 3 (Lukas *et al*., 2003; Bekker-Jensen *et al*., 2006). For delay or arrest of cell-cycle, CDC25 phosphatases, p53 and its negative regulator HDM2 (and CDC25A in case of S-checkpoints) are particularly important.

Phosphorylated CDC25 phosphatases are marked by SCF (SKP1-CUL1-F-box protein) ubiquitin ligase and consequently dispatched by proteolysis. Since they are necessary for activation of cyclin dependent kinases (CDKs), their dispatch promptly results in cell-cycle delay (Mailand *et al.,* 2000; extensively reviewed by Donzelli and Draetta, 2003).

Activation of p53 via its phosporylation (mediated by ATM, ATR, CHK1 and CHK2) and additional down-regulation of its inhibitor HDM2 leads to transcription activation of $p21^{waf1/cip1}$ gene (p21). The p21 acts as CDK inhibitor and thus contributes to cell-cycle delay (see below).

As shown before, the CHK1 and CHK2 share many substrates (Figure 3). Nevertheless, their roles are not mutually interchangeable. CHK1 regulates the physiological level of CDC25A, balancing its pro-replicative activity. Its effect represents a "housekeeping" mechanism, which is essential to maintain unperturbed replication (Sorensen *et al*., 2003). CHK2 conditionally amplifies the rate of CDC25A degradation in response to DSBs and thus causes S-phase delay (Bartek and Lukas,

2003). Different importance of these kinases is well reflected by mice knockout models. CHK1-deficiency leads to embryonic lethality due to p53-independent apoptosis (Liu *et al*., 2000). On the contrary, CHK2-deficient mice are viable and do not exhibit extensive development of tumors, unless they are exposed to carcinogenic agents (Hirao *et al*., 2002).

If the DNA lesions are correctly repaired, DDR is then shut down by remodeling of chromatin structure. The molecular mechanism of this action remains to be elucidated. However, when the DNA damage is excessively severe to be repaired during transient cell cycle arrest, persistent DDR can trigger permanent cell cycle arrest termed cellular senescence.

4. Cellular senescence

The cellular senescence is a state of irreversible cell cycle arrest. This phenomenon was first described by Hayflick and Moorhead (1961). They observed that in vitro cultured human diploid cells can undergo only a limited number of doublings. The proliferative limit (Hayflick limit) was determined at 50±10 cumulative doublings for human diploid fibroblast in culture (Hayflick and Moorhead 1961). Hayflick (1965) also proposed a hypothesis that limited proliferating capability may be a representation of aging on cellular level. However, cellular senescence should not be viewed as a physical state of the cell as it chronologically grows old. It is caused by an active response of cell to various stressors, as described below.

4.1. Characterization of senescent cells

Senescent cells exhibit various characteristic changes at all levels from gene expression to morphology. Their cytoplasm increases in volume and flattens (Figure 3; Bayreuther *et al*., 1988).

The expression of many genes throughout senescence is altered (extensive comparison of gene expression in various types of senescence was published by Pascal *et al*., 2005). In particular, overexpression of cell cycle inhibitors – cyclin dependent kinases (CDKs) inhibitors p21 (Shelton *et al* 1999) and $p16^{INK4a}$ (p16; Hara *et al*., 1996) is causal for cell cycle arrest. These CDKs inhibitors are key components of pathways that establish and maintain cell cycle arrest (see below; Sherr *et al*., 2002; Campisi and d'Adda di Fagagna, 2007). Interestingly, senescent cells upregulate either p21 or p16.

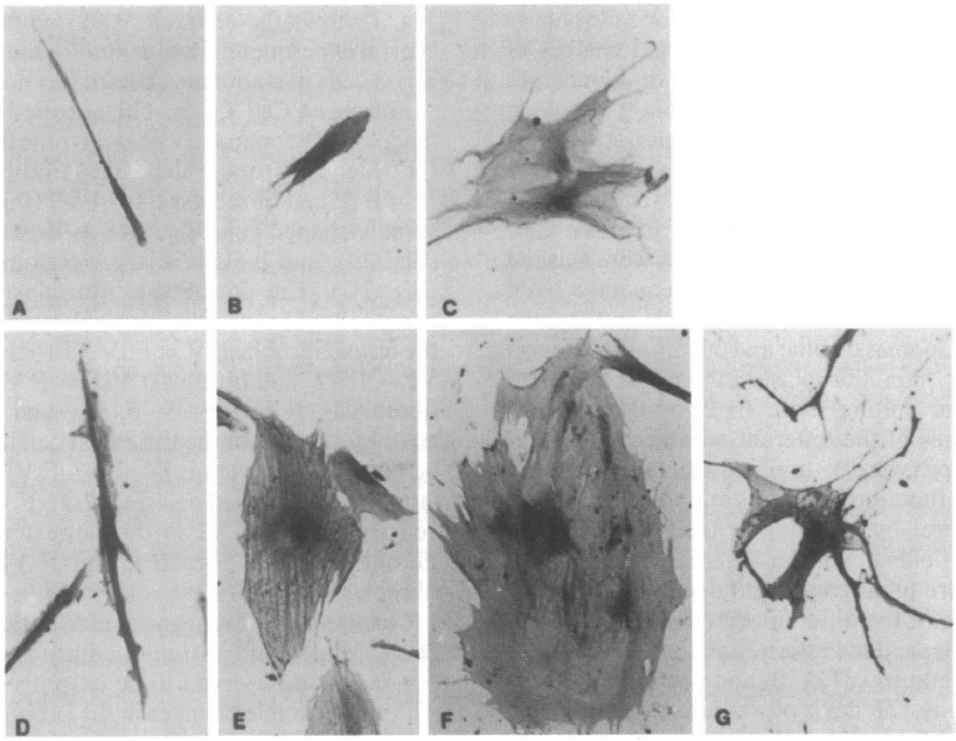

Figure 4 | Mitotic and postmitotic fibroblast morphotypes (×86)
Morphotypes of human diploid fibroblasts (HDF) during their ontogenesis, as described by Bayreuther and colleagues: (A) Mitotic (HDF) cell-type F I.; Mitotic HDF cell-type F II. (B); Mitotic HDF cell-type F III. (C); Postmitotic HDF cell-type F IV. (D); Postmitotic HDF cell-type F IV. (E); Postmitotic HDF cell-type F IV. (F); Postmitotic degenerating HDF cell-type F IV. (G). (C) and (D) well illustrate morphological changes of senescent cells.
Figure from Bayreuther *et al.*, 1988

Relationship between the regulation of expression of these two CDKs inhibitors is not fully elucidated. Presumably, some stimuli induce expression of p21, while others upregulate p16 (Herbig *et al.*, 2004).

Then again, Stein and colleagues (1999) demonstrated the different dynamics of p21 and p16 accumulation. Early increased levels of p21 exhibit a decrease after senescence is achieved. This is followed by a rise of p16 levels, suggesting that p16 overexpression may be crucial for stable maintenance of senescence. Another overexpressed protein that seems to have growth suppressive function is cyclin D2 (Meyyappan *et al.*, 1998; Shelton *et al.*, 1999).

In contrast, the proteins that stimulate cell proliferation are repressed. This repression is often facilitated by hypophosphorylated retinoblastoma protein (Rb), which inactivates transcription factor E2F (Hiebert *et al.*, 1992; Narita *et al.*, 2003).

However, not all changes in gene expression are linked directly to growth arrest. In addition senescent cells overexpress genes, whose secreted products can change the

tissue microenvironment: degradation enzymes – e.g. matrix metallopeptidase 3 (MMP3; Parrinello *et al*., 2005), growth factors – vascular endothelial growth factor, (VEGF; Coppe, *at al*., 2006), fibroblast growth factor 7 (FGF7), hepatocyte growth factor (HGF), amphiregulin (AREG; Bavik *at al*., 2006) and several cytokines such as IL-1, IL-6, IL-8, IL-11 etc. (Coppe *et al*., 2008). The findings of Krtolica and colleagues (2001) also suggest that this senescence-associated secretory phenotype (SASP) might paradoxically stimulate tumorigenesis of neighboring cells. They observed that senescent fibroblast can via SAPS stimulate preneoplastic and neoplastic epithelial cell proliferation, while normal epithelial cells do not respond to SAPS by hyperproliferation. These effects of SAPS dispute with the tumor-suppressive role of cellular senescence. Possible explanation of this contravention is proposed by the theory of antagonistic pleiotropy (see chapter 4.4.1.). Disruption of tissue homeostasis may likewise contribute to age-related pathologies (Campisi *et al.*, 2005). Moreover, recent studies reveal that secreted cytokines IL6 and IL8 can together with their receptors and transcription factors (e.g. NF-κB, C/EBPβ) form positive-feedback loops, which promote the establishment of senescence (Acosta *et al*., 2008, Kuilman *et al*. 2008; Bartek *et al*., 2008).

Senescent cells frequently acquire resistance to certain apoptotic stimuli (Wang e*t al*., 1995; Seluanov *et al*., 2001). The apoptotic resistance might partially explain why senescent cells accumulate in tissues with age. Nevertheless, the persistence of senescent cells in tissues may vary, depending on their cellular type. For example, senescent melanocytes from pigment naevi can continue in existence for years (Michaloglou *et al*., 2005). On the other hand, the senescent cells of liver carcinomas are promptly dispatched by phagocytes (Xue *et al*., 2007).

Until now, there are no markers exclusively specific for senescent cells. Presently, as a common but not universal senescence biomarker *in vitro* serves senescence-associated β-galactosidase activity (SA-β-GAL), detectable at pH 6 (Dimri *et al*., 1995). However, it reflects the enhanced activity of acidic lysosomal β-galactosidase (EC 3.2.1.23) at suboptimal pH, being detectable due to increased lysosomal mass in senescent cells. This activity is not directly involved in the development of senescence phenotype (Kurz *et al.*, 2000; Lee *et al*., 2006). β-galactosidase activity can be also induced by serum starvation and cell confluence. Yet, in these cases β-galactosidase activity can be reversed by re-culturing of cells in sufficient conditions, whereas SA-β-GAL activity cannot be reversed in this way (Yang and Hu, 2005).

4.2. Molecular pathways establishing senescence

Senescence is established and maintained by p53 and pRb pathways (Figure 5). Despite the possible interactions between these pathways, they are able to establish senescent growth arrest independently of each other. Activation of the particular pathway depends on primary stimuli and cell type. Yet an understanding why one pathway is chosen over the other is in many cases still incomplete.

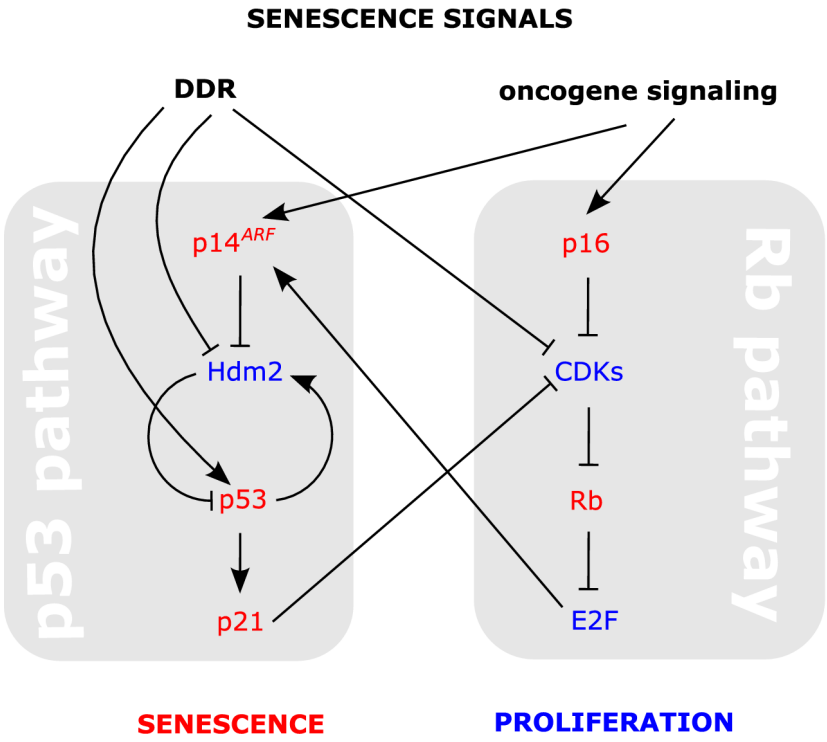

Figure 5 | p53 and pRb pathways
Molecular pathways establishing senescence. Growth-arrest promoting effectors are in red. Pro-proliferative effectors are in blue.

4.2.1. The p53 pathway

The p53 pathway can be activated by DNA damage response or by oncogene signaling. DDR kinases CHK1 and CHK2 activate p53 directly trough its phosphorylation and inhibition of its negative regulator Hdm2, homologue Mdm2 (mouse double minute 2) in mice. Oncogene signaling induces alternative reading frame protein $p14^{ARF}$ (alternatively called p14, ARF, and p19ARF in mice; oncogene-induced pathways demonstrated in figure 9, page 29) encoded at INK4 gene locus together with p16 (Stott *et al.,* 1998). Then $p14^{ARF}$ binds and blocks Hdm2 action (Chang *et al*., 2007). In addition, $p14^{ARF}$ transcription can be promoted by E2F. This represents one of the cross-talks between p53 and Rb pathways (Bates *et al.,* 1998).

Hdm2 is an E3 ubiquitin ligase, which directly interacts with p53 and facilitates its degradation (Kubbutat *et al*., 1997). This regulation has the character of a negative feedback loop, since increased activity of p53 results in increased production of Mdm2 (Lahav *et al*., 2004).

Active transcription factor p53 triggers transcription of p21 inhibitor of cyclin dependent kinases (Brown *et al*., 1997). Inhibition of CDKs (CDK4/6) results in hypophosphorylation of Rb, which can then suppress pro-proliferative transcription factor E2F. These actions lead to cell-cycle delay or arrest. An experimental inactivation of p53 is able to reverse p53-mediated growth arrest (Beausejour *et al*., 2003).

4.2.2. The Rb pathway

Initiation of Rb pathway comes usually second to p53 pathway (Stein *et al*.,1999). Interestingly, locus INK4 encodes $p14^{ARF}$ (p14-INK4b) and also p16 (also known as INK4a) (Stott *et al.,* 1998). Expression of p16 can be stimulated by oncogenes via activation of Ets transcription factors (Figure 9). Yet, it remains unclear how other stimuli can induce activation of the Rb pathway. The p16 acts as potent CDK inhibitor, keeping Rb in active hypophosphorylated form (Alcorta *et al*., 1996).

The Rb can repress E2F through formation of Rb-E2F complex, which is predominantly transcription repressive (Hiebert *et. al*., 1992). In addition, Rb promotes establishment of senescence-associated heterochromatin foci (SAFS) which provide stable repression of E2F target genes. Once established, SAPS no longer need activated Rb pathway (Narita *et al*., 2003). This is consistent with the observations that Rb mediated arrest cannot be reversed, as opposed to p53 mediated one (Beausejour *et al*., 2003).

4.3. Stimuli that induce cellular senescence

4.3.1. Replicative senescence - telomere-dependent senescence

The vertebrate telomeres are complexes of repetitive noncoding DNA sequences (5′ – (TTAGGG)n – 3′) (Moyzis *et al*., 1988) and associated proteins at the ends of linear chromosomes. They are terminated at protruding 3′ single-stranded DNA (sometimes referred to as G-overhang) that together with binding proteins forms the characteristic loop (Griffith *et al*., 1999; Grandin and Charbonneau, 2008; De Boeck *et al*., 2009) (Figure 6). These nucleoprotein structures serve as a protective cap, preventing chromosomal fusions (McClintock, 1941).

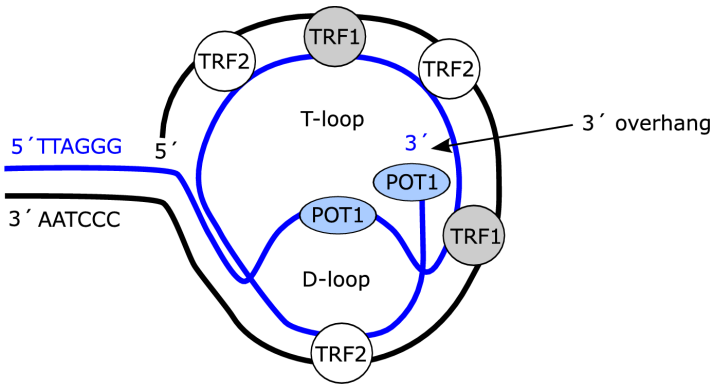

Figure 6 | Structure of vertebrate telomere
with depiction of main binding proteins – telomeric repeat-binding factor 2 (TRF2); telomeric repeat-binding factor 1 (TRF1), which directly bind double-stranded telomeric DNA; protection of telomeres 1 (POT1) binds to single-strand 3´extension and probably plays a role in formation of D-loop. These proteins together with other telomere binding proteins hold the telomere structure. Whole protein complex is also named shelterin or telosome.
Figure based on articles and accompanying diagrams of Grandin N., Charbonneau M. 2008; De Boeck, G. *et al*., 2009.

According to the Olovnikov's hypothesis (1971), the 5´ end of newly synthesized strand meets the "end replication problem" (Olovnikov, 1996) as there is no place for Okazaki fragment at a tip of the linear strand. This problem is overcame by the activity of specific enzyme, ribonucleoprotein telomerase reverse transcriptase (TERT), featuring the ability of 5´ → 3´ DNA synthesis, dependent on TERT own RNA template (Greider and Blackburn, 1987). The TERT is active in early development, but later on most cells lose an ability to express the TERT constitutionally or they express it at a very low level (Collins and Mitchell, 2002). This results in attrition of the ends of telomeres with every cell division (Olovnikov, 1996; Harley *et al.,* 1990). The homogeneous telomere shortening has been proposed as an intrinsic counting mechanism for the potential of cellular division. Finally, when telomeres reach critical length, they induce replicative senescence (Olovnikov, 1996).

More recently it was demonstrated that telomere shortening is not a strictly autonomous clock-like mechanism and that it can be also affected by physiological stress. The cells exposed to high oxygen conditions show more rapid shortening of telomeres than those which were exposed to lowered oxygen tension (von Zglinicki *et al.*, 1995; Forsyth *et al.*, 2003). The average length of telomeres in senescent cells ranges between 5 and 7.6 kbp (Allsopp and Harley, 1995). By contrast, the original length in "young" cells varies from 10 to 15 kbp (Lansdorp, 1995).

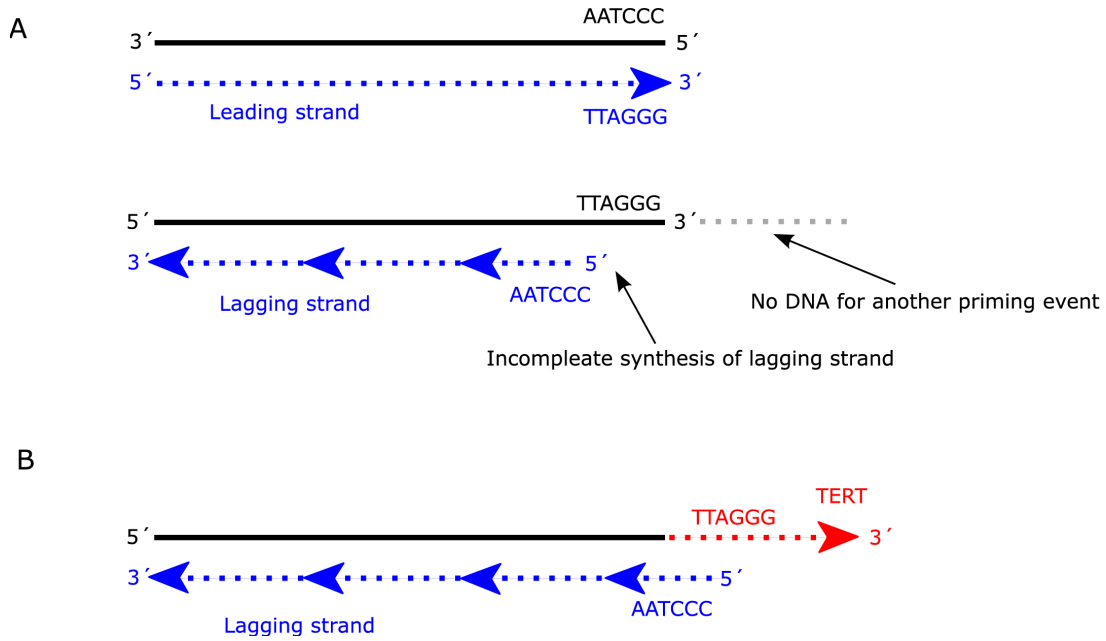

Figure 7 | The model of the end-replication problem
A - The end of newly synthesized 5´strand cannot be completed in full length as there is no place for Okazaki fragment at tip of the 3´ strand. (based on Shay and Wright, 2000)
B – TERT overcoming end-replication problem via prolongation of 3´strand

In addition, it has been proposed that it is not the average length of cellular telomeres that triggers senescence program, but the length of the shortest ones (Hemann *et al.,* 2001; Zou *et al*., 2004). The decisive length of shortest telomeres was suggested to be 2 kbp or even less (Martens *et al.,* 2000).

Based on the observation that telomere length not always stringently correlates with the establishment of senescence, another hypothesis has raised. It suggests that the determining feature is the structure of telomere, not its exact length. As shown in Figure 6, 3´overhang forms a particular protective cap. It seems that telomeres lose this cap at senescence due to the erosion of 3´overhang (Stewart *et al.*, 2003; Ben-Porath and Weinberg, 2004). Together with the loss of the overhang, there is also a loss of telomeric proteins, most notably the TRF2 and POT1, which inhibit the activity of checkpoint kinases ATM and ATR (Karlseder *et al*., 2004; Denchi *et al*.,2007). This results in exposure of chromosomal ends, which are recognized by DNA repair machinery as double strand breaks, triggering DNA damage response and permanent activation of cell cycle checkpoints evolving into cellular senescence (Herbig *et al*., 2004).

Telomeric structure can also be disrupted artificially by overexpression of a dominant negative TRF2 mutant or by G-rich telomeric oligonucleotides (Saretzki *et*

al., 1999). In both cases, disturbed telomeres lead to premature induced senescence, which supports the principal role of protective telomeric structure.

According to this view, the role of TERT should be also reconsidered. Its key effect is not extension of the whole telomere, but rather elongation of 3´overhang (Masutomi *et al*., 2003).

4.3.2. Stress-induced premature senescence (SIPS)

An irreversible cell cycle arrest prior to reaching Hayflick limit, triggered by various sub-lethal stresses, is referred to as stress-induced premature senescence (SIPS) (Toussaint *et al*., 2000). SIPS can be induced by excessive attrition of telomeric structure due to oxidative stress or other factors, as mentioned before. Notably, it can be activated also in telomere independent manner through nonspecific DNA damage (Gorbunova *et al*., 2002). In fact, the process of SIPS is established by the same DNA damage response pathways like in the case of replicative senescence.

Prematurely senescent cells display very similar phenotype to those which senesce replicatively. Nevertheless, it has been shown that they differ at the level of protein expression. The differences exhibited by cells undergoing SIPS have been linked to effects of subcytotoxic stress and termed "molecular scars" (Brack *et al*., 2000; Dierick *et al*., 2002; Pascal *et al*., 2005).

Activating agent of SIPS can be practically any stressor causing directly or indirectly DNA damage. Oxidative stress is likely the most common one. Oxidized base, like 8-oxodeoxyguanine, can result in transversion (G-C → T-A) and can be used as oxidative stress marker, as well. However, oxidations of the sugar-phosphate backbone are more significant for SIPS, as they can lead to DNA double strand breaks and hence to DNA damage response. Notably, agents which do not activate senescence via oxidative stress, often act through induction of common fragile sites and replicative stress. Numerous stressors, known for triggering SIPS, are summarized in Table 1 (based on Toussaint *et al*., 2002), including a mechanism of their DNA damage effect.

As the process of SIPS can be telomere independent, it can affect both normal cells and cancerous cells, expressing TERT (Kim *et al.,* 1994). The induction of SIPS is indeed one of the effects of anticancer treatments, like radiotherapy (Suzuki and Boothman, 2008) or chemotherapy (various anti-cancer drugs mentioned in Table 1, marked with "+", according to the list of approved drugs by U.S. Food and Drug Administration; FDA). Originally it has been thought that the main effect of these therapies is to cause cell death via apoptosis or mitotic catastrophe, but lately was

shown that nonlethal SIPS plays also important role (Schmitt *et al*., 2002; Roninson, 2003; Schmitt, 2007; Suzuki and Boothman, 2008). Senescence induced in normal cells is also plausibly responsible for some of side-effects of these treatments.

Table 1 | The stressors causing SIPS

stressor	***damage mechanism***
5-Bromo-2'-deoxyuridine (BrdU) (Michishita *et al*., 1999)	A thymidine analogue, which incorporates into the DNA during its synthesis and alters its structure, making it more sensitive to secondary stressors, therefore increasing danger of sister–chromatid exchanges, mutations and strand breaks (Taupin, 2007).
Actinomycin D (ActD) (Robles and Adami, 1998) +	ActD binds directly to DNA in the d(CpG) sequences, hence it interferes with replication as well as with transcription (Hou *et al.,* 2002).
Aphidicolin (APC) (Yogev *et al.,* 2006)	Specific inhibitor of DNA polymerases α and δ, capable of induction of common fragile sites (Tedeschi *et al.,* 2004).
Bleomycin (BLM) (Robles and Adami, 1998) +	The main active component, bleomycin A2, binds to DNA, which results in a DNA-bleomycin-Fe^{2+}-O_2 complex; later reduction of oxygen leads to reactive oxygen species (ROS), which cause deletions and strand breaks (Ackland *at al.,* 1988).
***Cis*-platin (cisplatinum; cis-diamminedichloridoplatinum(II); CDDP)** (Wang *et al.,* 1998) +	The DNA crosslinker specific for guanine or guanine/ adenine residues in the sequences d(GpG), d(ApG), less frequently than between d(GpNpG) and d(ApNpG), and between deoxyguanosines in opposite strands. The DNA crosslinks block replication, thus indirectly inducing strand breaks (Bernges and Holler, 1991).
Deficiency in glucose-6-phosphate dehydrogenase (G6PD) (Ho *et al.,* 2000)	G6PD is necessary for generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH). NADPH maintains the level of glutathione, which causes detoxification of free radicals. Deficiency in G6PD therefore indirectly results in increased oxidative stress (Ho *et al*., 2000).
Distamycin A (DM) (Suzuki *et al*., 2002)	DM alters DNA conformation by interaction with AT base pairs in the minor groove of DNA, acts synergistically with BrdU (Luck *et al*., 1977).
Ethanol (EtOH) (Dumont *et al*., 2002)	EtOH modifies membranes fluidity, which results in destabilisation of cellular homeostasis, also contributing to oxidative stress (Dierick *et al.*, 2002).
Etoposide **Doxorubicin (adriamycin)** (Robles *et al*., 1999) +	The topoisomerase II inhibitors, capable of inducing of DNA strand breaks. In addition, doxorubicin can induce free-radical formation, thus contributing to oxidative stress. (Hande, 1998)
Homocysteine (Xu, Neville and Finkel, 2000)	Various cytotoxic properties (reviewed by Perna *et al*., 2003), including formation of H_2O_2 through protein binding and causing oxidative stress.

Table 1 | The stressors causing SIPS

stressor	*damage mechanism*
Hydrogen peroxide (H_2O_2) (Frippiat *et al*., 2001)	H_2O_2 react in Fenton reaction to yield hydroxyl radical [·OH]: $H_2O_2 + Fe^{2+} \rightarrow Fe3^{+} + [\cdot OH] + OH^{-}$ Extremely reactive [·OH] causes most of oxidative stress (Henle and Linn, 1997) H_2O_2 indirectly induces higher order chromatin degradation (enzymatic excision of chromatin loops and their oligomers in matrix-attachment regions) (Konat, 2003)
Hydroxyurea (HU) (Yeo *et al*., 2000) +	The inhibitor of ribonucleotide reductase (RNR). The inhibition of RNR results in a lack of the deoxynucleotides required for DNA synthesis and accordingly leads to DNA single strand breaks (SSBs) (Krakoff *et al*., 1968).
Hyperoxia (von Zglinicki *et al.*, 1995)	O_2 giving rise to superoxide O_2^{-} and other reactive oxygen species (ROS); causing oxidative stress; moreover $O_2^{-\cdot}$ dismutases can produce H_2O_2 and can also reduce and release Fe_3^{+} from ferritin, both contributing to genesis of [·OH] (Henle and Linn, 1997).
Mitomycin C (MMC) (Yang and Hornsby, 1989) +	The DNA crosslinker exceedingly specific for guanine residue in the d(CpG) sequences (Tomasz, 1995).
Phleomycin D1 (Robles *et al*., 1999)	The bleomycin analogue, causes DSBs and SSBs through the production of free oxygen radicals (Robles *et al*., 1999)
Radiofrequency radiation (RFR) electromagnetic fields (EMF) (Rodemann *et al*., 1989)	The mechanism by which RFR EMF cause DNA damage remains currently unknown. However, the energy level of RFR EMF is not sufficient to cause direct breakage of chemical bonds, thus the effects are probably indirect through production of free radicals. Notably, not all papers observe DNA damage as a consequence of RFR EMF (Phillips *et al*., 2009).
***Tert*-butylhydroperoxide (t-BHP)** (Dumont *et al*., 2000)	A direct-acting oxidative stress-inducing agent (Adams *et al*., 1993).
Ultraviolet light A (UVA) (400–320 nm) (Wlaschek *et al*., 2000)	Ionizing radiation - damage DNA by its direct ionization (capability of bond breakage) or indirectly via production free radicals (major contribution). Yet the DNA bases absorb weakly above 320 nm, UVA can still produce at least some cyclobutane pyrimidine dimers (CPDs) directly (Jiang *et al*., 2009).
Ultraviolet light B (UVB) (320–280 nm) (Medrano *et al*., 1995; Chainiaux *et al*., 2002)	Ionizing radiation. Furthermore, UVB produces cyclobutane pyrimidine dimers (CPDs) at TT and TC sites, with low abundance also in CC and CT sites. Nevertheless, CC sites CPDs, even generated at low quantities, show high mutagenic potential (Cadet *et al*., 2005).
X-rays (Suzuki and Boothman, 2008)	Ionizing radiation.

Table 1 | The stressors causing SIPS

stressor	*damage mechanism*
γ-rays (Igarashi *et al*., 2007)	Ionizing radiation.

4.3.3. Oncogene-induced senescence (OIS)

An activation of certain oncogenes also belongs among stresses which can lead to senescent phenotype. Oncogenes are traditionally connected with cell transformation and cancer. Nevertheless, in normal cells the sole activation of oncogene does not end in cellular transformation. On the contrary, the initial burst of proliferation is succeeded by proliferative arrest. This fact was first observed on cultured fibroblasts, which were transduced with activated RAS allele H-RAS^{V12} (Serrano *et al*., 1997). Later on, oncogene-induced senescence (OIS) was described for numerous other oncogenes (summarized in Table 2). Not surprisingly, many of them represent RAS effectors in signaling pathways transducing mitogenic and surviving signals (e.g. Raf/MEK/Erk pathway; Akt/PKB pathway; Figure 9).

OIS occurrence *in vivo* has been questioned for a long time. Opponents demonstrated that in *in vitro* studies, oncogenes have been expressed ectopically in supraphysiological levels. On the contrary, murine embryonic fibroblasts which expressed a single activated K-RAS^{V12} allele driven by its own promoter, did not establish senescence phenotype and proliferate like immortal cells (Guerra *et al*. 2003). These findings led to the hypothesis that OIS is an exclusively artificial phenomenon caused by overexpression of oncogenes. Nonetheless, later studies presented clear evidence that OIS occurs *in vivo* as well. OIS presence has been independently detected in lung adenomas (Collado *et al*., 2005), T-cell lymphomas (Braig *et al*., 2005), prostate tumors (Chen *et al*., 2005) and benign melanocytic nevi (Michaloglou *et al*., 2005). Notably, OIS occurs in benign but not in advanced tumors. This supports the view of OIS as important tumor-suppressive barrier.

Dispute between studies with overexpressed mutant alleles and those with alleles expressed on lower levels pointed out the importance of oncogenic signal intensity. Moreover, this was later well demonstrated on MTB–TRAS mouse models. These models express an oncogenic H-RAS^{12V} allele under the control of a tetracycline-inducible promoter, which allows to titrate the levels of RAS activation. Results from these models showed that only high and not low levels of RAS activation were capable

of senescence induction (Sarkisian *et al*., 2007). Another problem which remains currently unclear is why some cell types are sensitive to induction of OIS, while other types are not.

Table 2 | Genes, whose altered expression triggers OIS
(based on Courtois-Cox *et al*., 2008; Di Micco *et al*., 2007)

Oncogenes	***Gene product function***	***Cell type observed to exhibit senescence response***
AKT (Miyauchi *et al*., 2004)	kinase in Akt/PKB pathway	endothelial cells and murine embryonic fibroblasts
BRAF (Michaloglou *et al*., 2005)	kinase in Raf/MEK/Erk pathway	melanocytes
CDC6 (Bartkova *et al*., 2006)	DNA replication licensing factor	human diploid fibroblasts
Cyclin E (Bartkova *et al*., 2006)	CDK2 modulator	U20S (cell line from osteosarcoma)
E2F1 (Dimri *et al.*, 2000)	pro-growth transcription factor	human diploid fibroblasts
IFN-β (Moiseeva *et al*., 2006)	cytokine	human diploid fibroblasts
MEK (Lin *et al*. 1998)	kinase in Raf/MEK/Erk pathway	human diploid fibroblast and murine embryonic fibroblast
MOS (Bartkova *et al*., 2006)	activator of the Raf/MEK/Erk pathway	human diploid fibroblast
MYC (Grandori *et al*., 2003)	pro- growth transcription factor	human diploid fibroblast
RAC1 (Debidda *et al*., 2006)	GTPase belonging to Ras superfamily of small GTP-binding proteins	murine embryonic fibroblast
RAF1 (Zhu *et al*., 1998)	protein kinase in growth-factor signaling pathway involved in the MAPK cascade.	human diploid fibroblast, murine embryonic fibroblast (require overexpression) and melanocytes
RAS (Serrano *et al*., 1997)	membrane-associated small GTPase activating the MAPK cascade.	human diploid fibroblast and murine embryonic fibroblast (require overexpression)
SMURF2 (Zhang and Cohen, 2004)	SMAD specific E3 ubiquitin protein ligase 2	human diploid fibroblast
STAT5A (Mallette *et al*., 2007)	pro- growth transcription factor	human diploid fibroblasts
TGF-β (Katakura *et al*., 1999)	growth-factor	A549 (cell line from human lung adenocarcinoma)

Table 2 | Genes, whose altered expression triggers OIS
(based on Courtois-Cox *et al*., 2008; Di Micco *et al*., 2007)

Tumor suppressors	***Gene product function***	***Cell type exhibiting senescence response***
NF1 (Courtois-Cox *et al*., 2006)	Ras GTPase activating protein (GAP)	human diploid fibroblasts
PML (Pearson *et al*., 2000)	regulator of p53	murine embryonic fibroblasts
PTEN (Courtois-Cox *et al*., 2006)	protein or lipid phosphatase - inhibitor of growth factors signaling pathway	murine embryonic fibroblasts and IMR90 (human diploid fibroblast-like cell line from lung tissue)

The whole picture how oncogenes induce senescence has not yet been fully elucidated. Several mechanisms of OIS induction have been proposed. They are not mutually exclusive and at least in some cases they may contribute to OIS together (Courtois-Cox *et al*., 2008).

4.3.3.1. Replicative stress model of OIS induction

Oncogenes induce the cellular proliferation through direct or indirect deregulation of cyclin-dependent kinases. In consequence, some replication origins can be fired repeatedly, causing hyper-replication. Hyper-replication leads to increase in replicative stress, which in result generates DNA damage foci. DNA damage triggers DDR that can induce senescence (Figure 8).

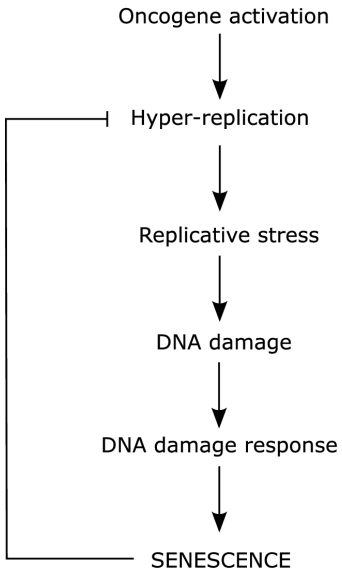

Figure 8 | Model of OIS induction through replicative stress
(based on model diagrams from Bartkova *et al*., 2006 and Di Micco *et al*., 2006)

This model was first proposed in two reports (Bartkova *et al*., 2005 and Gorgoulis *et al.,* 2005). Gorgoulis and colleagues observed in cancer cell lines (SAOS-2 osteosarcoma and HeLa cervical carcinoma lines) DDR, which were triggered even in the absence of exogenous stressor. They demonstrated that DDR is dependent on DNA replication. In addition, γH2AX, 53BP1, and ATR-ATRIP complexes were detected to colocalize with replicative stress foci, thus providing the evidence that oncogene induced-replicative stress is capable of triggering DDR. Moreover, foci of DNA damage correspond with common fragile sites. Similar results were independently obtained by Bartkova and colleagues on U2OS cells U2-OS cell with induced cyclin E expression. In further support of this hypothesis, inhibition of ATM kinase suppresses the senescence and leads to intense tumorigenesis (Bartkova *et al*., 2006; Di Micco *et al*., 2006). On the contrary, depletion of p16, which is also raised in response to oncogene activation, did not result in senescence suppression. This suggests that DDR has at least in the observed cases a primary role in senescence induction (Bartkova *et al*., 2006).

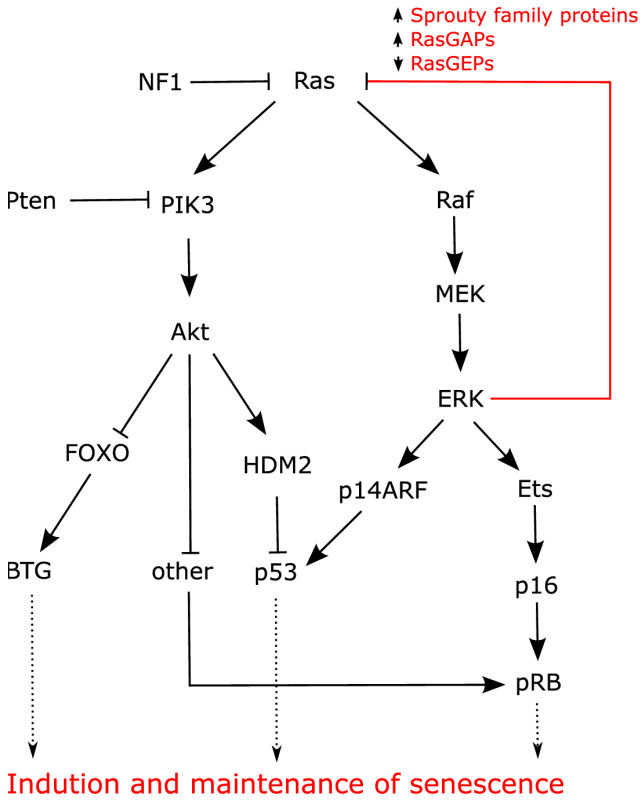

Figure 9 | Model of OIS induction via negative feedback signaling
Negative feedback signals are triggered by the Raf/MEK/ERK pathway. Hence the RAS is suppressed through suppression of Ras exchange factors, upregulation of Sprouty proteins and RasGAPs. In result, pro-proliferative signaling is ended and Rb and/or p53 pathways can be activated. (Adapted with slight modifications from Courtois-Cox *et al*., 2006).

4.3.3.2. Alternative models of OIS induction

Lee and colleagues (1999) suggested that expression of mutant RAS allele contributes to intracellular increment of ROS. As noted above, oxidative stress is indeed a potent DNA damaging agent. Nevertheless, it is not certain whether the increment of ROS is high enough to induce required DDR. Likewise, the mechanism by which activated RAS can elevate the levels of ROS stays ill-defined and not all oncogenes seem to contribute to DNA damage through ROS production (Ray *et al.*, 2006).A different point of view was brought to this issue by the report of Courtois-Cox and colleagues (2006). They proposed that the primary burst of mitogenic signaling sparks the negative feedback, which in result terminates the mitogenic signal and activates OIS (Figure 9).

4.3.4. Integrative scheme of proposed models

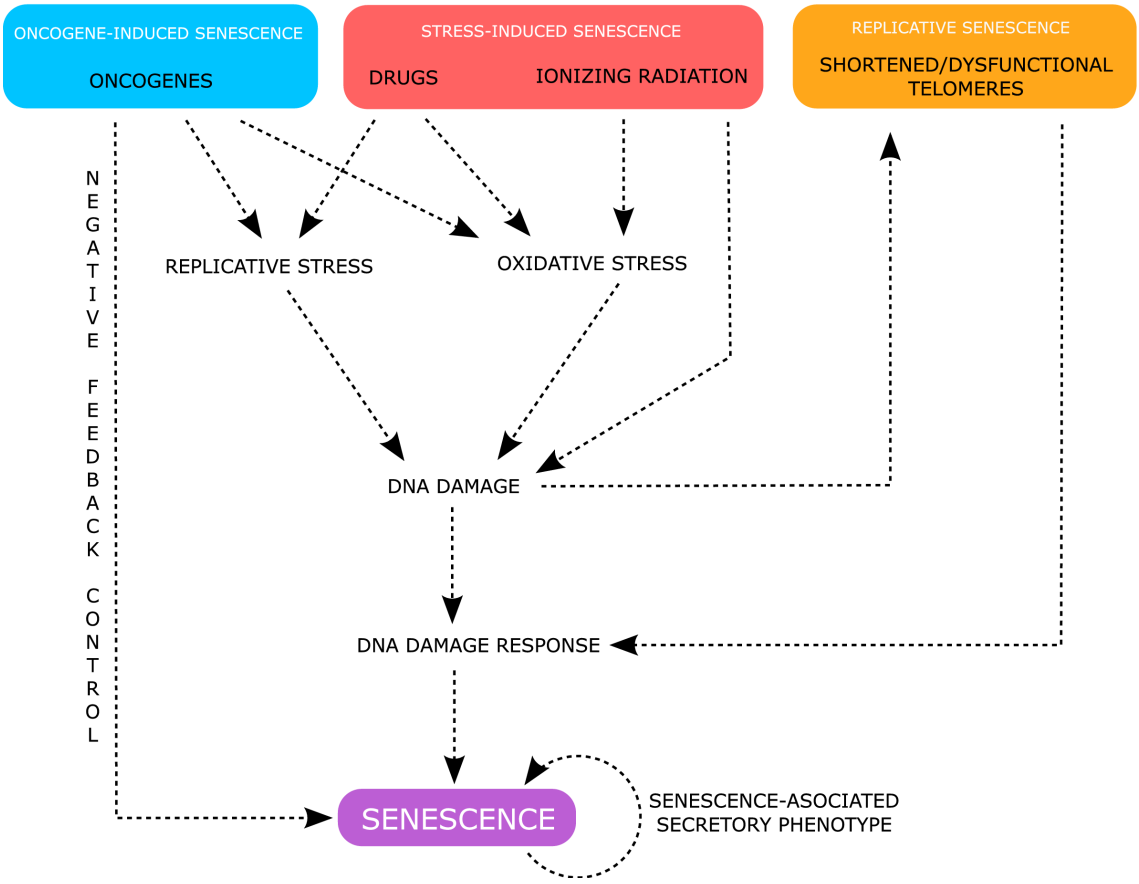

Figure 10 | Integrative scheme of proposed models
The scheme demonstrates mechanisms of action of various stimuli that are capable of inducing cellular senescence phenotype.

4.4. Cellular senescence in oncogenesis and aging

4.4.1. Current view – an evolutionary trade-off

As demonstrated above, cellular senescence plays a crucial role as an oncogenesis barrier. Particularly in preneoplastic lesions, it limits further cellular proliferation. Artificially induced senescence acts also as an important outcome of anticancer treatments. Tumor-suppressive mechanisms are necessary for surviving of complex multicellular organisms. However, it seems that they are not exclusively beneficial and rather represent a double-edged sword.

Senescent cells, which accumulate in tissues, no longer play a proper function and deplete the renewable capacity of the tissues. In addition, senescence-associated secretory phenotype (SAPS) disrupts tissue homeostasis through secretion of degradation enzymes and cytokines including inflammatory species, which can contribute to the development of age-related pathologies. Indeed, accumulation of senescent cells has been observed at sites of age-related atherosclerosis (Chang and Harley, 1995). Even more importantly, SAPS can also contribute to the development of cancer (Krtolica *et al*., 2001). This is in agreement with the observed general increase of cancer occurrence during aging (Meza *et al*., 2008).

The contradictory effect of senescence can be explained by evolutionary theory of antagonistic pleiotropy (Campisi, 2003; Kirkwood and Austad, 2000). This theory states that pleiotropic traits, which increase survival at young age but act deleteriously in old individuals, will be fixed in population, because the benefit in early life outweighs the deteriorative effect in old age.

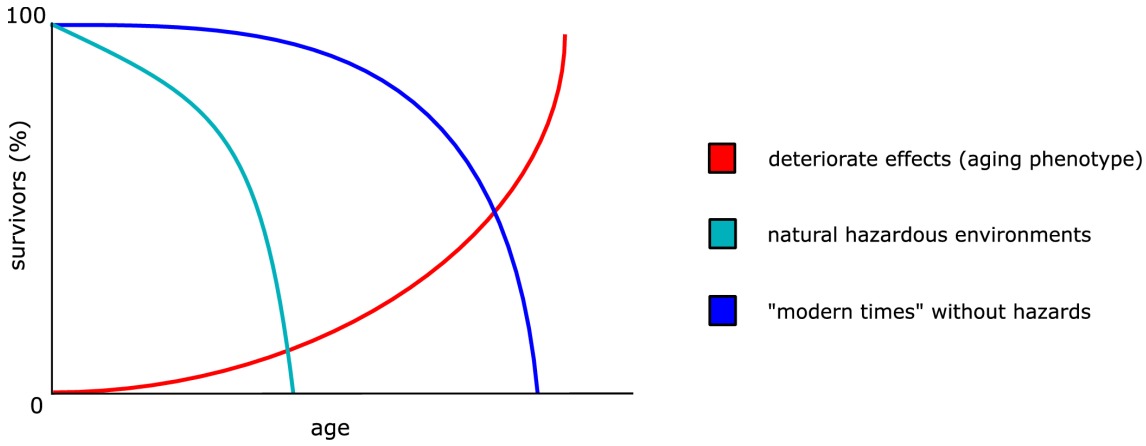

Figure 11: Evolution of aging
- deteriorate effects of pleiotropic traits were not eliminated through ages because of early death of our predecessors due to exogenous hazards (adapted from Campisi, 2003).

Additionally, in natural conditions most individuals die due to endogenous hazards before reaching advanced age (e.g. predation, infections and starvation). Deteriorating effects of pleiotropic traits, which are demonstrated in advanced age, therefore end in a “shadow zone”, where natural selection cannot act. However, humans in developed countries get rid of nearly all natural hazards, so we can now well observe negative effects of these pleiotropic traits on ourselves, demonstrated as age-related pathologies (Figure 11).

4.4.2. Original view – the death clock

From the very beginning of Hayflick discovery of replicative senescence, it has been proposed as a mechanism contributing to aging of organism. This is well reflected in the chosen term, the "senescence", derived from the Latin word "*senescere*", meaning "grow old". Later discovered telomere shortening was then suggested to be an intrinsic "death clock" - an active, teleological mechanism, which limits the renewable capacity of tissues and thus restrains the lifespan of complex multicellular organisms. Nevertheless, as described above, shortening of telomeres is not homogeneous, nor is the length of telomeres crucial for establishment of senescence. Even if the molecular mechanisms of "death clock" were not disproved, the programmed aging still has to face several challenges. In particular, what evolutionary reason would lead to evolution of such a trait?

Nusbaum (1996) suggests that aging inevitably directing to death is beneficial on the level of the species. According to his theory, shorter lifespan should be reflected in shorter duration of pregnancy, thus indirectly lead to increased number of generations in a time period. More frequent turnover of generations ought to result in better adaptive potential of the species to environmental changes. In addition, as old individuals exhaust the local resources, their death should enhance the living conditions of their offspring.

On the contrary, as in natural conditions extensive majority of individuals die young due to exogenous hazards, why would there be a selection pressure for the development of specific eliminating mechanism of the old ones? Besides, even if organisms would not die due to exogenous hazards, there would be a potent counter-pressure against the development of aging mechanism on the levels of individuals. Non-aging individuals would have a longer fertility span, therefore would in all probability leave more offspring than aging ones. This would necessarily lead to fixation of non-aging phenotype in population, regardless of potential benefits of aging on species level.

5. Conclusions

The field of cellular senescence has made a great leap forward in recent years. It has been demonstrated that DNA damage and corresponding DNA damage response signaling are causally linked to establishment of senescent phenotype. The model of oncogene-induce replicative stress proposes a plausible explanation how are activated oncogenes capable of cellular senescence induction. Observation of senescent cells in benign and preneoplastic lesions, preventing their further proliferation, supports a view that cellular senescence acts as a tumorigenesis barrier.

However, many questions still remain open. Numerous detailed mechanisms in mentioned pathways are waiting to be elucidated. Moreover, the relationship between parallel ways of senescence activation and significance of their impact in overall context are not yet fully understood.

Cellular senescence plays an important role in cancer and aging. Thus, it is of great clinical importance to further study its underlying mechanisms. Cancer is one of the leading causes of death in developed countries and nowadays the burden of cancer is constantly increasing even in many developing countries. In addition, as the aging population grows in developed countries, it raises hand in hand the prevalence of age-related pathologies, which causes an increasing demand for new efficient treatments. The research of cellular senescence may hopefully provide requisite knowledge for the struggle with these diseases.

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