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# Mechanisms of replicative, drug- and oncogene-induced cellular senescence

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# ABSTRACT

Cells continuously experience various forms of exogenous and endogenous stress stimuli. Cellular senescence, a state of permanent cell-cycle arrest, is a physiological response that prevents proliferation of damaged cells. It is an important tumor suppressive mechanism, because evading senescence in pre-malignant tumors leads to cancer progression. However, in the long term, senescence can also contribute to tissue aging. Our understanding of the causes and consequences of senescence can provide important insights into processes such as cancer development and aging and may help us design new therapeutic approaches.

In the first part of my thesis, we investigated a relationship between two known tumor suppressor pathways, the DNA Damage Response (DDR) and the Alternative Reading Frame (ARF), in response to oncogenic insults. By using several mouse models, human clinical samples and cell culture models we showed, that ARF activation occurs at a later stage of tumor progression than activation of the DDR. Moreover, ARF activation requires a higher level (threshold) of oncogenic stress than DDR. Therefore, we proposed, that ARF represents a delayed and complementary barrier to tumor progression.

Senescence-associated heterochromatin foci (SAHF) formation, representing pronounced changes in chromatin, has been previously established as a mechanism contributing to maintenance of the senescence phenotype in oncogene-induced and replicative senescence. In the second part of this thesis, we thus analyzed to what extent does SAHF formation represent a universal feature of the senescence program. We concluded that SAHF are dispensable for some types of cellular senescence and occur in an insult- and cell type-dependent manner.

Next, we investigated cytokine expression and signaling in cellular senescence evoked in tumor cells by diverse genotoxic drugs. We found, that senescent cells secrete a broad spectrum of cytokines/chemokines and persistently activate the JAK/STAT signaling pathway. Our data thus suggest autocrine/paracrine effects of cytokine signaling on senescence-associated gene expression, a phenomenon that is likely highly relevant to the outcome of cancer chemotherapy.

Finally, we provide novel insights into our current knowledge about regulation of the tumor suppressor PML in drug-induced senescence. According to our data, transcriptional upregulation of PML is independent of the p53 tumor suppressor pathway and is controlled by JAK/STAT signaling, via binding of the transcription factor STAT to the ISRE element in the PML gene promoter.

**Keywords:** ARF, cytokine, DNA damage response, oncogene, PML, SAHF, senescence

# ABSTRAKT

Buňky jsou trvale vystaveny různým formám stresu, který pochází jak z vnitřního, tak z vnějšího prostředí. Buněčná senescence, permanentní zástava buněčného cyklu, představuje fyziologickou odpověď organismu, která zabraňuje proliferaci poškozených buněk. Jedná se o významný mechanismus nádorové suprese, jelikož inaktivace této bariéry v pre-maligních stadiích tumoru vede k rozvoji rakovinného bujení. Na druhou stranu, v dlouhodobé perspektivě se senescence podílí na stárnutí tkáně organismu. Porozumění příčinám a důsledkům senescence nám může poskytnout důležité informace o procesech jako je rakovinné bujení a stárnutí organismu, což může přispět k nalezení nových terapeutických přístupů.

V první části dizertační práce jsme studovali vztah mezi dvěma tumor-suprimujícími signálními dráhami, DDR a ARF, v odpovědi na aktivaci onkogenu. S využitím několika myších modelů, lidských klinických vzorků a buněčných kultur jsme ukázali, že k aktivaci signální dráhy ARF dochází v pozdějším stádiu vývoje nádoru než k aktivaci dráhy DDR. Pro spuštění ARF signalizace je navíc zapotřebí vyšší hladiny onkogenního stresu než pro aktivaci DDR. ARF tedy představuje pozdní a komplementární bariéru nádorové suprese.

Změny chromatinu spojené se senescencí (SAHF) jsou obecně považovány za mechanismus přispívající k zástavě buněčného cyklu v onkogenní a replikativní senescenci. V druhé části této dizertační práce jsme proto analyzovali, do jaké míry jsou změny chromatinu univerzální u různých typů senescence. Zjistili jsme, že některé typy buněčné senescence nejsou doprovázeny změnami chromatinu a ty se tedy vyskytují v závislosti na povaze signálu spouštějícího senescenci a také v závislosti na buněčném typu.

Dále jsme studovali cytokinovou expresi a signalizaci v nádorových buňkách, které byly vystaveny účinku různých genotoxických látek. Naše data ukazují, že senescentní buňky sekretují široké spektrum cytokinů a trvale aktivují JAK/STAT signální dráhu. Tato cytokinová signalizace má autokrinní/parakrinní efekt na genovou expresi, což je relevantní vzhledem k chemoterapii používané při léčbě nádoru.

V poslední části práce poskytujeme nový vhled do současných znalostí o regulaci nádorového supressoru PML v chemickými látkami indukované senescenci. Dle našich výsledků je regulace transkripce PML nezávislá na dráze p53 a je kontrolována signální dráhou JAK/STAT prostřednictvím vazby transkripčního faktoru STAT na element ISRE, jež je lokalizován v promotoru genu PML.

Klíčová slova: ARF, cytokin, signalizace poškození DNA, onkogen, PML, SAHF, senescence

# Table of contents

**1. INTRODUCTION** ..... 12
1.1 Markers of senescent cells ..... 12
1.2 Triggers of cellular senescence ..... 14
1.2.1 Replicative cellular senescence ..... 14
1.2.2 Oncogene-induced senescence ..... 15
1.2.3 Drug-induced senescence ..... 15
1.3 Established senescence signaling pathways ..... 16
1.3.1 The DNA Damage Response pathway ..... 16
1.3.2 The p53-p21 pathway ..... 18
1.3.3 The $p16^{INK4a}$-RB pathway ..... 20
1.3.4 Senescence-Associated Heterochromatin Foci (SAHF) ..... 21
1.3.5 Senescence-Associated Secretory Phenotype (SASP) ..... 22
1.3.5.1 The JAK/STAT signaling pathway ..... 22
1.3.5.2 Cytokines and cellular senescence ..... 23
1.4 Role of the promyelocytic leukemia protein (PML) in senescence ..... 24
1.4.1 PML protein structure and PML nuclear bodies ..... 24
1.4.2 PML gene regulation ..... 25
1.4.3 Functions of the PML protein ..... 25
1.4.3.1 Role of PML in the DNA Damage Response pathway ..... 26
1.4.3.2 Roles of PML in the p53-p21 and $p16^{INK4a}$-RB pathways ..... 27
1.4.3.3 Role of PML in SAHF formation ..... 28
1.5 Cellular senescence and tumor suppression ..... 29
**2. AIMS OF THE STUDY** ..... 30
**3. METHODS** ..... 31
**4. RESULTS AND DISCUSSION** ..... 32
4.1 List of publications ..... 32
4.2 Linking of publications ..... 33

4.3 Publication 1 ..... 34
4.4 Publication 2 ..... 48
4.5 Publication 3 ..... 61
4.6 Publication 4 ..... 74
4.7 Discussion ..... 90
**5. CONCLUSIONS** ..... 99
**6. REFERENCES** ..... 101

# ABBREVIATIONS

| | |
|---|---|
| ARF | alternative open reading frame |
| ASF1a | anti-silencing function 1A histone chaperone |
| ATM | ataxia telangiectasia mutated |
| ATR | ataxia telangiectasia and Rad3 related |
| BLM | Bloom syndrome protein |
| BRAF | v-raf murine sarcoma viral oncogene homolog B |
| BRCA1 | breast cancer 1 |
| BrdU | 5-bromo-2-deoxyuridine |
| CDK | cyclin-dependent kinase |
| C/EBP-β | CCAAT/enhancer binding protein beta |
| CHK1 | checkpoint kinase 1 |
| CHK2 | checkpoint kinase 2 |
| CXCR2 | chemokine (C-X-C motif) receptor 2 |
| DAPI | 4`,6-diamidino-2-phenylindole |
| DDR | DNA damage response |
| DMA | distamycin A |
| DNA | deoxyribonucleic acid |
| DNA-PK | DNA-dependent protein kinase |
| DSB | double-strand break |
| ETS | ETS transcription factor |
| E1A | early region 1A |
| E2F1 | E2F transcription factor 1 |
| GAS | gamma activated site |
| γH2AX | phosphorylated histone H2AX |
| HdCDT | *Haemophilus ducreyi* Cytolethal Distending Toxin |
| HIRA | histone cell cycle regulator |
| HMGA | high mobility group AT-hook |
| HRAS | Harvey rat sarcoma viral oncogene homolog |

| | |
|---|---|
| HPV16 | human papilloma virus |
| HP1 | heterochromatin protein 1 |
| H3K9Me | methylated histone H3 on Lys 9 |
| IFN | interferon |
| IFNβ | interferon beta |
| IFNγ | interferon gamma |
| IL-6 | interleukin 6 |
| IL-8 | interleukin 8 |
| IL-10 | interleukin 10 |
| IL-24 | interleukin 24 |
| IR | ionizing radiation |
| IRIF | ionizing radiation-induced foci |
| ISG15 | ISG15 ubiquitin-like modifier |
| ISRE | interferon-stimulated response element |
| JAK | Janus kinase |
| KRAS | Kirsten rat sarcoma viral oncogene homolog |
| MDC1 | mediator of DNA-damage checkpoint 1 |
| MDM2 | E3 ubiquitin-protein ligase MDM2 |
| MEK | mitogen-activated protein kinase kinase 1 |
| MRE11 | meiotic recombination 11 |
| MX1 | myxovirus (influenza virus) resistance 1 |
| MYC | v-myc avian myelocytomatosis viral oncogene homolog |
| NBS1 | Nijmegen breakage syndrome 1 |
| NEK2 | NIMA-related kinase 2 |
| NF1 | neurofibromin 1 |
| NPM | nucleophosmin |
| PML | promyelocytic leukemia protein |
| PML-NBs | promyelocytic leukemia protein nuclear bodies |
| POT1 | protection of telomeres 1 |
| PP1 | protein-phosphatase 1 |

| | |
|---|---|
| PTEN | phosphatase and tensin homolog |
| $p14^{ARF}$ | alternative open reading frame protein 14 |
| $p16^{INK4a}$ | CDK inhibitor p16 |
| p21 | CDK-interaction protein 1 |
| p53 | p53 tumor suppressor |
| RAD50 | RAD50 homolog |
| RAD51 | RAD51 recombinase |
| RAF | v-raf murine sarcoma viral oncogene homolog |
| RARα | retinoic acid receptor α |
| RB | retinoblastoma protein |
| RECQL | RecQ protein-like |
| RPA | replication protein A |
| ROS | reactive oxygen species |
| SA-β-gal | senescence-associated β-galactosidase |
| SAHF | senescence-associated heterochromatin foci |
| SASP | senescence-associated secretory phenotype |
| SH2 | src homology 2 |
| ssDNA | single-strand DNA |
| STAT | signal transducer and activator of transcription |
| TOPBP1 | topoisomerase (DNA) II binding protein 1 |
| TRF2 | telomeric repeat binding factor 2 |
| TYK2 | tyrosine kinase 2 |
| 53BP1 | p53 binding protein 1 |

# 1. INTRODUCTION

Cellular senescence was described more than 40 years ago as a process that limits the growth of normal human cells in culture (Hayflick, 1965). Hayflick and coworkers demonstrated that these cells can proliferate *in vitro* for approximately 55 populations doublings before their proliferative capacity succumbs to proliferation arrest despite the presence of abundant nutrients and mitogens (Adams, 2009; Campisi and d'Adda di Fagagna, 2007). The term cellular senescence therefore denotes a stable and long-term loss of proliferative capacity, despite continued viability and metabolic activity (Kuilman et al., 2010).

## 1.1 Markers of senescent cells

An exclusive (specific) and universal marker of senescence has not been identified to date. However, senescent cells display several phenotypes, which in combination can define the senescent state:

a) **Growth arrest.** Senescent cells are unable to progress through the cell cycle. The senescence growth arrest is essentially permanent and cannot be reversed by known physiological stimuli (Di Leonardo et al., 1994; Serrano et al., 1997).

b) **Changes in morphology.** Senescent cells usually show altered cellular morphology. The most obvious is their increase in cell size. In some cases, cells can enlarge more than two-fold relative to the size of nonsenescent cells (Hayflick, 1965).

c) **Expression of SA- β-galactosidase.** A biomarker, which can be used for a more specific identification of senescent cells, is the senescence-associated β-galactosidase (SA- β-gal) (Dimri et al., 1995). The SA- β-gal probably derives from lysosomal β-D-galactosidase encoded by the GLB1 gene and reflects the

increased lysosomal biogenesis commonly occurring in senescent cells. It can be measured at suboptimal pH 6 (Lee et al., 2006).

d) **Lipofuscin accumulation.** Lipofuscin, an insoluble aggregate of oxidized proteins, accumulates in aged tissues (Jung et al., 2007). In contrast to SA- β-galactosidase, lipofuscin can be detected also in formalin-fixed paraffin-embedded (FFPE) archival tissues by a lipophilic histochemical stain Sudan Black B (SBB) (Georgakopoulou et al., 2013).

e) **DDR signaling.** Persistent activation of DNA Damage Response (DDR) enables cells to sense damaged DNA and respond by arresting cell-cycle progression. Many proteins participating in the DDR such as DNA damage sensors (e.g. MRE11-RAD50-NBS1), modified histones (γH2AX), adaptor proteins (MDC1 and 53BP1), protein kinases (ATM and CHK2) can be detected in senescent cells (Bartkova et al., 2005; di Fagagna et al., 2003; Herbig et al., 2004).

f) **Activation of tumor suppressor pathways.** Establishment of the senescence program is mediated by activation of tumor suppressor pathways such as p53-p21 and $p16^{INK4a}$-RB (Lowe et al., 2004). Therefore, accumulation (e.g. $p16^{INK4a}$, p21) or posttranslational modification (e.g. pSer15 p53, phosphorylated RB) of proteins involved in these pathways can be used (to some extent) as biomarkers of senescence (Chen et al., 2005; Lin et al., 1998; Serrano et al., 1997; Stein et al., 1990).

g) **Senescence-associated heterochromatin foci (SAHF).** Senescence can be accompanied by specific alterations of the chromatin, known as senescence-associated heterochromatic foci (Narita et al., 2003). In contrast to cycling human cells, some senescent cells display a punctuate nuclear pattern after staining of DNA by the 4',6-diamidino-2-phenylindole (DAPI) dye.

h) **Senescence-associated secretory phenotype (SASP).** Senescent cells exhibit profound changes in their secretome called the senescence-associated secretory phenotype: SASP (Coppe et al., 2008). Numerous inflammatory proteins and extracellular-matrix associated factors are induced in senescent cells and secreted to allow communication both within and also among cells (Acosta et al., 2008; Goldstein et al., 1994; Goldstein et al., 1991).

# 1.2 Triggers of cellular senescence

Although cellular senescence was first described in the context with excessive rounds of cell division (Hayflick, 1965), it can be caused also by various cellular stresses e.g. oncogene activation or tumor suppressor inactivation (Lin et al., 1998; Serrano et al., 1997; Zhu et al., 1998), oxidative stress (Adams, 2009; Chen and Ames, 1994), DNA-damaging agents such as ionizing radiation (Di Leonardo et al., 1994; Robles and Adami, 1998) and drugs (te Poele et al., 2002; Wang et al., 1998a).

## 1.2.1 Replicative cellular senescence

In 1990 it was shown that as human primary fibroblasts are cultured towards the end of their replicative lifespan, telomere length gradually decreases (Harley et al., 1990). Other authors later demonstrated that the activity of telomerase, which is the enzyme counteracting telomere shortening, is high in immortal cells and absent from human somatic cells. At the same time they have shown that human primary fibroblasts can be immortalized by transfection of the catalytic subunit of human telomerase (hTERT) (Bodnar et al., 1998). Telomere attrition was therefore established as a trigger for replicative cellular senescence originally described by Hayflick in 1965 (Hayflick, 1965, 2000).

## 1.2.2 Oncogene-induced senescence

Oncogene activation is a hallmark of cell transformation and cancer. However, early studies on mutant HRAS led to the discovery that it induces cell cycle arrest when it is introduced alone into primary cells (Land et al., 1983; Serrano et al., 1997). Subsequently, other oncogenes such as BRAF and MEK were shown to cause senescence when expressed as oncogenic versions or overexpressed (Lin et al., 1998; Michaloglou et al., 2005; Zhu et al., 1998). Unlike replicative senescence, oncogene-induced senescence can´t be bypassed by transfection of the catalytic subunit of human telomerase (hTERT), confirming its independence from telomere attrition (Wei et al., 1999).

This initial *in vitro* observation was validated *in vivo* by using mouse models. The conditional gene knock-in mouse model of KRAS was shown to trigger senescence during the early stages of pancreatic and lung tumorigenesis (Collado et al., 2005). Oncogenic BRAF expression in mouse leads to lung adenomas with features of cellular senescence (Dankort et al., 2007; Dhomen et al., 2009). Transgenic expression of β-catenin induces senescence in murine lymphocytes (Xu et al., 2008). In addition, loss of the tumor suppressor PTEN in mice prostate leads to development of benign lesions with presence of senescent cells (Chen et al., 2005).

Importantly, occurrence of senescence during tumor progression has been documented also in human premalignant lesions. Senescent cells were found in human colon adenomas (Bartkova et al., 2006), in benign melanocytic nevi carrying oncogenic mutant BRAF (Michaloglou et al., 2005), in neurofibromas from NF1 mutant patients (Courtois-Cox et al., 2006) and also in early-stages of human prostate cancer (Chen et al., 2005).

## 1.2.3 Drug-induced senescence

Anticancer drugs can usually induce either apoptosis or proliferation arrest, however, senescence can be achieved by using lower drug concentrations than those required to trigger apoptosis (Chang et al., 2002; Rebbaa et al., 2003). Importantly, drug-induced senescence, unlike most other types of premature senescence, can be established in cancer cells (Rebbaa, 2005). Many chemotherapeutic agents have been reported to force cells into senescence. The

first group of such drugs represent topoisomerase inhibitors such as campthotecin, etoposide and doxorubicin, which interfere with the ability of topoisomerases to re-ligate DNA (Chang et al., 1999a; Han et al., 2002). Other potent inductors of senescence are alkylating agents, platinum-based drugs causing crosslinking of DNA, and hydroxyurea, a well-known inhibitor of ribonucleotide reductase (Chang et al., 1999a; Yeo et al., 2000).

## 1.3 Established senescence signaling pathways

Several pathways have been shown to play roles in cellular senescence. These include among others the DNA-damage response (DDR), p53-p21 and $p16^{INK4a}$-RB pathways. Changes in chromatin (SAHF) (Narita et al., 2003) and proteins secreted during senescence (SASP) (Coppe et al., 2008) also contribute to establishment and maintenance of the senescence program.

### 1.3.1 The DNA Damage Response pathway

Ruptures of the DNA backbone can lead to the generation of single-stranded DNA (ssDNA) or DNA double-strand breaks (DSBs). Both structures compromise the structural stability of chromosomes (d'Adda di Fagagna, 2008). Signaling kinases, notably ATR, ATM and DNA-PK, are recruited to the sites of DNA damage and are activated. The recruitment leads to the phosphorylation of Ser-139 of histone H2AX adjacent to the site of DNA damage, which is necessary for focal assembly of checkpoint and DNA repair factors including NBS1, 53BP1 and BRCA1 (Celeste et al., 2002). An increase of local ATR and ATM activity also engage DDR factors which function far from the site of DNA damage such as transducer kinases CHK1 and CHK2. Both kinases then spread DDR signaling by phosphorylating its substrates throughout the cell (Bekker-Jensen et al., 2006; Lukas et al., 2003).

DNA damage signaling leads to establishment of transient cell cycle arrest or senescence depending on the severity of damage to the genome. Robust activation of the DDR pathway was shown to have a causative role in the establishment and also maintenance of

cellular senescence caused by different stimuli such as telomere shortering, oncogene activation or treatment by chemotherapeutic agents (Chang et al., 1999a).

In molecular terms telomeres consist of repetitive DNA sequences at the end of linear chromosomes that protects the DNA ends from recombination and degradation (Chan et al., 2001). Due to inability of the replication machinery to copy the ends of linear molecules each DNA replication cycle therefore reduces the number of telomere repeats (Blasco, 2005). When telomeres shorten below a certain threshold length, their protective structure consisting of telomere-associated proteins such as TRF2 and POT1 is disrupted and unrestrained DNA damage response (DDR) is activated (di Fagagna et al., 2003; Harley et al., 1990; Herbig et al., 2004; Karlseder et al., 2004). DDR enables the cells to sense damaged DNA and to respond by arresting cell-cycle progression and repairing the damage (di Fagagna et al., 2003; Herbig et al., 2004; Takai et al., 2003). However, if the DNA damage exceeds a certain threshold, cells are destined to undergo either apoptosis or senescence (Kuilman et al., 2010). Importantly, senescence is determined by the presence of a few short telomeres that are able to trigger the DDR and not by the average telomere length (Herbig et al., 2004). In summary, progressive telomere shortening eventually causes chromosome ends to be recognized as DNA breaks. That can activate the DNA damage response signaling pathway and cause senescence (d'Adda di Fagagna, 2008).

It has been observed that oncogene-induced cellular senescence, which is mediated by different oncogenes, is also associated with activation of the DDR, which is triggered by excessive replication caused by oncogenic signals (Bartkova et al., 2006; Di Micco et al., 2006).

Time-course studies have revealed that expression of the oncogenic HRAS leads to a transient initial hyperproliferation phase followed by proliferation slow-down and establishment of cellular senescence (Di Micco et al., 2006). The same response has also been observed after expression of MYC, BRAF and E2F1 (Dankort et al., 2007; Denchi et al., 2005; Dominguez-Sola et al., 2007; Michaloglou et al., 2005). Senescent cells have an augmented number of active replicons and exhibit defects in DNA replication fork progression. DDR is activated at the end of hyperproliferation phase and coincides with entry into senescence (Di Micco et al., 2006). Another evidence that DDR activation results from

altered DNA replication is that at least a subset of oncogenes can trigger senescence only if cells are allowed to enter S phase of the cell cycle (Bartkova et al., 2006; Di Micco et al., 2006). MYC is able to bind to DNA replication origins and activates the replication machinery (Dominguez-Sola et al., 2007).

The DNA damage response pathway is also activated by many chemotherapeutic drugs such as campthotecin, etoposide, doxorubicin, cisplatin and hydroxyurea which usually generates single- and double-strand DNA breaks via different mechanisms and leads to cellular senescence (Chang et al., 1999a; Han et al., 2002; Yeo et al., 2000).

### 1.3.2 The p53-p21 pathway

The p53 tumor suppressor pathway is inactivated in the majority of human cancers, leading to accumulation of damaged cells and progression of cancer (Petitjean et al., 2007). Mice with mutation in the p53 pathway develop spontaneous tumors at a juvenile age and Li-Fraumeni patients, who suffer from familial syndrome due to germ-line mutations in the same pathway, have an increased risk of early onset of numerous types of cancer (Donehower et al., 1992; Srivastava et al., 1990).

The key protein of this pathway is p53, also known as the “guardian of the genome”, which regulates numerous cellular processes such as apoptosis, autophagy, DNA repair and cellular senescence in response to various stress signals (Beckerman and Prives, 2010; Green and Kroemer, 2009; Lane, 1992; Yee and Vousden, 2005). Recent studies have postulated that the ability of p53 to regulate cellular senescence may contribute to its tumor-suppressive role (Chen et al., 2005; Xue et al., 2007).

In unstressed cells, p53 is a short-lived protein which is continuously targeted for proteasomal degradation via ubiquitination by E3 ubiquitin ligase MDM2 (Haupt et al., 1997; Kubbutat et al., 1997). Importantly, MDM2 itself is a product of a p53-inducible gene and thus forms a negative autoregulatory loop (Picksley and Lane, 1993). In order to establish cellular senescence under stressed conditions, p53 needs to escape the degradation-promoting effects of MDM2. Several pathways have been shown to activate p53.

First, p53 can be activated by the DDR pathway in response to DNA damage caused by different senescence stimuli such as short telomeres, oncogene activation or chemotherapeutic drugs (Bartkova et al., 2006; Chang et al., 1999b; Di Micco et al., 2006; Herbig et al., 2004). For example dysfunctional and uncapped telomeres occurring during replicative senescence became associated with DNA damage response factors such as γH2AX, 53BP1 and initiate activation of the DDR pathway (Takai et al., 2003; von Zglinicki et al., 2004). DDR signaling results in activation of serine/threonine kinases ATM and ATR and leads to phosphorylation of p53 (Herbig et al., 2004). p53 has been also shown to be phosphorylated upon oncogene expression, where DNA hyper-replication leads to collapse of replication forks and accumulation of DNA DSBs which activate the DDR (Di Micco et al., 2006). Similarly, treatment of cells with anti-cancer drugs, which are known to activate the DDR, leads to phosphorylation of p53 at $Ser^{15}$ (Liang et al., 2011). p53 phosphorylated by ATM became more resistant to inhibition by MDM2 (Canman et al., 1998; Shieh et al., 1997),

Interestingly, the DDR pathway can also modulate function of p53 through modification of MDM2. Upon ionizing radiation or neocarzinostatin treatment MDM2 is phosphorylated in an ATM-dependent manner. Moreover MDM2 is phosphorylated by ATM *in vitro* (Khosravi et al., 1999). For example phosphorylation of MDM2 on S395 by ATM impairs the ability of MDM2 to promote degradation of p53 (Maya et al., 2001).

In summary, the DDR pathway can promote p53 activity and stability by mediating phosphorylation of both p53 and E3 ubiquitin ligase MDM2, which prevents their interaction and protects p53 from proteasome-mediated degradation (Moll and Petrenko, 2003).

Activity of the p53 pathway can be also modulated via the $p14^{ARF}$ protein, which is the product of the INK4a/ARF locus (Sherr, 2012). This locus is located at chromosome 9p21 in human (chromosome 4 in mouse) and encodes two different proteins, $p14^{ARF}$ ($p19^{ARF}$ in mouse) and $p16^{INK4a}$, by reading a shared exon in a different translational reading frame. As a consequence $p14^{ARF}$ and $p16^{INK4a}$ don't share any amino acid homology (Nobori et al., 1994; Quelle et al., 1995; Serrano et al., 1993). $p14^{ARF}$ and $p16^{INK4a}$ proteins participate in two different tumor-suppressor pathways (p53 and $p16^{INK4a}$-RB, respectively) and both have been mechanistically linked to senescence (Evan and di Fagagna, 2009).

It has been shown that in mice ARF expression can be activated by oncogenic RAS/RAF signaling via Dmp1 and E2F1 (Palmero et al., 1998; Sreeramaneni et al., 2005). Both proteins are able to bind and activate ARF gene promoter (Bates et al., 1998; Inoue et al., 1999). Expressed ARF can modulate activity of the tumor suppressor p53 via inhibition of the E3 ubiquitin ligase MDM2 (Honda and Yasuda, 1999). ARF binds to and sequesters MDM2 in nucleolus, which therefore can't be in contact with p53 localized in the nucleoplasm, thereby resulting in stabilization of p53 (Tao and Levine, 1999). Major consequence of p53 stabilization, either by the DDR pathway or by ARF, is a sequence-specific binding of stabilized p53 to DNA and activation or repression of its target genes (Yee and Vousden, 2005). One of many examples of genes regulated by p53 is a cyclin-dependent kinase inhibitor p21, which plays a critical role in inducing the cell cycle arrest (Campisi and d'Adda di Fagagna, 2007).

### 1.3.3 The $p16^{INK4a}$-RB pathway

Another protein encoded by the INK4a/ARF locus is $p16^{INK4a}$, a critical regulator of senescence acting through protein RB (Adams, 2009; Quelle et al., 1995). Under non-stressed conditions, cyclin D-CDK4/6 complexes phosphorylate the RB protein, which in turn releases tethered E2F transcription factors. E2F subsequently activates transcription of genes which are necessary for the progression from G1 to S phase of the cell the cycle (Harbour et al., 1999; Sherr and Roberts, 1999).

It has been shown that oncogenic RAS induces $p16^{INK4a}$ expression by activating transcription factors ETS (Ohtani et al., 2001). In turn, the elevated $p16^{INK4a}$ negatively regulates CDK4/6 and inhibits its kinase activity (Gil and Peters, 2006). The RB protein, which can't be phosphorylated by CDK4/6, remains in its hypophosphorylated state and binds the transcription factor E2F1. The RB-E2F1 binding subsequently prevents transcription of E2F1 target genes that are necessary for the progression from G1 to S phase and leads to the cell cycle arrest (Rayess et al., 2012).

### 1.3.4 Senescence-Associated Heterochromatin Foci (SAHF)

Importance of chromatin changes in cellular senescence has been discussed for a long time (Ryan and Cristofalo, 1972). A significant mechanistic insight into the role of chromatin in permanent cell cycle arrest maintenance was brought in 2003, when global heterochromatinization of senescent nuclei was described as Senescence-associated heterochromatin foci (SAHF) (Narita et al., 2003).

SAHF can be defined as discrete domains of heterochromatin that form due to large-scale chromosome condensation (Narita et al., 2003). Each SAHF focus is thought to be an individual chromosome (Funayama et al., 2006). Senescence-associated heterochromatin foci can be easily visualized by the DNA dye 4',6-diamidino-2-phenylindole (DAPI). While cycling or quiescent cells show largely homogenous DAPI staining for DNA, senescent cells positive for SAHF display bright positive nuclear foci (Narita et al., 2003).

SAHF, which were initially described in replicative and oncogene-induced senescence in human fibroblasts, contain several common markers of heterochromatin such as methylated histone H3 on Lys 9 (H3K9Me), hypoacetylated histones and Heterochromatin Protein 1 (HP1) (Narita et al., 2003). At the same time, depletion of a linker histone H1 and enrichment of a histone variant macroH2A or HMGA are considered to represent SAHF-specific marks (Funayama et al., 2006; Narita et al., 2006). Several pathways have been demonstrated to contribute to the SAHF formation.

The HIRA/ASF1a pathway is necessary for SAHF formation. The chromatin regulator HIRA is translocated into PML (Promyelocytic leukemia nuclear) bodies where it colocalizes with HP1 proteins. HIRA/HP1 together with another chromatin regulator ASF1 are subsequently incorporated into chromatin. ASF1a depletion leads to a senescence bypass (Zhang et al., 2005).

The HIRA/ASF1 pathway appears to converge with the $p16^{INK4a}$-RB pathway, which plays a key role in inducing SAHF formation in oncogene-induced senescence (Narita et al., 2003; Ye et al., 2007). It was published that oncogene-induced SAHF formation is impaired once RB pathway is disrupted by the adenoviral oncoprotein E1A (Narita et al., 2003), by expression of an shRNA against RB (Narita et al., 2003) or by expression of the SV40 virus large T antigen (Ye et al., 2007). Proliferation-promoting genes such as Cyclin A, known

targets of the E2F transcription factor which is regulated by RB, are incorporated into SAHF. At the same time RB colocalizes with SAHF (Narita et al., 2003). These results suggest that growth arrest in senescent cells can be partly maintained by heterochromatinization (and hence silencing) of proliferation-promoting genes (Funayama et al., 2006; Narita et al., 2003; Zhang et al., 2007).

The mechanistic contribution of the p53 pathway to cell cycle arrest via SAHF formation is not entirely clear. Knockdown of a chromatin remodeler p400 results in p53-dependent and at the same time RB-independent SAHF formation (Chan et al., 2005). This result suggests that p53 and RB form distinct pathways leading to SAHF formation.

## 1.3.5 Senescence-Associated Secretory Phenotype (SASP)

Senescent cells show pronounced changes in gene expression of many secreted proteins such as metalloproteinases or cytokines (Bavik et al., 2006; Coppe et al., 2008; Kuilman et al., 2008; Shelton et al., 1999; Wajapeyee et al., 2008). The first clue of changes in secretome of senescent cells was revealed by microarray analyses of replicatively senescent fibroblasts (Shelton et al., 1999). Since that it has been shown that the secretory phenotype occurs in different cell types such as human melanocytes, human breast epithelial cells, human endothelial cells and mouse lymphoma cells, and it has been termed senescence-associated secretory phenotype (SASP) (Chien et al., 2011; Coppe et al., 2008; Kuilman et al., 2008; Maier et al., 1990).

### 1.3.5.1 The JAK/STAT signaling pathway

Cytokines are key mediators of the immune response and play important roles also in processes such as autoimmune diseases, cancer and cellular senescence (Aringer et al., 1999; Dinarello, 2007; Kuilman et al., 2008). They are produced by many different types of cells and can be subdivided into several subgroups including interferons, interleukins, mesenchymal growth factors, tumor necrosis factors, adipokines and chemokines (Dinarello, 2007).

Cytokines bind to their cognate receptor and trigger intracellular signaling through Janus tyrosine kinases (JAKs) and their transcription factors STATs (signal transducers and activators of transcription). There are four members of JAKs family (JAK1, JAK2, JAK3 and TYK2) and seven members of STAT known. Cytokine signaling via the JAK/STAT pathway impacts and changes gene expression (Jatiani et al., 2010).

Particulary, ligand binding to cytokine receptor localized at the cytoplasmatic membrane induces receptor dimerization/oligomerization. Subsequently, JAKs undergo autophosphorylation and this activation of JAKs results in phosphorylation of receptors on tyrosine residues. STATs are recruited via their SH2 domains to activated receptors and become phosphorylated by Janus kinases. Phosphorylated STATs can dimerize and their translocation from cytoplasm to the nucleus facilitates activation or suppression of target genes (Baker et al., 2007).

### 1.3.5.2 Cytokines and cellular senescence

It was found that more than fifty different cytokines are secreted at higher levels by senescent cells (Davalos et al., 2010). Moreover, several publications showed that cytokines are able to amplify the senescence phenotype in an autocrine manner (Acosta et al., 2008; Kuilman et al., 2008).

The first cytokine which was causally implicated in the induction and maintenance of oncogene-induced and replicative senescence is IL-8. This cytokine signals via the CXCR2 receptor. Depletion of CXCR2 abolishes cell cycle arrest and at the same time ectopic expression of CXCR2 results in premature senescence via the p53 pathway (Acosta et al., 2008). This work thus showed that senescent cells reinforce their growth arrest by activation of a self-amplifying secretory network (Bartek et al., 2008).

Another cytokine, IL-6, is secreted by senescent cells and required together with its receptor for establishment of oncogene-induced senescence. The transcription factor C/EBP-β is an essential regulator of IL-6 and both proteins cooperate to amplify the oncogenic signal (Kuilman et al., 2008).

Cytokines have been also shown to cause DNA damage-induced senescence by ROS production upon prolonged exposure of cultured cells. For example, treatment with IFNβ causes activation of DNA damage signaling with accumulation of phosphorylated ATM, CHK2 and p53 (Moiseeva et al., 2006).

# 1.4 Role of the promyelocytic leukemia protein (PML) in senescence

The promyelocytic leukemia (PML) tumor-suppressor was initially identified in acute promyelocytic leukemia (APL), where PML gene (on chromosome 15) can be subject to fusion with the retinoic acid receptor α (RARα) gene (on chromosome 17). The chromosomal translocation t(15;17) results in proeduction of the PML-RARα fusion protein, which plays a driving role in acute promyelocytic leukemia (APL) (de The et al., 1990; Melnick and Licht, 1999).

## 1.4.1 PML protein structure and PML nuclear bodies

The PML protein consists of N-terminally located RING domain, two RING-like domains and an α-helical coiled-coil motif (Duprez et al., 1999; Kastner et al., 1992; Tao et al., 2008). Alternative splice variants of PML exist, resulting in seven different PML isoforms, named PML I–VII. Those variants all contain an identical N-terminal region and at the same time differ in their C-terminal sequences (Jensen et al., 2001). Beside the isoforms localized in the nuclear matrix or cytoplasm, some isoforms are found to be part of PML nuclear bodies (PML-NBs), which represent macromolecular ‘objects’ of a doughnut shape. Cells usually contain about 10-30 PML-NBs depending on the cell cycle stage (Jensen et al., 2001; Zhong et al., 2000). While the PML protein is usually localized on the outside of the PML-NBs, other partner proteins are usually inside of this structure (Guiochon-Mantel et al., 1995).

To date, more than 100 proteins involved in a variety of molecular pathways have been found to localize either constitutively or transiently with PML-NBs (Lallemand-

Breitenbach and de The, 2010). Thus, PML NBs appear to be plurifunctional structures containing an assembly of diverse proteins, post-translationally modified, or anchored.

## 1.4.2 PML gene regulation

To date, several reports showed, that PML expression and formation of PML NBs increase upon oncogenic expression and DNA damage (Ferbeyre et al., 2000; Pearson and Pelicci, 2001; Scaglioni et al., 2012). In oncogene-induced senescence, PML transcription is controlled by the tumor suppressor protein p53, through p53 response element in the PML gene (de Stanchina et al., 2004).

PML promoter also contains IFN-response elements (ISREs) and IFNγ activated sites (GAS), which are responsive to interferons (Stadler et al., 1995).

## 1.4.3 Functions of the PML protein

The tumor suppressive functions of PML were originally suggested by the identification of a PML-RARα fusion protein in human acute promyelocytic leukemia (APL), which can be characterized by a differentiation block of the myeloid progenitor cells at the promyelocytic stage (de The et al., 1990; Kakizuka et al., 1991). The RARα protein regulates transcription of genes, which are critical for the induction of myeloid cell terminal differentiation. The PML-RARα fusion protein, which is the product of translocation of the PML gene to the RARα gene, acts as a double dominant-negative oncoprotein blocking RARα-dependent transcriptional functions and also the PML pathway. Aberrant function of the RARα pathway due to interference with PML-RARα is considered to be one of the major causes of the differentiation block and an essential feature of leukemogenesis (Melnick and Licht, 1999; Pandolfi, 2001; Yang et al., 2006).

At the same time, dominant-negative mutants of RARα fail to cause leukemia with APL features in transgenic mice, suggesting that functional disruption of PML is also critical in the process of malignant transformation (Pandolfi, 2001). PML null mice are also prone to

develop T and B cell lymphomas (Wang et al., 1998b) and in a mouse model of lung cancer, PML inactivation leads to increased tumorigenesis (Scaglioni et al., 2006).

Numerous reports suggest that the PML protein plays a critical role in several cellular processes related to tumor suppression such as regulation of apoptosis, neoangiogenesis, cell migration, DNA damage response and cellular senescence (Chen et al., 2012). Interestingly, PML has been also found to act in concert with all main pathways, which are involved in mediating cellular senescence. Upregulated PML expression and increase in the number and size of PML-NBs have been for the first time observed upon expression of oncogenic RAS and also in replicative senescence (Ferbeyre et al., 2000; Pearson et al., 2000). At the same time, oncogene-induced senescence is impaired in PML depleted cells (Pearson et al., 2000).

### 1.4.3.1 Role of PML in the DNA Damage Response pathway

Many senescence-inducing stimuli have been shown to generate a persistent DNA damage response (DDR), which is very often associated with DNA double-strand breaks (DSB). DDR signaling plays an important role in establishing and maintaining senescent phenotypes (d'Adda di Fagagna, 2008).

PML NBs represent dynamic structures which are known to change morphology in response to DNA damage (Carbone et al., 2002; Conlan et al., 2004). A growing number of DNA repair factors such as MRE11, NBS1, BLM, RAD50, ATR, RPA and RECQL, have been reported to localize to PML-NBs. Moreover, their translocation to and from PML NBs is regulated in response to DNA damage (Barr et al., 2003; Johnson et al., 2000; Lombard and Guarente, 2000; Mirzoeva and Petrini, 2001). This data strongly suggest that PML may play a role in DNA repair and indeed, several publications support such concept.

First, PML NBs are sites of BLM, RAD51 and the replication protein A (RPA) assembly during the late S-G2 phase of the cell cycle and upon ionizing radiation (IR). At the same time, the BLM protein, a member of the RECQ DNA helicase family, doesn’t form Ionizing Radiation-Induced Foci (IRIF) in PML defective cells upon IR (Bischof et al., 2001). These data indicate that the localization of proteins such as BLM to PML NBs is somehow required for their normal function. Following IR, PML NBs also associate with MRE11 and

the tumor suppressor p53 at sites of ssDNA and DSB breaks. PML NBs thus can represent structures where DNA repair activities and cell cycle checkpoints are co-ordinated (Carbone et al., 2002).

Evidence of a functional relationship between PML and one of the main DDR kinases, ATM, has also been shown. Thus, PML became phosphorylated at S117 by CHK2, which is an ATM-activated kinase (ATM substrate), in response to IR (Yang et al., 2002). Furthermore, after CHK2 phosphorylation by ATM at the site of DSB, PML activates CHK2 by mediating the autophosphorylation step of CHK2 (Yang et al., 2006). Finally, inhibition of ATM and CHK2 signaling abrogates PML NBs formation in response to DSB (Dellaire et al., 2006).

Another major DDR kinase, ATR, has been found to be localized in PML NBs together with the RPA protein prior to damage. Upon exposure of cells to ionizing radiation, ATR and RPA leave PML NBs and translocate to IRIF. This relocalization requires kinase activity of ATR (Barr et al., 2003). Furthermore, PML modulates expression of TOPBP1, which physically interacts with ATR and is required for stimulation of ATR in response to damage. Inhibition of PML expression results in a significantly decreased expression of TOPBP1. At the same time, TOPBP1 is unable to form IRIF in PML deficient cells. PML thus regulates activation of ATR, a kinase critical for DNA damage and replication stress resistance (Xu et al., 2003).

### 1.4.3.2 Roles of PML in the p53-p21 and p16$^{INK4a}$-RB pathways

The mechanism(s) by which PML contributes to senescence involves both the p53 and the RB pathway (Ferbeyre et al., 2000; Pearson et al., 2000). Both proteins have been shown to directly interact with PML (Alcalay et al., 1998; Fogal et al., 2000).

The relationship between PML and p53 is complex and PML is able to regulate the p53 pathway in several ways. First, upon oncogenic RAS expression, PML modulates p53 acetylation and its transcriptional activation, resulting in p21 tumor suppressor expression and cell cycle arrest. Second, it has been shown, that PML can protect p53 from proteasome-

mediated degradation. PML interacts with the E3 ubiquitin ligase MDM2 and sequesters it in the nucleolus, thereby leading to p53 stabilization (Bernardi et al., 2004).

Although PML is able to induce senescence in cells with inactivated p53, expression of HPV16 protein E7, which binds to RB and blocks the E2F-dependent gene expression, causes senescence bypass (Mallette et al., 2004). At the same time PML overexpression leads to increased levels of $p16^{INK4a}$ and hypophosphorylated RB (Ferbeyre et al., 2000). One mechanism how PML can regulate senescence upon oncogenic RAS expression or extensive rounds of cell division via the RB/E2F pathway was published recently (Vernier et al., 2011). PML controls the activity of E2F transcription factors via relocalization of RB/E2F complexes into PML-NBs. This mechanism contributes to cell cycle arrest (Vernier et al., 2011).

### 1.4.3.3 Role of PML in SAHF formation

The promyelocytic leukemia (PML) tumor-suppressor has been implicated in regulation of heterochromatin changes in oncogene-induced and replicative senescence. Several SAHF (Senescence-Associated Heterochromatin Foci) components transiently colocalize with PML-NBs as cells approach senescence (Ye et al., 2007; Zhang et al., 2005). Specifically, the chromatin regulator HIRA and heterochromatin protein HP1 enter into PML-NBs, prior to deposition of HP1 into SAHF. HIRA relocalization is considered to be one of the earliest events in the senescence program, which precedes appearance of other markers of senescence such as cell cycle exit, changes in morphology, SA-β-gal activity and importantly SAHF formation. It has been also shown, that recruitment of HIRA to PML bodies correlates with incorporation of histone macroH2A (a transcription-silencing histone H2A variant) into chromatin and that ectopic expression of HIRA accelerates SAHF formation (Zhang et al., 2005).

A strong link between PML bodies and SAHF formation was supported in two ways. First, expressions of mutant HIRA, which blocks localization of endogenous HIRA into PML-NBs, abolished SAHF formation. Next, formation of SAHF was also blocked once PML-NBs are disrupted by the PML-RARα fusion protein, a hybrid protein with altered functions (Ye et

al., 2007). Overall, the PML bodies seem to play a catalytic role in assembly and modification of proteins prior to their translocation to chromatin and formation of SAHF (Zhang et al., 2005). However, once cells approach senescence, PML-NBs and SAHF form distinct nuclear structures (Vernier et al., 2011).

## 1.5 Cellular senescence and tumor suppression

As mentioned previously, presence of senescent cells has been identified both in mouse and human premalignant lesions such as in the prostate of mouse lacking the tumor suppressor PTEN (Chen et al., 2005) and in human colon adenomas, respectively (Bartkova et al., 2006). Importantly, senescent cells commonly found in premalignant or early cancer lesions, can be rarely found in the later stages of cancer (Bartkova et al., 2006; Chen et al., 2005; Collado et al., 2005).

Moreover, bypass of cellular senescence upon inactivation of senescence-inducing pathways, such as p53/p21 and RB, can result in accelerated cancer development in mice and humans. For example, inactivation of p53 in benign lesions of the murine prostate lacking PTEN leads to progression into adenocarcinomas (Chen et al., 2005). Similarly, expression of oncogenic K-Ras stimulates the development of mammary epithelial hyperplasias in mice, while inactivation of senescence-inducing pathways results into malignant tumors (Sarkisian et al., 2007). In human, patients with Li-Fraumeni syndrome are cancer-prone and their cells with p53 mutations overcome senescence more readily than normal (p53 wild-type) cells (Shay et al., 1995).

Thus, based on the observations that senescent cells can be found only in premalignant lesions and that inactivation of senescence-inducing pathways leads to cancer development, cellular senescence is considered to be an important physiological tumor suppressing mechanism *in vivo*, which can restrain the growth of potentially dangerous cells (Bartkova et al., 2006; Chen et al., 2005; Collado et al., 2005).

# 2. AIMS OF THE STUDY

The main objective of this study is to provide novel insights into several aspects of cellular senescence, which are important from not only mechanistic and conceptual points of view, but also highly relevant to cancer therapy. The aims are sub-divided into four parts, each represented by a separate study, dealing with the following issues and open questions:

- **Publication 1:** Activation of DDR and ARF pathways in response to oncogenes
  - Are DDR and ARF triggered concurrently or at different stages of cancer progression in response to oncogenic signaling?
  - Is there any difference in activation thresholds between DDR and ARF?
  - What is the relative contribution of DDR and ARF tumor suppressor pathways during oncogene-induced senescence?

- **Publication 2:** Insights into chromatin changes during cellular senescence
  - Is Senescence-associated heterochromatin foci formation (SAHF) a universal feature of cellular senescence?
  - What is the correlation between SAHF formation and $p16^{INK4a}$ pathway activation?
  - Is detection of SAHF applicable as a marker for studies *in vivo*?

- **Publication 3:** Role(s) of the senescence-associated secretory phenotype (SASP) in drug-induced senescence
  - Is cellular senescence induced by diverse genotoxic compounds accompanied by protein secretion analogous to replicative and oncogene-induced senescence?
  - If yes, what is the spectrum of induced secreted proteins?
  - Which specific signaling pathways are activated?

- **Publication 4:** Regulation of the tumor suppressor protein PML in cellular senescence
  - What is the molecular mechanism of PML induction in genotoxic drug-induced senescence?

# 3. METHODS

The list below represents methods used in the following four publications, which are parts of this thesis. Detailed protocols and other information (cell lines, mouse models, human samples, chemicals, antibodies, shRNA sequences, plasmids etc.) can be found in the section Material and Methods in each of the enclosed publications.

- Allelic imbalance analysis
- BrdU proliferation assay
- ChIP assay
- DNA copy number and gene expression data
- ELISA
- Flow cytometry
- Gel retardation assay
- Immunohistochemistry
- Indirect immunofluorescence
- Luciferase reporter assay
- Preparation of stable cell lines using lentivirus transduction
- Quantitative real time RT-PCR (qRT-PCR)
- SDS-PAGE and western blotting
- Senescence associated-β-galactosidase assay
- Statistical analysis

# 4. RESULTS AND DISCUSSION

Results are presented in the form of the four original publications. Each of them is discussed in the context of relevant literature.

## 4.1 List of publications

Publication 1: Evangelou, K., Bartkova, J., Kotsinas, A., Pateras, I.S., Liontos, M., Velimezi, G., **Kosar, M.**, Liloglou, T., Trougakos, I.P., Dyrskjot, L., Andersen, C., Papaioannou, M., Drosos, Y., Papafotiou, G., Hodny, Z., Sosa-Pineda, B., Wu, X.R., Klinakis, A., Orntoft, T., Lukas, J., Bartek, J., Gorgoulis V.G. (2013). The DNA damage checkpoint precedes activation of ARF in response to escalating oncogenic stress during tumorigenesis. Cell Death Differ. 20, 1485-1497.

Publication 2: **Kosar, M.**, Bartkova, J., Hubackova, S., Hodny, Z., Lukas, J., Bartek, J. (2011). Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16 (ink4a). Cell Cycle 10:3, 457-468.

Publication 3: Novakova, Z., Hubackova, S., **Kosar, M.**, Janderova-Rossmeislova, L., Dobrovolna, J., Vasicova, P., Vancurova, M., Horejsi, Z., Hozak, P., Bartek, J., Hodny, Z. (2010). Cytokine expression and signaling in drug-induced cellular senescence. Oncogene 29, 273-284.

Publication 4: Hubackova, S., Novakova, Z., Krejcikova, K., **Kosar, M.**, Dobrovolna, J., Duskova, P., Hanzlikova, H., Vancurova, M., Barath, P., Bartek, J., Hodny, Z. (2010). Regulation of the PML tumor suppressor in drug-induced senescence of human normal and cancer cells by JAK/STAT-mediated signaling. Cell Cycle 9:15, 3085-3099.

## 4.2 Linking of publications

**Publication 1** provides mechanistic insights into the cellular response to oncogenic stress, which can lead to premature senescence, cell death or to cancer progression in the case of impaired anticancer barriers. Particularly, we have investigated orchestration and relative contributions of the DNA damage response (DDR) and Alternative Reading Frame (ARF) pathways, which can both activate the p53 pathway and play a role in tumor suppression upon oncogene activation.

**Publication 2** focuses on heterochromatin changes in senescent cells upon oncogene activation and other insults known to cause premature senescence. Since heterochromatinization was previously established as one of the mechanisms that help to maintain the senescent state, we investigated how universal feature of senescence phenotype is Senescence-associated heterochromatin foci (SAHF) formation. In addition, we analyzed whether or not are SAHF applicable to studies *in vivo*.

**Publication 3** contributes to our understanding about senescence-associated secretory phenotype (SASP) in response to treatment with genotoxic drugs, and thus extends recent analogous studies on replicative and oncogene-induced senescence. We have analyzed the spectrum of cytokines secreted by tumor cells and examined pathways involved in the maintenance of cytokine signaling.

**Publication 4** characterizes regulation of the tumor suppressor Promyelotic leukemia protein (PML), which was previously mechanistically linked to Senescence-associated heterochromatin foci (SAHF) formation in oncogene-induced senescence. Particularly, we examined contribution of cytokine signaling to PML regulation in response to diverse drugs including clinically used anti-cancer chemotherapeutics.

# 4.3 Publication 1

Evangelou, K., Bartkova, J., Kotsinas, A., Pateras, I.S., Liontos, M., Velimezi, G., **Kosar, M.**, Liloglou, T., Trougakos, I.P., Dyrskjot, L., Andersen, C., Papaioannou, M., Drosos, Y., Papafotiou, G., Hodny, Z., Sosa-Pineda, B., Wu, X.R., Klinakis, A., Orntoft, T., Lukas, J., Bartek, J., Gorgoulis V.G. (2013). The DNA damage checkpoint precedes activation of ARF in response to escalating oncogenic stress during tumorigenesis. Cell Death Differ. 20, 1485-1497.

# 4.4 Publication 2

**Kosar, M.**, Bartkova, J., Hubackova, S., Hodny, Z., Lukas, J., Bartek, J. (2011). Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16 (ink4a). Cell Cycle 10:3, 457-468.

# 4.5 Publication 3

Novakova, Z., Hubackova, S., **Kosar, M.**, Janderova-Rossmeislova, L., Dobrovolna, J., Vasicova, P., Vancurova, M., Horejsi, Z., Hozak, P., Bartek, J., Hodny, Z. (2010). Cytokine expression and signaling in drug-induced cellular senescence. Oncogene 29, 273-284.

# 4.6 Publication 4

Hubackova, S., Novakova, Z., Krejcikova, K., **Kosar, M.**, Dobrovolna, J., Duskova, P., Hanzlikova, H., Vancurova, M., Barath, P., Bartek, J., Hodny, Z. (2010). Regulation of the PML tumor suppressor in drug-induced senescence of human normal and cancer cells by JAK/STAT-mediated signaling. Cell Cycle 9:15, 3085-3099.

# 4.7 Discussion

1. *The DNA damage checkpoint precedes activation of ARF in response to escalating oncogenic stress during tumorigenesis*

Oncogene activation can lead to premature senescence or apoptosis via activation of the p53 pathway (Collado and Serrano, 2010; Halazonetis et al., 2008). Although the DNA damage response (DDR) and alternative reading frame (ARF) pathways are known to trigger p53 during oncogenic stress (Bartkova et al., 2005; Bartkova et al., 2006; Gorgoulis et al., 2005; Sherr, 2012; Sherr and Weber, 2000), precise mechanisms of any functional interplay between these two anti-cancer barriers remain unclear.

To shed light on this issue, in the first part of this study we asked whether DDR and ARF are triggered concurrently or at different stages of cancer development. *In vivo* analysis of several experimental mouse models showed that DDR markers are expressed earlier than ARF during progression of premalignant lesions to malignancy. Analysis of human samples of head and neck, skin, urinary bladder and pancreatic lesions during cancer progression lead us to the same conclusion. Overall, results in the first part of our study showed that DDR activation preceded ARF induction and ARF thus likely provides a delayed checkpoint-barrier during cancer development.

Next, we asked why ARF signaling is not active during initial steps of cancer development. To answer this question we employed human bronchial epithelial cells (HBECs) and BJ fibroblast models *in vitro*, into which various oncogens were sequentially introduced. While DDR activation was evident upon expression of single oncogene (e.g. a mutant, p16-insensitive CDK4) in HBECs, ARF activation required an additional oncogenic event, here provided by mutant KRAS. Experiments with BJ fibroblasts led to the same conclusion, because even though DDR was activated upon expression of one oncogene (β-catenin or HRAS, respectively), ARF was induced only upon combined (concomitant) transformation by β-catenin together with HRAS. Importantly, even prolonged expression of the single oncogene (HRAS) in BJ fibroblasts was not sufficient to induce ARF activation, in contrast to efficient induction of the DDR. These data indicate that ARF provide a less-

sensitive tumor suppressor barrier than DDR. At the same time, late upregulation of ARF, which was observed during cancer development *in vivo*, could be a consequence of accumulating oncogenic insults.

To examine the relative 'biological' contributions of DDR versus ARF pathways under conditions of increased oncogenic load, we first co-expressed β-catenin and HRAS in BJ fibroblasts. Next, we silenced either DDR or AFR or both pathways at the same time. While inhibition of single pathway led to the partial bypass of cellular senescence, concomitant silencing of ARF and DDR was more potent.

Overall, our results suggest that ARF provides complementary and delayed barrier against the risk of malignant transformation and, when activated, it acts in concert with the DDR.

A potential crosstalk between the DDR and ARF pathways has been recently described in two studies. First of them focused on the role of MRE11 in oncogene induced senescence (Gupta et al., 2013). MRE11 is a known sensor of DNA double-strand breaks, which plays a role in the activation of the DDR (Stracker and Petrini, 2011). Authors showed that in response to oncogene expression, the MRE11 complex mediates the G2 arrest in mouse mammary hyperplasia via DDR activation. Impairment of MRE11 functions, and thus DDR, lead to progression of hyperplasias into aggressive and metastatic breast cancers. Importantly, hypomorphic mutations in the MRE11 complex components caused oncogene-dependent activation of the ARF pathway and selection for Ink4a-ARF locus inactivation was observed at later stages of tumor development (Gupta et al., 2013).

The other recent study also suggests a close interplay between the DNA damage response and ARF pathways (Velimezi et al., 2013). ATM, the prominent DNA damage response kinase, can suppress ARF protein levels and activity in oncogene-transformed and cancer cells. The authors proposed that ATM activates protein phosphatase 1 (PP1), which antagonizes NEK2-dependent phosphorylation of nucleophosmin (NPM). PP1-dependent dephosphorylation of NPM weakens the interaction between ARF and NPM, and the liberated ARF was subsequently susceptible to proteasome degradation. On the contrary, ATM inhibition enhanced ARF levels and promoted p53-dependent tumor suppressor functions of ARF. Data from human clinical samples showed that reduced expression of ATM at advanced

stages of tumor progression correlated with increased ARF protein levels in lung carcinomas. In conclusion, this publication supports the role of ARF as a secondary anti-cancer barrier, which can be particularly activated upon loss of ATM (Velimezi et al., 2013).

*2. Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16 (ink4a)*

Senescence-associated foci (SAHF) were first described by Narita and Lowe, who observed SAHF formation upon oncogenic RAS activation in human diploid fibroblasts IMR90 (Narita et al., 2003). This pioneering work was later followed by several other studies, which were mostly focused on the mechanism of SAHF formation in oncogene-induced senescence (Ye et al., 2007; Zhang et al., 2005).

Since the previous publications used only few strains of human fibroblasts, data regarding SAHF formation in a broader spectrum of cell types, especially the cancer highly relevant human epithelial cells, were lacking. At the same time there was lack of information about any significance of SAHF formation in senescence caused by diverse stimuli. Since a universal marker of cellular senescence has not been identified yet, another important issue was the potential applicability of SAHF as a biomarker of senescence *in vivo*.

In order to extend the studies published previously and to answer the above-mentioned open questions we decided to systematically compare the ability to form SAHF in various types of normal human cells exposed to different senescence-inducing stimuli. Moreover, we have analyzed SAHF formation in premalignant and malignant human lesions.

Senescence-associated heterochromatin foci formation accompanied by silencing genes that drive cell cycle progression has been considered to be critical for induction of senescence (Narita et al., 2003). However, our data implies that SAHF are not a common feature of the senescence program and that there is a strong variation in SAHF formation depending on cell type and senescence stimulus. Although SAHF formation upon oncogenic RAS expression was obvious in all tested cell strains (BJ, MRC5 and normal human keratinocytes), only MRC5 were able to form SAHF in response to chemotherapeutic drugs (hydroxyurea, etoposide, doxorubicin), excessive rounds of cell division or bacterial

intoxication (HdCDT). Importantly, markers of cellular senescence (SA-β-gal expression, diminished BrdU incorporation, DDR activation) were present in all strains upon all senescence insults. Therefore this part of our study lead us to the conclusion that some types of cellular senescence can be established in the absence of SAHF formation.

This conceptually important observation was recently confirmed also by several other authors. First of them noticed that although BJ fibroblasts became senescent following telomere shortening, exposure to ionizing radiation or $H_2O_2$ treatment, detectable SAHF were missing (Di Micco et al., 2011). Variable and partly cell-type specific compaction of DNA was observed also by Contrepois (Contrepois et al., 2012), who studied the interplay between regulation of deacetylation of histone H4-K16Ac and heterochromatin assembly during senescence.

Finally, a recent study that focused on evaluation of markers and their development and specifity in replicative senescence, revealed the lack of SAHF formation in BJ fibroblasts in contrast to MRC5 (Schauble et al., 2012).

The second key aspect of our study was the critical role of the $p16^{INK4a}$-RB pathway in SAHF formation. Importance of the $p16^{INK4a}$-RB pathway in SAHF development upon oncogene activation had been proposed previously (Narita et al., 2003; Ye et al., 2007). We have systematically tested any correlation between SAHF and $p16^{INK4a}$ induction in MRC5 and BJ fibroblasts, undergoing senescence induced by different stimuli. Biochemical analysis of $p16^{INK4a}$ expression in BJ cells revealed increased $p16^{INK4a}$ expression only upon oncogenic RAS activation, the only type of cellular senescence where we detected SAHF. In contrast, MRC5 showed increased $p16^{INK4a}$ levels in all types of senescence. Moreover, there was a perfect correlation between the level of $p16^{INK4a}$ expression and SAHF formation. These results are in line with the proposed role of the $p16^{INK4a}$-RB axis in SAHF development (Narita et al., 2003).

Finally, our data are also relevant to the biological role of senescence as anti-cancer barrier (Bartek et al., 2007; Campisi, 2005). Enhanced heterochromatinization in clinical samples of premalignant human lesions detected by H3K9Me and HP1 was reported previously (Bartkova et al., 2006). Whether heterochromatinization is accompanied by the presence of DNA/DAPI-defined SAHF in such human samples has not been investigated

before. Although examination of paraffin sections of formalin-fixed pellets of senescent BJ cells with oncogenic RAS expression revealed presence of DNA/DAPI-defined SAHF (used as a positive control scenatio), we were unable to detect any clear DNA/DAPI-defined SAHF on tissue sections of normal human colon, adenomas and carcinomas. At the same time heterochromatin markers H3K9Me and HP1 were present in these tissue samples. Thus, heterochromatin domains observed in human tissues appear to be different from the DNA/DAPI-defined SAHF which can be detected in cell culture models. Although precise mechanistic understanding of differences in heterochromatin biology in premalignant human lesions and cell culture models will require more studies, it is clear that identification of senescent cells through detection of the DNA/DAPI-defined SAHF may not be applicable in human lesions.

3. *Cytokine expression and signaling in drug-induced cellular senescence*

One of the features of cellular senescence is the striking change in gene expression of many secreted proteins (Bavik et al., 2006; Wajapeyee et al., 2008). To date, more than fifty cytokines have been found to be secreted by senescent cells (Davalos et al., 2010). Senescence-associated secretory phenotype (SASP) has been previously investigated mainly in replicative and oncogene-induced senescence (Acosta et al., 2008; Kuilman et al., 2008; Shelton et al., 1999). In order to extend our current knowledge about the role of SASP in premature senescence, we decided to examine cytokine secretion in cells treated by various genotoxic drugs, including those used in cancer chemotherapy.

In agreement with previously published data (Michishita et al., 1999; Suzuki et al., 2002), long-term administration of BrdU and DMA led to premature senescence of cancer cells. Interestingly, we found that treatment with those chemicals persistently activates the IFNβ-JAK/STAT signaling pathway. This pathway was previously shown to be transiently evoked in response to virus infection, where infection leads to secretion of interferons which contributes to establishment of a positive regulatory loop via an autocrine/paracrine mechanism (Honda et al., 2005). Beside prolonged activation of the JAK/STAT signaling pathway, we found up-regulated expression of many other secreted proteins such as ISG15,

PML, MX1, IL-6, IL-8, IL-10 and IL-24. Our results inspired two conceptually important conclusions. First, the spectrum of proteins secreted by senescent cells is much broader than published previously (Acosta et al., 2008; Kuilman et al., 2008). Second, secretion of diverse proteins is probably common in all major types of cellular senescence.

It has been shown, that cytokines IL-6 and IL-8 are directly involved in the establishment and maintenance of oncogene-induced senescence and depletion of both cytokines abolished the senescence phenotype (Acosta et al., 2008; Kuilman et al., 2008). Whereas we did find IL-6 and IL-8 expressed in genotoxic drugs-induced senescence, in our hands depletion of IL-6 was unable to overcome senescence. Moreover, markers of senescence such as pS15p53, p21 and RB, were present even after inhibition of the JAK/STAT signaling pathway through depletion of kinases of JAK family or treatment with a JAK specific inhibitor. Thus, in contrast to the previously published role of IL-6 in oncogene-induced senescence, our data indicate that IL-6 and JAK mediated signaling are not essential for maintenance of drug-induced senescence. It has been shown, that DNA damage signaling is essential for maintenance of diverse types of senescence (Campisi and d'Adda di Fagagna, 2007; Halazonetis et al., 2008). At the same time, a certain threshold level of DNA damage is necessary for activation of cellular senescence (Bartek et al., 2007). Therefore, we speculate, that the observed different outcomes of IL-6 depletion in drug-induced senescence versus senescence evoked by the BRAF oncogene (Kuilman et al., 2008), could be explain by different DNA damage thresholds in such distinct biological settings. Particularly, if DNA damage level is lower in BRAF-induced senescence, one possibility is, that cells need to boost the cell cycle arrest using other pathways such as IL-6 signaling. In contrast, the high level of DNA damage after drug administration is sufficient to maintain genotoxic-stress induced senescence without IL-6 signaling.

Taken together, findings presented in this study extend our knowledge about proteins secreted during genotoxic drug-induced cellular senescence. Because many of the secreted proteins that we found are known to play roles in the immune system and tumor suppression, our data are highly relevant to cancer therapies.

4. *Regulation of the PML tumor suppressor in drug-induced senescence of human normal and cancer cells by JAK/STAT-mediated signaling*

Critical roles of the tumor suppressor PML in several processes related to tumor suppression have been described extensively (Chen et al., 2012). Beside the other PML functions, such as regulation of apoptosis or cell migration, there are several reports showing that PML induction and formation of PML NBs is a common feature of replicative, oncogene- or bacterial-induced senescence (Blazkova et al., 2010; Ferbeyre et al., 2000; Janderova-Rossmeislova et al., 2007).

Our previous work, where we reported induction of a senescence-associated secretory phenotype upon cell exposure to diverse genotoxic compounds, revealed prolonged activation of JAK/STAT signaling and upregulation of numerous important tumor suppressors including PML (Novakova et al., 2010). At the same time, it is known that the PML promoter contains sequences (ISREs, GAS) which can bind STAT transcription factors (Stadler et al., 1995). Therefore we decided to investigate whether PML expression is directly regulated by the JAK/STAT signaling pathway in drug-induced senescence.

First, we showed that treatment with diverse genotoxic drugs (BrdU, Thymidine, Distamicin, Etoposide, Aphidicoli and Campthotecin) leads to higher numbers of PML NBs in normal fibroblasts and also in human cancer cell lines. Multiplication of PML NBs was accompanied by increases in both PML protein and also mRNA levels. At the same time, there was no difference in PML protein stability as revealed by treatment with cycloheximide, a known inhibitor of de novo protein synthesis. Taken together, those data showed that the observed increase of PML NBs can be attributed to enhanced PML expression after treatment with genotoxic drugs.

Next we asked, whether expression of PML in drug-induced senescence requires JAK/STAT signaling. Indeed, chemically or genetically down-regulated signaling via JAK led to decreased levels of PML mRNA in drug-induced senescent cells. Two STAT binding sites (ISREs and GAS) in the PML gene promoter were published previously (Stadler et al., 1995), therefore we asked, which (if any) of them is required for transcription of PML after

genotoxic stress. Data obtained by gel retardation assay revealed that the STAT binding that contributes to PML expression requires ISRE-mediated transcription. This result was also confirmed by a reporter assay with the PML promoter containing or lacking the ISRE element, respectively.

It has been published that the PML promoter contains, besides the STAT binding sites, also a p53 response element and, furthermore PML transcription can be controlled by p53 in oncogene-induced senescence (de Stanchina et al., 1998; Stadler et al., 1995). Therefore, we decided to investigate whether PML expression via JAK/STAT signaling relies on transcriptional activity of p53. For that, we used two different systems. First, human cell lines HCT-116 with wild-type or deleted p53 were treated with thymidine in the presence or absence of a JAK inhibitor. In this setup, PML expression was inhibited by JAK inhibition in both cell lines, showing that indeed JAK/STAT signaling is crucial for regulation of PML transcription upon genotoxic stress, regardless of p53 expression. Second, we used the U2OS cell line conditionally expressing (or not) the dominant-negative tumor suppressor p53, which were published previously (Thullberg et al., 2000). Because also in this case there was no significant difference in levels of PML expression, we conclude, that in this case, transcriptional activation of PML upon genotoxic stress is not dependent on p53.

Many types of cellular senescence are maintained by persistent DNA damage signaling, which can reflect irreparability of some types of DNA lesions (von Zglinicki et al., 2004). Previous studies showed that PML NBs co-localized with sites of persistent DNA damage foci after ionizing radiation (Carbone et al., 2002; Xu et al., 2003). These types of lesions are usually positive for markers of DNA double strand breaks such as γH2AX and 53BP1 (Rogakou et al., 1998; Schultz et al., 2000). Although in our experiments cells were treated with genotoxic drugs instead of ionizing-radiation, consistently with previous studies PML NBs also co-localized with persistent DNA damage foci.

The exact purpose for the co-localization of PML NBs with persistent DNA damage foci needs to be investigated. One possible scenario is that PML NBs, known sites of localization of chromatin-modifying proteins such as HP1 (Zhang et al., 2005) and HDACs (Wu et al., 2001), can promote changes in chromatin architecture surrounding DSB to impact persistent DNA damage signaling. In support of this idea, recently published work showed

that PML NBs can represent the site of chromatin remodeling that regulates transcriptional activity of the E2F promoters through their recruitment into the close proximity of high concentrations of histone deacetylases (HDACs) (Vernier et al., 2011).

Overall, our study provides novel mechanistic insights into regulation of the tumor suppressor PML in cellular senescence caused by several genotoxic drugs used in clinical chemotherapy. These emerging links between cytokine signaling and PML can be important especially in the light of the fact that, unlike other forms of cellular senescence, drug-induced senescence can be evoked even in tumor cells and hence be exploited therapeutically.

# 5. CONCLUSIONS

The major aim of this work was to contribute to better understanding of several key aspects of cellular senescence. From a broader perspective, our data are highly relevant for complex processes such as development of cancer, aging, and response to clinical chemotherapy. We hope that our findings will positively stimulate further research in this exciting area of biomedicine.

The main conclusions of four publications presented in this PhD thesis can be shortly summarized as follows:

- **Publication 1:** Activation of DDR and ARF pathways in response to oncogenes
  - DDR activation occurs at earlier stage of tumor progression than ARF
  - DDR requires a lower threshold of oncogenic stress for its activation
  - ARF thus represents a delayed and complementary barrier to tumor progression

- **Publication 2:** Insights into chromatin changes during cellular senescence
  - Senescence-associated heterochromatin foci formation (SAHF) is not a universal feature of cellular senescence
  - SAHF occur in insult- and cell type-dependent manner
  - SAHF formation correlates well with $p16^{INK4a}$ accumulation
  - SAHF are not markers applicable to studies *in vivo*

- **Publication 3:** Role of senescence-associated secretory phenotype (SASP) in drug-induced senescence
  - Senescence induced by diverse genotoxic compounds is accompanied by secretion of a broad spectrum of secreted proteins including pro-inflammatory cytokines
  - Autocrine/paracrine signaling mediated by persistent activation of the JAK/STAT pathway regulates gene expression in senescent cells and contributes to maintenance of cytokine signaling

- **Publication 4:** Regulation of the tumor suppressor protein PML in drug-induced senescence
  - PML is regulated at the transcriptional level by JAK/STAT signaling via binding of the transcription factor STAT to the ISRE element in the PML gene promoter

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