Charles University in Prague

Faculty of Science

Study programme: Biology

Curriculum: Genetics, Molecular Biology and Virology - Molecular Biology and Genetics of Eukaryots

Bc. Lucie Knoblochová

Role of the tumour suppressor PML in DNA damage response and cellular senescence after genotoxic stress

Úloha nádorového supresoru PML v odpovědi na poškození DNA a buněčné senescenci po genotoxickém stresu

Diploma thesis

Supervisor: Zdeněk Hodný, M.D., Ph.D.

Prague, 2015

Declaration

I hereby declare that I completed this master thesis independently under the guidance of Zdeněk Hodný, M.D., Ph.D. and Hana Hanzlíková, Ph.D. It documents my own work if not explicitly otherwise mentioned. I have properly acknowledged and cited all sources used. The thesis was not used to obtain another degree.

Prague, August 13, 2015

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 13. srpna 2015

Podpis

Acknowledgement

I would like to thank my supervisor Zdeněk Hodný, M.D., Ph.D. and my consultant Hana Hanzlíková, PhD. for guidance and recommendations that were a great help for me during my work on this thesis. Many thanks belong also to Jana Dobrovolná, Ph.D., Soňa Hubáčková, Ph.D., Ján Valášek, M.Sc. and Blanka Gabajová, M.Sc. for advising me during the experiments and in writing the thesis. Another big acknowledgement belongs to all members of the Laboratory of Genome Integrity (IMG, Prague), who were never reluctant to help me. A big acknowledgement comes also to Michael Janský, M.A. for never ending corrections of my English texts. Last but not least, many thanks belong to my family and my boyfriend for their support during my studies and in writing this thesis.

My work was supported by GA13-17555S grant of the Grant Agency of the Czech Republic.

Abstract

The promyelocytic leukemia protein (PML) is a tumour suppressor. It has been reported that PML interaction with the p53 protein is involved in the activation of cell cycle checkpoints and, when persistent, may lead to the premature onset of cellular senescence. Cellular senescence is a state of permanent cell growth arrest that is associated with characteristic morphological and metabolic changes and persistent DNA damage signalling. Importantly, PML nuclear bodies coassociate with persistent DNA damage foci in senescent cells; however, the role of this interaction is still obscure. My goal was to characterize the role of PML in DNA damage response (DDR) and the induction of premature cellular senescence after genotoxic stress, namely X-radiation, using both siRNA-mediated PML knock down (PML KD) and complete PML knock out (PML KO) in human cells. The dynamics of DNA damage foci, levels of various proteins involved in DDR, and proliferation rate were measured in both PML KD and KO cells. No significant changes in the formation of DNA damage foci, activated DDR (p53 and Chk2), activated $p21^{CIP1/WAF1}$ cyclin-dependent kinase inhibitor, senescent morphology, and SA-β-galactosidase activity in PML KO cells were observed. However, PML KO cells displayed higher levels of retinoblastoma protein (Rb) and diminished proliferation. In conclusion, PML protein has no direct role in the development of ionizing radiation-induced premature cellular senescence but could be important for the regulation of cell cycle progression via modulation of Rb protein level.

Key words: PML, PML nuclear bodies, DNA damage response, DNA damage foci, Rb, cellular senescence, genotoxic stress, ionizing radiation, X-radiation

Abstrakt

Promyelocytický leukemický protein (PML) je nádorovým supresorem. Důležitým aspektem je jeho interakce s proteinem p53, která může způsobit aktivaci kontrolních bodů buněčného cyklu a při dlouhodobé signalizaci i rozvoj předčasné buněčné senescence. Buněčná senescence je stav trvalé zástavy růstu, který je spojen s morfologickými a metabolickými změnami a permanentní signalizací odpovědi na poškození DNA. Role proteinu PML při asociaci s lézemi poškození DNA a ve vývoji senescence je zatím nejasná. Cílem mé práce bylo charakterizovat protein PML v souvislosti s odpovědí na poškození DNA (DDR) a rozvojem senescence po genotoxickém stresu, a to rentgenovém záření, díky využití deplece PML pomocí technik siRNA-zprostředkovaného „knock-downu“ a pomocí kompletního „knock-outu“ (PML KO) PML v lidských buňkách. Dynamika lézí DNA, hladiny proteinů souvisejících s DDR a proliferační křivka byly měřeny pomocí metod imunoblotu a nepřímé imunofluorescence. Nebyly pozorovány žádné významné změny ve vytváření lézí DNA, aktivaci DDR (p53 a Chk2), aktivaci inhibitoru cyklin dependentních kináz $p21^{CIP1/WAF1}$, senescentní morfologii a aktivitě SA-β-galaktozidázy u PML KO buněk. Na druhou stranu, PML KO buňky vykazovaly vyšší hladinu retinoblastomového proteinu (Rb) a pomalejší proliferaci. Závěrem, PML protein hraje pravděpodobně jen nepřímou roli v předčasné buněčné senescenci indukované ionizujícím zářením, ale může být důležitý pro regulaci průchodu buněčným cyklem díky modulaci hladiny proteinu Rb.

Klíčová slova: PML, jaderná tělíska PML, odpověď na poškození DNA, léze DNA, Rb, buněčná senescence, genotoxický stres, ionizující záření, rentgenové záření

Contents

List of abbreviations:

53BP1	tumour suppressor p53-binding protein 1
APS	ammonium persulfate
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and rad3-related protein
ATRIP	ATR-interacting protein
BCA	bicinchoninic acid
BJ	human foreskin BJ fibroblasts
Brca1	breast cancer susceptibility protein 1
BrdU	5-bromo-2'-deoxyuridine, a nucleotide analogue
BSA	bovine serum albumin
CBP	CREB-binding protein
Cdc25	dual specificity phosphatase cdc25
CDKIs	cyclin-dependent kinase inhibitors
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
Chk2 pThr68	checkpoint kinase 2 phosphorylated on threonine 68
CIP/KIP	cyclin-dependent kinase interacting protein/the kinase inhibitor protein
CREB	cAMP response element-binding protein
ctrl	control
DAPI	4', 6-diamidino-2-phenylindole
DAXX	death domain-associated protein 6
ddH_2O	double distilled H_2O
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
DNA-SCARS	DNA segments with chromatin alterations reinforcing senescence
DSBs	double strand breaks
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EDTA	ethylenediaminetetraacetic acid, 99%
EdU	5-ethynyl-2´-deoxyuridine, a nucleotide analogue
FBS	foetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLH	glutaraldehyde
Gy	gray (a derived unit of ionizing radiation dose in the International System of Units)
H2AX	histone H2AX
HR	homologous recombination
HRP	horseradish peroxidase
hTERT	catalytic subunit of the human telomerase reverse transcriptase
IMG	Institute of Molecular Genetics of the ASCR, v. v. i., Vídeňská 1083, 142 20 Prague 4, Czech Republic
INK4	inhibitors of cyclin-dependent kinase 4
IR	ionizing radiation
MDC1	mediator of DNA damage checkpoint protein 1
Mdm2	E3 ubiquitin-protein ligase Mdm2
MEFs	mouse embryonic fibroblasts
mono	monoclonal antibody
MRN	mre11–rad50–nbs1 complex

NCS	neocarzinostatin
NHEJ	nonhomologous end joining
No.	number
OIS	oncogene-induced premature cellular senescence
p15	$p15^{INK4b}$ (cyclin-dependent kinase 4 inhibitor b)
p16	$p16^{INK4a}$ (cyclin-dependent kinase inhibitor 2a, isoforms 1/2/3; cyclin-dependent kinase 4 inhibitor a)
p18	$p18^{INK4c}$ (cyclin-dependent kinase 4 inhibitor c)
p21	$p21^{CIP1/WAF1}$ (cyclin-dependent kinase inhibitor 1)
p19	$p19^{INK4d}$ (cyclin-dependent kinase 4 inhibitor d)
p27	protein $p27^{Kip1}$ (cyclin-dependent kinase inhibitor p27; cyclin-dependent kinase inhibitor 1b)
p300	histone acetyltransferase p300
p53	tumour suppressor protein 53
p53 pSer15	tumour suppressor protein 53 phosphorylated on serine 15
p57	$p57^{Kip2}$ (cyclin-dependent kinase inhibitor 1c)
PARP-1	poly (ADP-ribose) polymerase 1
PBS	phosphate-buffered saline
PBST	1 x PBS + 0.1% Tween® 20
PD	population doubling
PFA	paraformaldehyde
PML	promyelocytic leukemia protein
PML KD	PML knock down
PML KO	knock out in the PML gene
PML NBs	PML nuclear bodies
PML wt	PML wild type; with an intact PML gene
ppRb	hyperphosphorylated retinoblastoma protein
poly	polyclonal antibody
pRb	hypophosphorylated retinoblastoma protein
Rad51	DNA repair protein rad51 homolog 1
RARα	retinoic acid receptor alpha
Rb	retinoblastoma protein
RNA	ribonucleic acid
RPA	replication protein a
RPE-1 hTERT	human retinal pigment epithelial cells immortalized by the expression of catalytic subunit of the human telomerase reverse transcriptase
Ser	serine
SAHFs	senescence-associated heterochromatin foci
SASP	senescence-associated secretory phenotype
SA-β-galactosidase	senescence-associated beta-galactosidase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate -polyacrylamide gel electrophoresis
SUMO	small ubiquitin-related modifier
TEMED	N,N,N',N'-tetramethylethylenediamine
Thr	threonine
TopBP1	DNA topoisomerase 2 binding protein 1
Tris	tris(hydroxymethyl)aminomethane
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
γH2AX	gamma-H2AX, histone H2AX phosphorylated on serine 139

1. Introduction

Cellular senescence is a state of an irreversible cell cycle arrest which is accompanied by many metabolic and morphological features that distinguish senescent cells from quiescent ones. Cellular senescence was first described by Leonard Hayflick, who observed that a cell culture can reach only a limited number of population doublings and after exceeding that threshold the culture remains alive but unable to proliferate. This state was called replicative cellular senescence. Later on, a similar cellular state was also observed after inflicting various types of stress, namely oxidative stress, DNA damage, treatment with genotoxic drugs or activated oncogenes. This state was termed premature cellular senescence, as the cells were unable to proliferate even though they had not reached the critical amount of population doublings. Senescent cells often display activated DNA damage response (DDR) signalling, indicating impaired genome integrity.

The promyelocytic leukemia protein (PML) is considered a tumour suppressor. Its role in malignant disease has been discovered first in acute promyelocytic leukemia. Moreover, PML protein was shown to be deregulated in many types of cancer indicating its widespread tumour suppressive role. PML homodimerizes utilizing SUMO-modification and SUMO-interaction domain and in this way it forms PML nuclear bodies (PML NBs). PML acts as a scaffold component of PML NBs, a nuclear compartment composed of several dozen molecules of various proteins. One of the possible functions of PML NBs is a storage site for other proteins, which would then influence various cellular processes such as DDR signalling, cell cycle arrest, or apoptosis.

PML is involved in the stabilization of p53, supporting its acetylation and facilitating the p53 dependent expression of $p21^{CIP1/WAF1}$ cyclin dependent kinase inhibitor. This results in the activation of cell cycle checkpoints and, when persistent, in the development of premature cellular senescence. Therefore, the PML NBs were suggested to play a role in the regulation of cell proliferation, cell cycle arrest, response to viral infections, and, last but not least, cellular senescence.

The regulation of PML protein expression in cellular senescence has been studied by the members of the Laboratory of Genome Integrity (IMG, Prague) in the past. Nowadays, the role of PML protein in IR-induced premature cellular senescence is explored as the coassociation of PML nuclear bodies with unrepaired double strand breaks representing DNA damage foci, an intriguing but not yet functionally

characterized feature of the IR-induced premature cellular senescence. The DNA damage foci can be induced by genotoxic stress, for example by X-irradiation or by chemotherapeutic drugs. For reasons yet unclear, some DNA damage foci remain unrepaired. It is believed that such DNA damage foci result in DDR signalling, persistent activation of cell cycle checkpoints, and development of the senescent phenotype.

Based on the coassociation of PML NBs with DNA damage foci and on the fact that PML was shown to induce premature cellular senescence, I attempted to characterize the role of PML in DDR and IR-induced premature cellular senescence using an *in vitro* human cell model with manipulated levels of PML expression. The new insights found in this work are based mainly on the utilization of human cells bearing a complete knock out in the PML gene (PML KO), which have not been used in the investigation of cellular senescence before.

2. Literature review

2.1 DNA damage response

The DNA damage response (DDR) is a signalling pathway which coordinates the repair of various types of DNA damage with several cellular processes, mainly with the progression of cell cycle (Fig. I); for a review, see (Branzei and Foiani 2008). The reason to stop the progression of the cell cycle is to avoid subsequent DNA damage by interference with e.g. DNA replication or cellular division during the repair of the damage, which could result in a genomic instability.

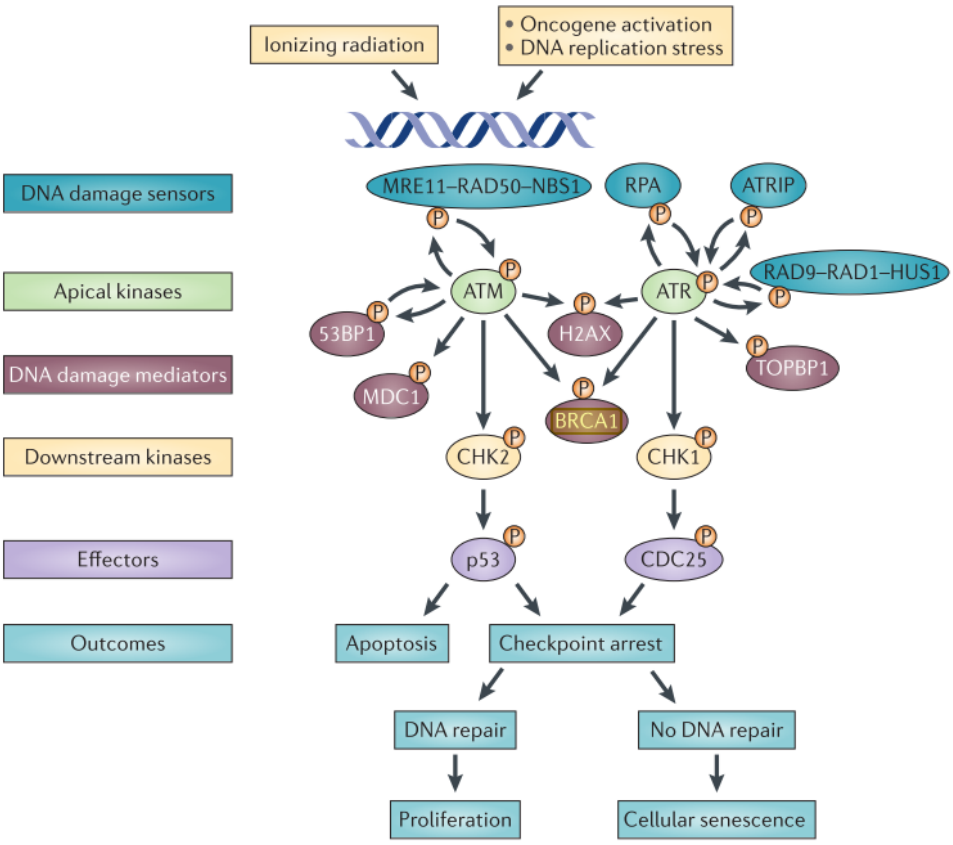


Figure I. A simplified depiction of the DNA damage response signalling pathway and phosphorylation events. Sensors (MRN complex, RPA, 9-1-1 complex and ATRIP), mediators (53BP1, H2AX, MDC1, Brca1 and TopBP1), transducing kinases (ATM and ATR), downstream kinases (Chk2 and Chk1 kinases) and effectors p53 and Cdc25 (Sulli et al. 2012).

DNA damage can result from various types of stress and so it can manifest in various ways – single and double stranded breaks, chemical or spontaneous modification of bases, incorporation of nucleotide analogues, replication mistakes, interstrand cross links or damage caused by intercalating agents. The amplification of the DDR signalling leads to the formation of so-called DNA damage foci (Haaf et al. 1995). My aim is to focus on DNA damage foci which are formed around DNA double strand breaks (DSBs). The signal from DNA damage foci then triggers DDR signalling and influences the fate of the cell.

The focus of this thesis is on genotoxic stress, namely ionizing radiation (IR; in particular X-radiation), being one of the stimuli which can cause DNA DSBs. The master regulator of DDR signalling of DNA DSBs after IR is the ATM (ataxia telangiectasia mutated) kinase, which phosphorylates many downstream proteins, among others histone H2AX, checkpoint kinase 2 (Chk2), tumour suppressor protein 53 (p53), breast cancer susceptibility protein 1 (Brca1) and others (Lee and Paull 2005). H2AX is one of the special variants of histone H2A and represents about 2 - 25% of the total H2A amount. It is spread throughout the genome as a sensing component of DNA DSBs and is phosphorylated on serine 139 in the response to DNA DSBs triggered, among other phenomena, by IR. The phosphorylated form of histone H2AX was termed γH2AX (Rogakou et al. 1998). Another important mediator is tumour suppressor p53-binding protein 1 (53BP1) which mediates DNA damage checkpoint signalling, DNA repair and activation of Chk2 and p53 (Wang et al. 2002).

After exposure of the cell to IR, p53 is phosphorylated by ATM on serine 15. This phosphorylation leads to a diminished interaction of p53 with its negative regulator, E3 ubiquitin-protein ligase Mdm2 (Mdm2) (Fig. II) (Shieh et al. 1997; Banin et al. 1998). Mdm2 links ubiquitin to p53 a thus targets it to proteosomal degradation (Kubbutat et al. 1997). Therefore, the diminished p53 interaction with Mdm2 leads to a reduced p53 turnover and to an increase in the p53 pool. What is more, it was found that Chk2 phosphorylates p53 on serine 20 after DNA damage, which is also known to impair the binding of p53 negative regulator Mdm2 (Hirao et al. 2000).

The coordination and maintenance of the signalling and repair proteins at the sites of DNA damage is effected through several posttranslational modifications, such as ubiquitination, poly(ADP-ribosyl)ation, phosphorylation, acetylation and SUMOylation; for a review, see (Lukas et al. 2011). One of the most important mechanisms is the ubiquitination cascade (Mailand et al. 2007).

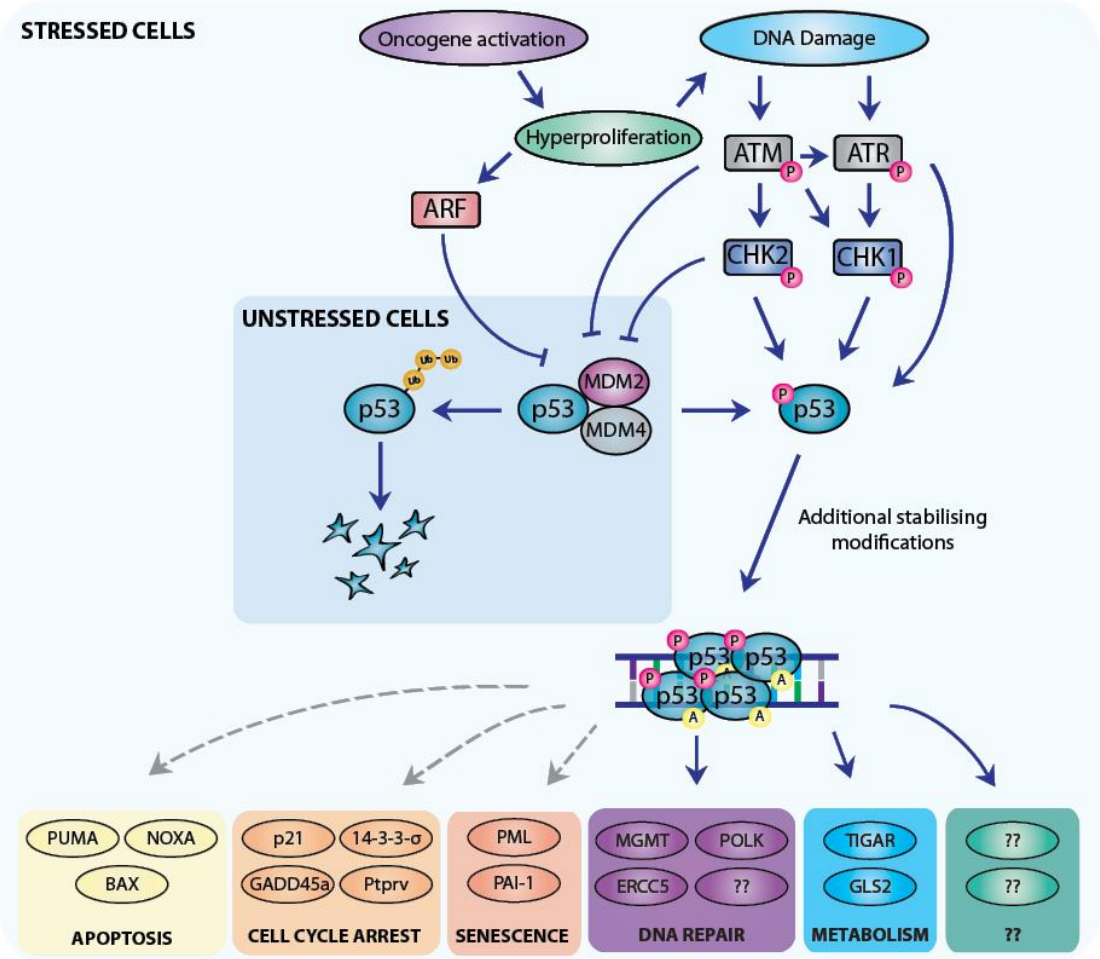


Figure II. Regulation of p53 stability. Mdm2 E3 ubiquitin ligase links ubiquitin to p53 that target p53 for proteosomal degradation. Oncogene activation or DNA damage induces ATM activation leading to posttranslational modifications of p53 which impair the p53 interaction with Mdm2. Thus the nuclear p53 pool is increased and enables regulation of other cellular processes such as apoptosis, cell cycle arrest, cellular senescence, DNA repair, metabolism, and probably also others, not yet discovered (Valent and Strasser 2013).

The main effect of the DDR signalling in connection with premature cellular senescence lies in the induced cell cycle arrest by activated cyclin dependent kinase inhibitors. The cyclin-dependent kinase inhibitors counteract the cell cycle progression resulting in cell cycle arrest (Vermeulen et al. 2003). Due to the ATM dependent signalling, the p53 is posttranslationally modified and therefore stabilized (Banin et al. 1998). Because p53 works as a transcriptional factor and induces $p21^{CIP1/WAF1}$ expression, the stabilisation of p53 leads to the accumulation of $p21^{CIP1/WAF1}$ cyclin dependent kinase inhibitor (Stein et al. 1999).

2.2 Cellular senescence

Cellular senescence is a specific cellular state characterized by essentially irreversible cell cycle arrest. Its purpose is thought to withdraw damaged cells from the cell cycle so they are not able to transduce their damage to the daughter cells and thus it prevents the cells from malignant transformation. Apart from tumour suppression, cellular senescence has also been identified as a factor in tissue repair, wound healing mechanisms and foetal development (Mendez et al. 1999; Muñoz-Espín et al. 2013).

2.2.1 Features of cellular senescence

The senescent cells display a plethora of various features which distinguish them from proliferating or quiescent cells. These features typically represent persistent DDR signalling, p16INK4a expression, an irreversible cell cycle arrest at the G1/S or at G2/M cell cycle checkpoints, enlarged and flattened cellular morphology, enhanced senescence-associated β-galactosidase (SA-β-galactosidase) activity, senescence-associated secretory phenotype (SASP), resistance to some forms of apoptosis, chromatin alterations such as the formation of senescence-associated heterochromatin foci (SAHF) or DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS), increased size and amount of PML nuclear bodies and also altered transcriptome and proteome (Dimri et al. 1995; Kulju and Lehman 1995; Alcorta et al. 1996; McConnell et al. 1998; Ferbeyre et al. 2000; Chang et al. 2002; Dierick et al. 2002; Hardy et al. 2005; Bartkova et al. 2006; Zhang et al. 2007; Rodier et al. 2009; Hubackova et al. 2010; Rodier et al. 2011; Lupa et al. 2014). It was shown that for the establishment and maintenance of cellular senescence, the indispensable feature is the activation of the persistent DDR signalling pathway (Bartkova et al. 2006; Di Micco et al. 2006; Masterson and O'Dea 2007; Fumagalli et al. 2014). However, no marker has yet been identified which would be specific to senescent cells and which would distinguish senescent cells from quiescent cells (Rodier and Campisi 2011).

Morphological changes in senescent cells

The morphological changes associated with the development of cellular senescence are generally the most easily observable features (Fig. III). The senescent cells are flattened, larger with higher cytoplasmic content and often multinuclear (Hayflick and Moorhead 1961).

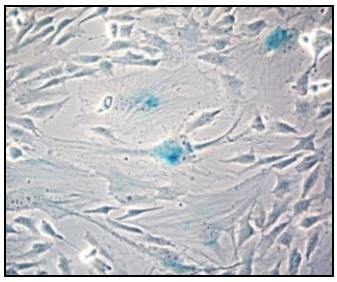


Figure III. Senescent cells. Senescent cells generally display an enlarged cellular morphology and express SA-β-galactosidase (blue staining). RPE-1 hTERT drug-induced senescent cells by neocarzinostatin in the middle surrounded by RPE-1 hTERT proliferating cells. (Knoblochova, unpublished data)

Activity of SA-β-galactosidase

In the senescent cells, a higher activity of a lysosomal β-galactosidase was described and this lysosomal β-galactosidase was termed SA-β-galactosidase (Dimri et al. 1995). The increased detection of SA-β-galactosidase in senescent cells was found to be due to a growth in lysosomal mass. Lee et al. (2006) showed its nonessential role for cellular senescence and the higher expression of SA-β-galactosidase was attributed to a consequence of cellular senescence, rather than as a cause. The activity of SA-β-galactosidase is detected by a cleaved substrate termed X-gal (5-bromo-4-chloro-3-indolyl-B-galactopyranoside), which shows an indigo colour after cleavage (Fig. III) (Kurz et al. 2000; Lee et al. 2006). Despite the nonessentiality, the senescence-associated β-galactosidase activity is an acceptable indicator of the senescence state, especially in *in vitro* culture model systems.

Persistent DDR signalling

Cellular senescence is often triggered by DDR signalling which persists (Bartkova et al. 2006; Di Micco et al. 2006; Masterson and O'Dea 2007; Fumagalli et al. 2014). The DDR can be caused by various genotoxic stimuli - by eroded telomeres, DNA DSBs, various drugs or strong mitogenic signals (Maser et al. 1997; Michishita et al. 1998; Takai et al. 2003; Bartkova et al. 2006; Novakova et al. 2010). However, cellular senescence can occur without an apparent DDR signalling, e.g. after increased expression of cyclin-dependent kinase inhibitors (CDKIs) from both the CIP/KIP ($p21^{CIP1/WAF1}$, $p27^{KIP1}$, $p57^{KIP2}$) and INK4 families ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{NK4c}$, $p19^{INK4d}$) (Kamijo et al. 1997; McConnell et al. 1998; Tresini et al. 1998; Fuxe et al. 2000).

Irreversible cell cycle arrest

Irreversible cell cycle arrest is caused by a suprathreshold permanent activity of at least one of the CDKIs. The permanent activity of CDKIs after DNA damage is triggered mostly by persistent DDR signalling and is mediated, for example, via p53 or $p16^{INK4A}$/retinoblastoma protein (Rb) tumour suppressor signalling pathways (Kulju and Lehman 1995; McConnell et al. 1998; Narita et al. 2003). Particularly, p53 regulates CDKIs from both CIP/KIP and INK4 families (see above). The CDKIs prevent the cyclin-dependent kinases from phosphorylating the Rb protein and thus disable in consequence the dissociation and activation of E2F transcription factors, which initiate the cell cycle progression (Weintraub et al. 1992). Thus, the effector protein which is indirectly regulated by the CDKIs (also by $p21^{CIP1/WAF1}$) is the Rb protein. The Rb can be found in the cells in two forms. In its hypophosphorylated (pRb) state, Rb interacts with the E2F transcription factors. This interaction disables the E2F transcription factors to function as a transcription factors and thus inhibits the cell cycle progression. In its hyperphosphorylated (ppRb) state, i.e. phosphorylated by the cyclin-dependent kinases, the Rb protein dissociates from the E2F transcription factors and so enables the E2F proteins to function as transcription factors and to induce cell cycle progression. The $p21^{CIP1/WAF1}$ is induced after DNA damage via DDR signalling to cause an immediate cell cycle arrest, in contrast to $p16^{INK4a}$ induction which is still not well understood (Robles and Adami 1998; Stein et al. 1999; Rodier et al. 2009; Coppé et al. 2011).

Heterochromatin formation

It is believed that the permanent cell cycle arrest in senescent cells is triggered by the persistent DDR signalling effecting the activation of CDKIs (as described above) accompanied by changes in chromatin remodelling – the formation of facultative heterochromatin, namely SAHFs (Narita et al. 2003; Zhang et al. 2007). However, cellular senescence can be established even in the absence of SAHFs (Kosar et al. 2011; Di Micco et al. 2011).

Another type of heterochromatin structure found in senescent cells are DNA-SCARS. DNA-SCARS are persistent nuclear foci that trigger DDR signalling and harbour activated DDR proteins. They result from various types of cellular stress such as DNA damage, dysfunctional telomeres or robust mitogenic signalling (Rodier et al. 2011).

Senescence-associated secretory phenotype

Apart from cell cycle arrest, persistent DDR also triggers events related to the induction of SASP. The SASP is characterized by the secretion of various cytokines, growth factors and other factors stimulating the microenvironment around the senescent cells in an autocrine and paracrine manner (Rodier et al. 2009). It was shown that the induction of the Janus kinase-signal transducer and activator of transcription (JAK/STAT) signalling pathway, possibly induced by the various cytokines, evokes PML expression in drug-induced premature cellular senescence (Hubackova et al. 2010). However, Coppé et al. showed that the SASP is a common but unessential feature of senescent cells (Coppé et al. 2011).

It should be noted that the abovementioned features of cellular senescence may or may not be present according to the cell type and the nature of the primary stimulus which has caused the senescent state.

2.2.2 Types of cellular senescence

Replicative cellular senescence

Two main types of cellular senescence are recognized – replicative cellular senescence and premature cellular senescence. Replicative cellular senescence was first described by Leonard Hayflick, who observed that human primary diploid cells are able to undergo only a finite number of cell divisions, and after reaching this limit they do not divide any further. Despite this inability to divide these cells are vital and metabolically active (Hayflick and Moorhead 1961). The replicative senescence was thought to be caused by telomere erosion, where the reduced telomeres trigger persistent DDR signalling causing a cell cycle arrest (d'Adda di Fagagna et al. 2003). However, it was shown that the DNA damage foci on the telomeres were probably not caused by telomere shortening but by some other type of DNA damage, because telomeric proteins inhibit DNA repair (Fumagalli et al. 2012; Zimmermann et al. 2013).

Premature cellular senescence

Premature cellular senescence occurs after diverse types of stress, most of them triggering persistent DDR signalling by various mechanisms (Fig. IV). Among such stressors are ionizing radiation (X and γ radiation) or genotoxic drugs, activation of oncogenes or oxidative stress. According to the type of such stimulus, there can be distinguished e.g. IR-induced premature cellular senescence,

drug-induced premature cellular senescence or oncogene-induced premature cellular senescence. Although the morphology of the various types of senescent cells looks similar, the gene expression varies according to the type of the stimulus (Chang et al. 2002; Dierick et al. 2002) .

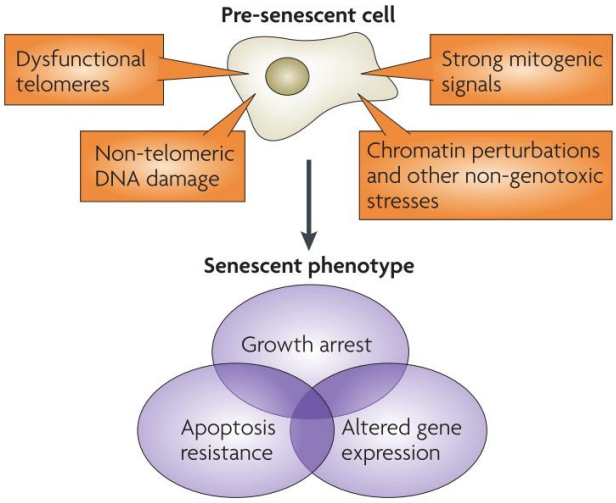


Figure IV. The induction of senescent phenotype by various stimuli (Campisi and d'Adda di Fagagna 2007).

IR-induced premature cellular senescence

Ionizing radiation causes various types of DNA damage; most important are DNA DSBs. The DNA DSBs arise both from the direct impact of ionizing radiation and from enzymatic processing of other types of DNA damage caused by the free radicals, which mostly arise from the radiolysis of water molecules (mainly hydroxyl radicals) (Ward 1985; Sachs et al. 1992; Wallace 1998). The DNA DSBs are detected as DNA damage foci (or ionizing radiation-induced foci, IRIF), where many DDR and DNA damage repair proteins can be found. The DNA damage foci were first reported to occur after irradiation in connection with the immunofluorescence staining of Rad51, a component of homologous recombination (Haaf et al. 1995). Moreover, later it was shown that the DNA damage foci are precisely organized dynamic structures, created by the multiplied DDR signalling factors which spread from the DNA DSBs sites to the flanking chromatin with substantial help from phosphorylation and ubiquitination (Bekker-Jensen et al. 2006; Mailand et al. 2007; Stewart et al. 2009). The DDR signalling triggers the activation of cell cycle checkpoints and hence causes a cell cycle arrest through the decreased activity of cyclin dependent kinases in order that the DNA DSBs repair processes do not interfere with replication, cell division or other cellular processes, which would cause more damage. If the DNA damage proves irreparable, the

DDR signalling triggered by the DNA damage becomes persistent and results in permanent growth arrest and so in premature cellular senescence or apoptosis, depending on the type or amount of the damage (Noda et al. 2012).

Ionizing radiation was reported to induce premature cellular senescence in both malignant transformed and nontransformed cell types after various doses ranging from 5 Gy to 20 Gy (Brown et al. 1997; Rodier et al. 2009; Noda et al. 2012; Münch et al. 2014; Rossiello et al. 2014).

Drug-induced premature cellular senescence

Drug-induced premature cellular senescence can occur after treatment of cells with various genotoxic drugs and chemicals, e.g. 5-bromo-2'-deoxyuridine (BrdU) or neocarzinostatin (NCS) (Dedon and Goldberg 1992; Michishita et al. 1998). A frequent consequence of the treatment with genotoxic drugs is the accumulation of reactive oxygen species (ROS) and the generation of oxidative stress which in turn influence the development of cellular senescence. BrdU is a nucleotide analogue which was shown to incorporate into DNA in place of thymidine and it is thought to change interaction of DNA with proteins and thus to change the expression of some genes by an unknown mechanism (Bick and Devine 1977; Morris 1991). The role of BrdU as a trigger of cellular senescence was first described by Michishita et al. (1998) and confirmed by several following studies (Michishita et al. 1998; Janderova-Rossmeislova et al. 2007; Masterson and O'Dea 2007; Hubackova et al. 2010; Novakova et al. 2010). NCS is an antibiotic which binds into a DNA minor groove and it inflicts single- and double-stranded DNA breaks in a sequence-specific manner (Dedon and Goldberg 1992; Smith et al. 1994). NCS was shown to be a senescence-inducing agent in ViBo cervical cancer cell line and MRC5 fibroblasts (Bañuelos et al. 2003; Hewitt et al. 2012).

Oncogene-induced premature cellular senescence

Oncogene-induced premature cellular senescence (OIS) is triggered by an overexpression or a mutation in some oncogenes which become more active. It was first discovered on mutant protein $HRAS^{V12}$ which in the primary cells caused a cell cycle arrest instead of a malignant transformation as in immortalized cells (Serrano et al. 1997). The cause of the cell cycle arrest and thus of cellular senescence seems to be replicative stress caused by elevated ROS or repeated firing of replicons during the S phase resulting in DNA DSBs (Bartkova et al. 2006; Di Micco et al. 2006).

As mentioned above, cellular senescence is a state of permanent cell cycle arrest accompanied by many other features, which can also differ according to the type of induction agent. One such feature is the already mentioned connection of cellular senescence with PML. The possible role of PML in premature cellular senescence is the main investigative aim of my work.

2.3 Promyelocytic leukemia protein

The promyelocytic leukemia protein (PML) is known to be an important tumour suppressor; its role in cancer was discovered in acute promyelocytic leukemia, where the PML gene (on chromosome 15) is translocated to the gene encoding the retinoid acid receptor α (RARα) receptor (on chromosome 17) and thus causes an aberrant signalling (de The et al. 1990). PML is also deregulated in a broad range of cancers and its tumour suppressor function was also confirmed by studies made on $PML^{-/-}$ mice (Koken et al. 1995; Terris et al. 1995; Wang et al. 1998; Gurrieri et al. 2004). Although the $PML^{-/-}$ mice showed a normal and fertile phenotype, they developed more tumours upon treatment with tumour-promoting drugs than their $PML^{+/+}$ siblings (Wang et al. 1998). Later it was shown that PML deficiency even alone leads to spontaneous tumorigenesis in the prostate (Trotman et al. 2006).

Further studies showed that PML is significant in many other cellular processes such as transcriptional regulation, DDR, apoptosis, cellular senescence, and viral defence; for a review, see (Bernardi and Pandolfi 2007; Everett and Chelbi-Alix 2007).

2.3.1 PML structure and isoforms

The PML gene is approximately 35 kb long and contains 9 exons which can be spliced into seven main alternative splicing forms (PML isoforms I-VII), unmodified with molecular weight from ca. 70 to 95 kDa (Fig. V) (Fagioli et al. 1992; Jensen et al. 2001). All isoforms contain an N-terminal domain within the first three exons, with a tripartite motif consisting of a zinc finger called the RING domain, two additional zinc fingers called the B-box motif, and a coiled-coil domain (RBCC/TRIM motif). The RBCC motif is important in multimerization and thus in the formation of PML nuclear bodies (Jensen et al. 2001). The PML isoforms are placed predominantly in the nucleus but some of them can be found also in the cytoplasm.

Intriguingly, it was shown that PML is in contact with nucleolar material creating large ring-shaped structures (termed PML doughnuts) embracing small nucleoli in senescent cells after encountering stress (Condemine et al. 2007; Janderova-Rossmeislova et al. 2007).

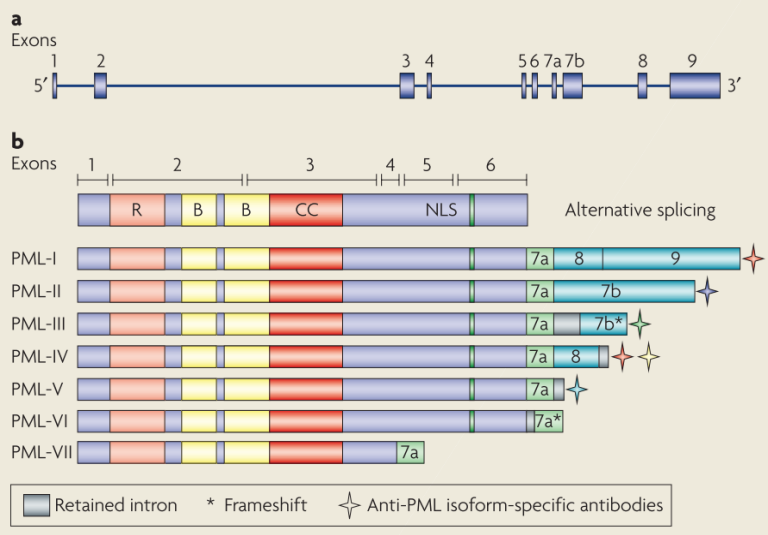


Figure V. The human PML gene containing 9 exons (a) and 7 isoforms formed by alternative splicing (b). The localisation of the RBCC motif and NLS is shown (Bernardi and Pandolfi 2007).

2.3.2 Transcriptional regulation of PML

PML upregulation was shown after oncogene activation, DNA damage, treatment with cytokines such as interferons and IL6, and viral infections (Maul et al. 1995; Stadler et al. 1995; Chan et al. 1997; Ahn and Hayward 2000; Ferbeyre et al. 2000; Adamson and Kenney 2001; Pearson and Pelicci 2001; Hubackova et al. 2012). Correspondingly, PML was found to include IFNγ-activated sites and IFN-response elements (ISREs) (Stadler et al. 1995). PML transcription is also controlled by the p53 protein due to a p53 responsive element in the PML gene (de Stanchina et al. 2004). In normal cells, PML protein is expressed diversely in various cell types or tissues (Flenghi et al. 1995).

2.3.3 Posttranslational modifications of PML

PML has been found to be phosphorylated on many sites. Two phosphorylation events were reported in connection with DNA damage: Firstly, after phosphorylation by Ataxia telangiectasia and Rad3-related (ATR) kinase, PML is translocated into the nucleolus, followed by its negative regulator Mdm2 (Bernardi et al. 2004). Secondly, PML is phosphorylated also by Chk2; this event is considered to trigger apoptosis within the ATM-Chk2-PML signalling pathway in a p53-independent manner (Yang et al. 2002). PML was also reported to be negatively regulated by the casein-kinase 2 (CK2) and concurrently inhibition of CK2 increased the tumour-suppressive function of PML (Scaglioni et al. 2006).

SUMOylation (a covalent binding of proteins from the SUMO family; small ubiquitin-related modifier) is another important posttranslational modification. PML contains SUMO-interacting motifs and binds SUMO-1, SUMO-2, SUMO-3, and SUMO-conjugating enzyme, which catalyses SUMOylation on lysine residues (Kamitani et al. 1998; Duprez et al. 1999; Shen et al. 2006). The covalent modifications especially by SUMO-2 and -3 were found to be crucial for the regulation of PML accumulation and formation of PML nuclear bodies (Zhong et al. 2000; Mukhopadhyay et al. 2006). It was proposed that the nucleation event of PML nuclear bodies is dependent on the PML covalent binding of SUMO proteins and on noncovalent interactions of PML SUMO-interacting motifs with other SUMOylated PML molecules (Shen et al. 2006).

2.3.4 PML nuclear bodies

PML can be found in most mammalian cells in the form of small nuclear speckles, PML nuclear bodies (PML NBs). Normal human cells contain 1 to 30 PML NBs, mostly ranging in size from 0.1 to 1 µm (Zhong et al. 2000; Jensen et al. 2001).

PML NBs (also known as the POD – PML oncogenic domain, ND10 – nuclear domain 10, or Krüppel bodies) are highly heterogeneous and dynamic structures which change in various cell cycle phases under normal circumstances and in response to cellular stress (Fig. VI) (Muratani et al. 2002). They are ring-shaped and do not contain any nucleic acid in the core (as was shown by electron microscopy) (Boisvert et al. 2000). The PML NBs are in extensive contact with the surrounding chromatin which also influences their morphology and dynamics (Eskiw et al. 2004). Moreover, PML NBs are not randomly positioned in the nucleus; they are detectable near other nuclear bodies such as Cajal bodies or nucleoli and were shown to bind to the nuclear matrix (Stuurman et al. 1992; Grande et al. 1996). Although only PML is indispensable for the formation of PML NBs, more than 150 other proteins may accumulate in the PML NBs, such as SUMO-1, Death domain-associated protein 6 (DAXX), CREB-binding protein (CBP), transcription factor Sp100 and p53 (Dyck et al. 1994; Ishov et al. 1999; Weidtkamp-Peters et al. 2008).

It was shown that the number of PML NBs increased with cell cycle progression from G0 to G1, S, and G2. PML NBs respond to the changes in chromatin structure in the S phase where they undergo fission and thus probably monitor the chromatin state (Dellaire, Ching et al. 2006). Immunofluorescence

in situ experiments also revealed a close association of PML NBs with histone gene clusters during the S phase (Wang et al. 2004). More than 50% of PML NBs associate with replicative genes during the middle-late S phase (Grande et al. 1996). During the M phase PML NBs form aggregates and are spread around the cytoplasm, although a small fraction remains bound to the chromosomes (Dellaire, Eskiw et al. 2006). It was suggested that the tied PML NBs form seeds for new PML NBs in the G1 phase (Dellaire, Eskiw et al. 2006).

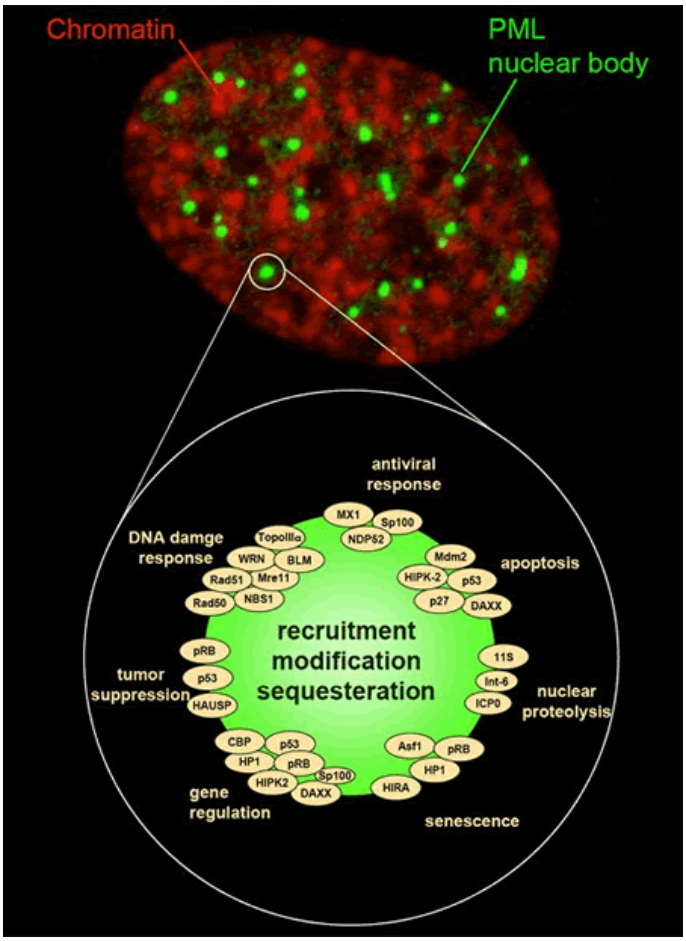


Figure VI. PML nuclear bodies are highly dynamic structures containing proteins participating in various cellular processes. (http://www.fli-leibniz.de/www_imaging/PML_en.php)

The PML NBs grow also in size and number after cellular stress, after induction by cytokines (e.g. interferons type I and II), and in senescent cells, which has led to the conclusion that PML NBs are stress-responding structures (Stadler et al. 1995; Ferbeyre et al. 2000; Hubackova et al. 2010).

2.3.5 PML functions

Although many findings point to PML role in tumour suppression or cellular senescence, the exact function of PML remains to be elucidated. Several explanations of PML NBs' function have been proposed: In the first model, PML NBs are seen as a storage site for various proteins both in normal conditions and after stress. According to the second model, PML NBs can act as platforms for posttranslational modifications, considering the putative function of PML as an E3 SUMO ligase and the role of PML NBs in SUMOylation (see above). The third model presents the idea that PML NBs could serve as sites for ongoing cellular processes such as chromatin regulation or transcription, principally due to the spatially proximate assembly of the required factors (Bernardi and Pandolfi 2007).

The role of PML in transcription

It has been shown that PML NBs are found in transcription rich regions, but PML knock down had no impact on the respective gene expression (Wang et al. 2004). Although PML was assumed to play an important role in transcription regulation, it has not been confirmed yet. There are only few pieces of evidence supporting the PML role in transcription; one of them is the nonrandom association of PML NBs with a cluster of major histocompatibility complex (MHC) genes at the p53 gene locus (Shiels et al. 2001; de Stanchina et al. 2004). It has been proposed that PML can modulate transcription by the availability of transcriptional factors or by playing a role in chromatin remodelling (Zhong, Delva et al. 1999). However, a transcriptional repressive role was also proposed for PML NBs because of the binding of heterochromatin protein 1 (HP1) and because of PML NBs participating in the formation of SAHFs in senescent cells (Seeler et al. 1998; Zhang et al. 2007). The kinetics of the formation of SAHFs is by all evidence a multistep process, which includes the recruitment of HP1 and HIRA to the PML NBs in the first step (Zhang et al. 2007). Explanation for these contradictory findings could be a more complex function of PML NBs, where e.g. PML NB function could be cell cycle- or stress-dependent or PML isoform-specific (Bernardi and Pandolfi 2007).

PML as a tumour suppressor

As already mentioned PML is aberrantly regulated in many types of cancer (Koken et al. 1995; Terris et al. 1995; Gurrieri et al. 2004). Also, $PML^{-/-}$ mice developed more tumours upon treatment with tumour-promoting agents than $PML^{+/+}$ mice (Wang et al. 1998). Moreover, it has been described that PML deficiency itself promotes spontaneous tumorigenesis in the prostate (Trotman et al. 2006).

The overexpression of PML leads to growth suppression and in consequence to establishment of premature cellular senescence or apoptosis (Ferbeyre et al. 2000; Lin et al. 2004; Mallette et al. 2004; Bischof et al. 2005). Also, PML-overexpressing prostate cancer cells showed a lower growing potential *in vivo* (He et al. 1997). Interestingly, most of the PML tumour suppressive effects are ascribed to the PML effect on p53 and Rb signalling pathways (Alcalay et al. 1998).

The role of PML in DDR

The exact role of PML in DDR is not known; however, there is evidence that PML interacts with many DDR components and that it could regulate them or be regulated by them. As already mentioned, PML is phosphorylated by Chk2 and by ATR after genotoxic stress (Yang et al. 2002; Bernardi et al. 2004). Also, increased expression of PML and an increased number of PML NBs was observed in relation with ATM signalling after genotoxic stress (Dellaire, Ching et al. 2006). Moreover, PML protein interacts with many DDR components, mostly inside of PML NBs (Carbone et al. 2002). Dellaire et al. suggested that PML could play a role in sensing DNA damage (Dellaire, Ching et al. 2006). However, the interactions of DDR and repair proteins with PML NBs are highly complex; some proteins associate with PML NBs before stress and consequently are activated and change localisation (Chk2) or dissociate and re-associate (Mre11); other proteins localize to PML NBs only after encountering stress (ATM, γH2AX, Werner syndrome helicase, TopBP1 or Brca1) (Mirzoeva and Petrini 2001; Blander et al. 2002; Xu et al. 2003; Dellaire, Ching et al. 2006). A possible explanation for the role of PML in the DDR could lie in the fact that PML was found to interact with p53 in several ways. PML facilitates p53 acetylation by CBP acetyltransferase and hence helps to induce OIS in a p53-dependent manner (Pearson et al. 2000). PML NBs contain various proteins, including proteins regulating p53 stability via posttranslational modifications - Mdm2, CBP, $p14^{Arf}$, etc.; for a review, see (Takahashi et al. 2004). Also, PML can activate p53 and increase the transcription of p53-responsive genes by sequestering p53 into PML NBs, which implies positive feedback (de Stanchina et al. 2004). Another connection with the p53 pathway lies in the fact that PML recruits Chk2 to the PML NBs after encountering stress and thus promotes the phosphorylation of p53 by Chk2 (Yang et al. 2006). Moreover, HIPK2 kinase, a regulator of p53 and promoter of apoptosis in a p53-dependent manner, was also found in PML NBs (D'Orazi et al. 2002; Hofmann et al. 2002). Importantly, PML NBs were shown to colocalize with DNA damage foci as soon as 24 h after a genotoxic event, and this coassociation can persist for weeks (Carbone et al. 2002; Rodier et al. 2011). However, the exact nature of the PML NBs coassociation with

DNA damage foci in senescent cells remains still unclear. The evidence of the PML role in DDR signalling is extensive; on the other hand, unfortunately only indirect.

The role of PML in cellular senescence

The connection of the PML protein with cellular senescence has been proposed on the basis of the following observations: Upregulated PML and an increase in the number and size of PML NBs have been found in oncogene-induced senescent human diploid fibroblasts and also mouse embryonic fibroblasts (MEFs) (Ferbeyre et al. 2000; Pearson et al. 2000). The increased number of PML NBs and PML upregulation was also described in drug-induced or replicative senescent human diploid fibroblasts (Ferbeyre et al. 2000; Janderova-Rossmeislova et al. 2007; Hubackova et al. 2010). On the other hand, PML overexpression itself can cause premature cellular senescence (Ferbeyre et al. 2000; Pearson et al. 2000). Specifically, PML isoform IV was shown to be indispensable to induce premature cellular senescence in $PML^{-/-}$ cells. However, PML isoform IV alone was not sufficient to induce premature senescence, implying an important role of the cooperation of more PML isoforms (Bischof et al. 2002). Recently, Münch et al. (2014) showed that PML is dispensable in cellular senescence in cells with a stable shRNA-mediated PML knock down. Although a gene knock down is a commonly used tool in molecular biology, it is not always successful to inhibit fully the expression of the gene of interest, as a residual protein level can be sufficient to cover the gene's function. Therefore, it is worth to explore the role of PML in senescent cells also bearing a PML KO.

PML role in cellular senescence has also been implied by several other indirect observations. As seen above, PML interacts functionally with p53 in several ways. However, overexpression of E6 and E7 from human papillomavirus oncoproteins caused an onset of PML-dependent premature cellular senescence, which was reliant on the Rb tumour suppressor pathway but not on the p53 signalling pathway in human fibroblasts (Mallette et al. 2004). Furthermore, PML NBs were shown to participate in heterochromatin formation (SAHFs, described above) in senescent cells (Zhang et al. 2007).

Although there is indirect evidence that PML affects cell cycle regulation via p53 activity, it was suggested that the key mediator is in consequence the Rb protein (Alcalay et al. 1998). It has been shown that pRb localizes into PML NBs and that PML interacts with Rb during the development of cellular senescence (Alcalay et al. 1998; Ferbeyre et al. 2000; Vernier et al. 2011; Talluri and Dick 2014). A growing body of evidence suggests the importance of E2F target genes silencing in the cellular

senescence in a PML- and Rb-dependent manner (Vernier et al. 2011). It the OIS was found that PML interacts with E2F target genes and that this interaction is dependent on the Rb LXCXE motif, inducing the silencing of E2F target genes via heterochromatinization (Talluri and Dick 2014). However, PML was shown to be nonessential for the activation of Rb growth suppressive effects (Alcalay et al. 1998).

2.3.5. The differences between human and murine cell cultures in connection with PML protein and cellular senescence

The mouse PML KO MEFs were first used by Wang et al. in 1998. Although the parental mice had impaired immune response, their sensitivity to carcinogenesis manifested no earlier than after one year (Wang et al. 1998). In several experiments, MEFs were used as a model of PML depletion (de Stanchina et al. 2004; Vernier et al. 2011). However, there are some findings which show that murine and human cells show some differences in connection with cellular senescence and PML protein. It was reported that the mechanisms controlling cellular senescence differ between humans and mice, mainly in the role of the $p21^{CIP1/WAF1}$ cyclin dependent kinase inhibitor, where the role of $p21^{CIP1/WAF1}$ in the cellular senescence in murine cells is only indirect (Itahana et al. 2004). Also, $PML^{-/-}$ MEFs showed faster proliferation than $PML^{+/+}$ MEFs (about one additional population doubling of the PML KO cells in a period of 3 days). This stands in stark contrast to the diminished proliferating capacity of PML depleted human cells as reported by Münch et al. (Münch et al. 2014). Apart from that, only PML isoforms I and V are identical in humans and mice, implying differences in the PML itself (Condemine et al. 2006). It was also reported that MEFs without PML showed a decreased rate of induction of apoptosis and cellular senescence despite the activation of p53 responsive genes (de Stanchina et al. 2004). Altogether, the mouse is a potent model for several human diseases but I chose the human model due to differences in the function of PML in the cellular senescence of human and mouse cells.

As described above, various findings indicate a functional link of PML to the development of cellular senescence. Characterization of the role of PML in DDR and IR-induced premature cellular senescence after genotoxic stress in human cells *in vitro* is the main objective of my work.

3. Aims of the thesis

The main goals of the thesis were:

1) find the best conditions for the development of premature cellular senescence induced by ionizing radiation *in vitro* and
2) use this model for the characterization of the role of PML protein in DNA damage response and premature cellular senescence after genotoxic stress

The specific aims of the thesis were:

Aim 1:

Characterization of the impact of glucose concentration in the culture medium.

Hypothesis:

- It was found that high glucose concentration in the culture medium can promote the development of premature replicative senescence. My goal was to test whether a high glucose concentration in culture medium influences the development of IR-induced premature cellular senescence.

Aim 2:

Analysis of the effect of different types of X-radiation and identification of the most effective radiation dose.

Hypothesis:

- X-radiation is characterized by a broad range of energies dependent not only on intensity (dose) but also wavelength. The shorter wavelength (higher frequency) results in higher energy, and therefore penetration depth in matter. The goal was to test the effect of the total dose, as well as 'soft' versus 'hard' irradiation, on the formation of DNA damage foci and activity of DDR, cell cycle arrest, morphological changes characteristic of cellular senescence, such as the increase in the cytoplasmic amount, and the activity of SA-β-galactosidase, for the induction of IR-induced premature cellular senescence in RPE-1 hTERT human epithelial cells.

Aim 3:

Examination of the role of PML protein in DDR.

Hypothesis:

- The p53 tumour suppressor is often is called the "genome integrity guardian" and is described as a key component of the DDR signalling pathway. The IR-induced premature cellular senescence is known to be established due to p53-dependent persistent DDR activation and p53-induced cell cycle arrest, and correspondingly PML and PML NBs were reported to interact with p53 in many ways. Based on the complex interplay of PML and the p53 protein, I examined the possible role of PML in DDR. My hypothesis is that if PML positively interacts with p53 in promoting DDR, there are likely to be some changes in the DDR after PML KD or PML KO in IR-induced senescent cells.

Aim 4:

Investigation of the role of PML in the coassociation of PML NBs with DNA damage foci.

Hypothesis:

- Since the majority of DNA damage foci are known to coassociate with PML NBs in IR-induced premature cellular senescence, the working hypothesis was that PML NBs are functionally involved in DNA repair. To test this hypothesis, I utilized a cell model with PML KD or complete PML KO challenged with genotoxic stress.

Aim 5:

Exploration of the role of PML in the establishment of IR-induced premature cellular senescence.

Hypothesis:

- Based on the fact that PML overexpression has been found to promote premature cellular senescence and the observation that the level of PML changes in senescent cells, which implies a positive role of PML protein in cellular senescence, the effectiveness of the establishment of IR-induced cellular senescence in a PML KO cell type was examined.

4. Materials and methods

4.1 Materials

4.1.1 Chemicals

Chemical	Manufacturer/Vendor	Catalogue No.
2-Mercaptoethanol	Sigma-Aldrich	M3148-25 ML
10 x TG Buffer (Tris/Glycine Buffer - 25 mM Tris, 192 mM glycine, pH 8.3)	BioRad	161-0771
10 x TGS Buffer (Tris/Glycine/SDS - 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3)	BioRad	161-0772
Acrylamide/Bis-acrylamide, 29:1, 30% solution	Serva	1068701
APS (Ammonium persulfate, 98%)	Sigma-Aldrich	A3678-25 G
Aqua for injectione	B. Braun Melsungen AG	87/024/98-C
BrdU (5-bromo-2'-deoxyuridine, 99%)	Sigma-Aldrich	B5002
Bromophenol blue (3',3",5',5"-tetrabromophenolsulfonphthalein)	Lachema	
BSA (Bovine Serum Albumin)	Sigma-Aldrich	A9647_50 G
Click-iT® EdU Alexa Fluor® 488 Imaging Kit	Invitrogen (Life Technologies)	C10337
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Aldrich	D9542-5 MG
DMEM (Dulbecco's Modified Eagle's Medium)	Biochrom/Media and Glass Washing Department, IMG	
DMEM, Low Glucose, GlutaMAX™, Pyruvate	Gibco/Invitrogen	21885-025
DPBS (Dulbecco's Phosphate-Buffered Saline)	Sigma	D8662-6 X 500 ML
Dithiothreitol	Sigma-Aldrich	43817
Double distilled H_2O	Media and Glass Washing Department, IMG	
EDTA (Ethylenediaminetetraacetic acid, 99%)	Sigma-Aldrich	E5134-50 G
EdU (5-Ethynyl-2´-deoxyuridine)	Invitrogen (Life Technologies)	C10337
Ethanol 96%	Penta	
FBS (Foetal bovine serum)	Gibco (Life Technologies)	10270
Glutaraldehyde solution (Grade I, 25% in H_2O, specially purified for use as an electron microscopy fixative)	Sigma-Aldrich	G5882
Glycerol (99%)	Sigma-Aldrich	G-5516-1 L

Lipofectamine® RNAiMAX Transfection Reagent	Life Technologies	13778030
Methanol	Penta	
$MgCl_2$ (Magnesium chloride)	Sigma	M1028-100 ML
NCS (Neocarzinostatin)	Sigma-Aldrich	N9162
Opti-MEM® I Reduced Serum Media	Gibco (Life Technologies)	51985
PageRuler™ Prestained Protein Ladder, 10 to 180 kDa	Life Technologies	26616
PBS (Phosphate-Buffered Saline)	Gibco (Life Technologies)	20012
Penicillin-Streptomycin (10,000 units penicillin and 10 mg streptomycin per ml in 0.9% NaCl, sterile-filtered), 100 x	Sigma-Aldrich	P0781-100 ML
FA (formaldehyde), 4% (m/v) pH 7.2	Vitrum	
Pierce™ BCA Protein Assay Kit	Life Technologies	23227
Pierce™ ECL Western Blotting Substrate	Life Technologies	32209
Ponceau S	Fluka	
Sanolait Magermilchpulver 350 g	Coop, Basel, Switzerland	
SDS (Sodium dodecyl sulfate)	Serva	20765
Sodium azide	Koch-Light Laboratories Ltd.	91789
SuperSignal West Femto Chemiluminescent Substrate	Life Technologies	34095
TEMED (N,N,N',N'- Tetramethylethylenediamine)	Fluka	87689
Tris (tris(hydroxymethyl)aminomethane)	Serva	37186
Triton X-100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether)	Fluka	93426
Trypsin EDTA (1 x) 0.05% / 0.02% in DPBS	PAA	L11-004
Tween20 (Polyoxyethylene (20) sorbitan monolaurate)	Serva	37470
VECTASHIELD® Mounting Medium	Vector Laboratories	H-1000
X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside ≥98%, powder)	Sigma-Aldrich	B4252-100 MG

Table 1: List of chemicals

4.1.2 Antibodies

Primary Antibodies	Species	Type	Vendor	Catalogue No.	Dilution
Immunofluorescence					
53BP1	rabbit	poly	Santa Cruz	sc-22760	1:1,000
PML	mouse	mono	Santa Cruz	sc-966	1:300
H2AX pSer139	mouse	mono	Millipore	05-636	1:500
H2AX pSer139	rabbit	mono	Cell Signaling	9718S	1:300
Western blot					
Chk2 pThr68	rabbit	poly	Cell Signaling	2661	1:1,000
GAPDH	mouse	mono	GeneTEX (from EXBIO)	GTX3066	1:8,000
H2AX pSer139	mouse	mono	Millipore (from Scintila)	05-636	1:1,000
H2AX pSer139	rabbit	poly	Cell Signaling	2577	1:1,000
Pan-actin (C-terminus: alfa, beta, gamma-actin)	rabbit	poly	Sigma-Aldrich	A2066	1:1,000
p21waf1 (DCS-60)	mouse	mono	Santa Cruz	sc-56335	1:1,000
p53 (D.01)	mouse	mono	Santa Cruz	sc-126	1:1,000
p53 pSer15	rabbit	poly	Cell Signaling	9284	1:1,000
PML	rabbit	poly	Santa Cruz	sc-966	1:1,000
Rb (Retinoblastoma protein)	mouse	mono	BD Pharmingen	554136	1:1,000

Table 2: List of primary antibodies

Secondary Antibody	Species	Type	Vendor	Number	Dilution
Immunofluorescence					
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate	goat	anti-mouse, poly	Life Technologies (Invitrogen)	A11029	1:1,000
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 568 conjugate	goat	anti-rabbit, poly	Life Technologies (Invitrogen)	A11036	1:1,000
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 647 conjugate	goat	anti-mouse, poly	Life Technologies (Invitrogen)	A21236	1:1,000
Western blot					
Goat Anti-Mouse IgG (H + L)-HRP Conjugate	goat	anti-mouse	BioRad	170-6516	1:10,000
Goat Anti-Rabbit IgG (H + L)-HRP Conjugate	goat	anti-rabbit	BioRad	170-6515	1:10,000

Table 3: List of secondary antibodies

4.1.3 Oligonucleotides

Oligonucleotides	Sequence, sense	Vendor	Catalogue Number
Silencer® Select Negative Control No. 1 siRNA (siNC)	Ambion proprietary information	Ambion	4390843
siPML#3	GGCAGAUUGUGGAUGCGCAtt	Ambion	s194692
siPML#4	GGAGCAGGAUAGUGCCUUUtt	Ambion	s194691

Table 4: List of oligonucleotides

4.1.4 SDS-PAGE gels

	1 mm 8%	1 mm 14%	1.5 mm 8%	1.5 mm 14%
ddH_2O (ml)	2.4	1.4	3.6	2.1
30% Acrylamide (ml)	1.3	2.3	2.0	3.5
Buffer A (ml)	1.3	1.25	1.9	1.9
APS 10% (µl)	35	35	50	50
TEMED (µl)	8	8	12.5	12.5

Tables 5: Separating SDS-PAGE gels

1 mm	1 mm	1.5 mm
ddH_2O (ml)	1.2	1.8
30% Acrylamide (ml)	0.3	0.45
Buffer B (ml)	0.5	0.75
APS 10% (µl)	16	24
TEMED (µl)	4	6

Table 6: Stacking SDS-PAGE gels

	1 mm 6%	1 mm 20%	1.5 mm 6%	1.5 mm 20%
ddH_2O (ml)	1.715	0	2.57	0
Buffer A (µl)	781	781	1.17	1.17
30% Acrylamide (ml)	0.639	1.767	0.959	2.65
Glycerol (µl)	-	625	-	938
APS 10% (µl)	15	15	22.5	22.5
TEMED (µl)	2	2	3	3

Table 7: Separating SDS-PAGE gradient gels (for stacking gels for SDS-PAGE gradient gels, see Table 6)

4.2 Methods

4.2.1 Cell cultures

Human retinal pigment epithelial cells immortalized by the expression of catalytic subunit of the human telomerase reverse transcriptase RPE-1 hTERT PML wild type (PML wt) were purchased from ATCC™. RPE-1 hTERT cells bearing a complete PML KO (RPE-1 hTERT LC-D10A-PML2a-#2 single clone-derived cells bearing a knock out in PML gene; hereinafter referred to as PML KO cells) were prepared by J. Kosla (Laboratory of Genome Integrity, IMG Prague) using lentiCRISPR Cas9-D10A technology. Both RPE-1 hTERT cell types were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium with a glucose concentration of 4.5 g/l. The medium was obtained from the Media and Glass Wash Department at IMG. It was supplemented with 10% foetal bovine serum (FBS) by Gibco® and with antibiotics (Penicillin 100 U/ml, Streptomycin 100 ng/ml). Cells were cultured at 37°C in 5% CO_2 on 6 cm dishes (with 5 ml of medium), on 10 cm dishes (with 12 ml of medium) or in 6-well dishes (with 2 ml of medium per well). The cells were subcultured with trypsin/EDTA (0.05%/0.02%) in Dulbecco's Phosphate-Buffered Saline (DPBS).

Human foreskin BJ fibroblast cells were purchased from ATCC™ and cultured in a Gibco®DMEM medium with a glucose concentration of 1.0 g/l.

4.2.2 Induction of cellular senescence

Cellular senescence was induced by ionizing irradiation (in particular X-radiation) or by a specific drug, as specified below. The cells were seeded the day before treatment to reach approximately 60% confluency at the day of treatment, and then harvested at various time points, as indicated.

1) BJ fibroblasts were irradiated with a dose of 10 Gy by an X-ray generator (Pantak, Berkshire, United Kingdom). In a pilot experiment, RPE-1 hTERT cells were irradiated with doses of 5, 10, 20, 50, and 100 Gy and the development of senescence was assessed. For further experiments, a dose of 20 Gy was chosen both for RPE-1 hTERT PML wt and PML KO cells.

2) RPE-1 hTERT PML wt and PML KO cells were treated with 100 µM BrdU (a nucleotide analogue) (Michishita et al. 1998)**.** The fresh drug was added each second day during medium exchange.

4.2.3 Other treatments

RPE-1 hTERT PML wt and PML KO cells were treated with a nucleotide analogue 5-ethynyl-2´-deoxyuridine (EdU) to detect their proliferation rate. A concentration of 10 µM was used for 24 h. The cells were cultivated on round glass coverslips with a diameter of 12 mm.

4.2.4 Lipofection

Senescent cells were transfected with two consecutive transfections at a final siRNA concentration of 20 nM using Lipofectamine® RNAiMAX Transfection Reagent diluted in Opti-MEM® I Reduced Serum Medium. First, 300,000 cells were seeded on a 6 cm dish and transfected by a reverse transfection. The following day the medium was replaced with a fresh medium. On day 3, a direct transfection was used for the second transfection. On day 4 day the medium was replaced again. The cells were harvested on day 7.

4.2.5 Immunoblotting

Preparation of cell lysates

Cells on 6 cm or 6-well dishes were washed twice with 2 ml of PBS (pH 7.2) at room temperature, lysed with 60 - 100 µl 1 x sample buffer (250 mM Tris-HCl, 40% Glycerol, 8% SDS, pH 6.8) heated to 93°C and scraped into 1.5 ml Eppendorf tubes. The tubes were placed into a freezer with a constant temperature of -80°C. After harvesting all lysates, the lysates were thawed, then denatured at 93°C for 3 min, and then sonicated with an amplitude of 2-3 microns in 3 cycles of 15 seconds with pauses of 7 seconds. Protein concentration in the samples was determined by bicinchoninic acid (BCA) assay using the Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions, and absorbance of the samples was measured with the wavelength of 560 nm on Microplate Photometer Multiscan® EX.

SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins in lysates. The samples contained 16 - 35 µg of protein and were diluted with 1x sample buffer as needed to contain all an equal amount of proteins. 1 M dithiothreitol and bromphenol blue were added to the samples to achieve final concentrations of 1% and 0.02%, respectively. The samples were loaded onto 1 or 1.5 mm thick polyacrylamide gels of 8%, 14%, or 6 - 20% gradient gels. The electrophoresis unit Mini-PROTEAN® Tetra Handcast System was filled with 1x TGS buffer (100 ml 10x TGS buffer, 900 ml ddH_2O) and set to 25 mA per gel.

Western blotting

The proteins from the gels were transferred to nitrocellulose sheets (Amersham™ Hybond ECL, GE Healthcare Life Sciences) using Hoeffer TE 22 Mini Tank Transfer Unit (400 mA, 150 V, 1.5 h) filled with 1x TG Buffer with 20% methanol (100 ml 10x TG Buffer, 200 ml methanol, 700 ml H_2O). The membranes were stained with Ponceau Red to visualize proteins, cut to stripes, washed in ddH_2O, and blocked in 5% powdered nonfat milk dissolved in PBS with 0.1% Tween (PBST) for 20 min.

Immunodetection

The nitrocellulose membrane stripes were washed in PBST for 3x5 min and put into Falcon tubes with solutions of primary antibodies. They were incubated overnight on a tube roller at 4°C. On the following day, the stripes were washed in PBST for 3x5 min, incubated in a solution with a secondary antibody for 45 min and washed in PBST for 3x5 min. Chemiluminescent substrate Pierce™ ECL Western Blotting Substrate (Life Technologies) and SuperSignal West Femto Chemiluminescent Substrate were used for protein detection. The immobilized proteins were detected on a medical screen film CP-BU NEW (high-speed, medium- to high-contrast blue-sensitive, universal film for general radiography; Agfa Health Care) and developed in OPTIMAX 2010 (PROTEC).

Semiquantification of proteins on immunoblots by densitometry

Densitometry of Rb and its respective loading control from the same immunoblot was performed utilizing ImageJ (NIH) software. Relative optical density was calculated as a ratio of the density of the signal of Rb protein and the density of the protein signal used as a loading control.

4.2.6 Immunofluorescence

Direct and an indirect immunofluorescence

Cells cultivated on glass coverslips (diameter 12 mm) and treated with EdU for 24 h were fixed in 4% formaldehyde (FA) in 24-well dishes for 15 min. The coverslips were stored in PBS with 0.1% sodium azide at 4°C. The cells were permeabilized by 0.2% Triton X-100 in PBS for 18 min. An EdU staining solution was prepared during these 18 min, from the Click-iT® EdU Alexa Fluor® 488 Imaging Kit following the manufacturer´s protocol. The amount of the reaction solution was recalculated for an incubation amount of 30 µl per a glass. The coverslips were blocked in 250 µl 10% FBS/PBS solution for 10 min. The coverslips were then incubated first in 25 µl of the primary antibody and subsequently in 100 µl of the secondary antibody at room temperature for 60 min. Both the primary and secondary antibodies were diluted in 10% FBS in PBS. After each step, the coverslips were washed three times with 250 or 500 µl PBS. The coverslips were then incubated in 100 µl 4', 6-diamidino-2phenylindole (DAPI, 1 µg/ml dissolved in aqua pro inj. for 2 min), left to dry off, and mounted on slides using 5 µl of Vectashield. The images were acquired on a LEICA DM 6000 microscope or on an Olympus Scan^R Screening Station microscope.

Indirect immunofluorescence

Cells cultivated on glass coverslips (diameter 12 mm) were fixed in 4% FA in 24-well dishes for 15 min. The coverslips were stored in PBS with 0.1% sodium azide at 4°C. The cells were permeabilized by 0.2% Triton X-100 in PBS for 10 min and blocked in 250 µl by 10% FBS in PBS solution for 10 min. The coverslips were then incubated first in 25 µl of the primary antibody and subsequently in 100 µl of the secondary antibody at room temperature for 60 min. Both the primary and secondary antibodies were diluted in 10% FBS in PBS. After each step, the coverslips were washed three times with 250 or 500 µl PBS. The coverslips were then incubated in 100 µl DAPI (1 µg/1 ml) for 2 min, left to dry off, and mounted on slides using 5 µl of Vectashield. The images were acquired on a LEICA DM 6000 microscope.

4.2.7 SA-β-galactosidase staining

Cells cultivated on glass coverslips (diameter 12 mm) were washed three times with 0.5 ml PBS and fixed in 0.5% glutaraldehyde in 24-well dishes for 12 min. The coverslips were stored in PBS/$MgCl_2$ with 0.1% sodium azide (pH 6.0) at 4°C before further processing. The fixed cells were then stained with an X-gal solution containing 1x X-Gal (40x X-Gal solution was dissolved in N,N-dimethylformamide), 0.02 mM $K_3Fe(CN)_6$, 0.12 mM $K_4Fe(CN)_6$ x $3H_20$ in PBS/$MgCl_2$ (pH 6.0) for 2 - 4 h. After each step following the fixation, the coverslips were washed three times with 250 or 500 µl PBS/$MgCl_2$ (pH 6.0). The coverslips were then incubated in DAPI (1 µg/1 ml) for 2 min, left to dry off, and mounted on slides using 5 µl of Vectashield. The images were acquired on a LEICA DM 6000 microscope.

5. Results

5.1 The effect of glucose concentration and spectrum of X-radiation on the induction of IR-induced premature cellular senescence in human cells *in vitro*

The first task of my diploma thesis was to test the effect of various conditions (i.e. glucose concentration in the culture medium and the spectrum of the X-radiation used) on the development of IR-induced premature cellular senescence.

Normal human BJ fibroblasts are a frequently used cell model for the development of all forms of cellular senescence *in vitro*. Although the culture medium recommended for BJ fibroblasts by the ATCC® (American Type Culture Collection, vendor of various cell types) is the DMEM with a low level of glucose, I used two formulations of DMEM medium – one with a low concentration of glucose (1 g/l, 5.5 mM, representing normoglycemia), and one with a high concentration of glucose (4.5 g/l, 25 mM, hyperglycemic medium used usually for the cultivation of cancer cells). I tested these two DMEM formulations because both were normally used to culture BJ fibroblasts in my laboratory. As high glucose concentration in the culture medium can cause a premature replicative senescence (Blazer et al. 2002), my aim was to test whether a high glucose level can also act as a stress factor facilitating the onset of IR-induced premature cellular senescence.

Another condition I focused my attention on was the quality of the X-radiation used. X-radiation is electromagnetic radiation with a wavelength between ultraviolet and γ-radiation (Fig. 1). It is capable of breaking down chemical bonds, either directly or by ionization; for the latter reason X-radiation is considered a form of ionizing radiation. The dose of X-radiation is expressed in gray (Gy) units, where one gray represents the energy of one joule absorbed by one kilogram of matter.

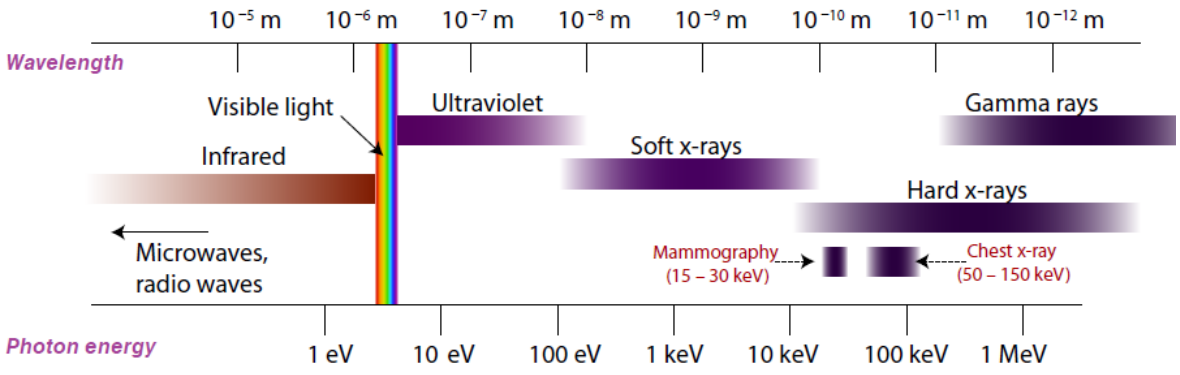


Figure 1. The electromagnetic spectrum.
(http://www.sciencetechnologyaction.com/perch/resources/lessons/sta9-a4-rpii-mq.pdf)

The spectrum of X-radiation produced by an X-ray tube is usually modified by metal filters. For example, a thorium filter removes low energy wavelengths and provides the so-called "hard" radiation, characterized by a higher proportion of higher energy photons, resulting in a higher penetration depth in tissue, while the effect of an aluminium filter is substantially weaker, providing "soft" radiation. For the treatment of patients, hard irradiation is used because of the insufficient penetration depth of soft irradiation resulting in superfluous damage. That is in line with the standard irradiation technique for cell cultures, where hard radiation is usually used. However, by administering high radiation doses the cells encounter stress due to the massive decrease in temperature and the resulting pH change (because the cells, which are normally cultured at 37°C, are irradiated at room temperature). Also, hard irradiation causes more stress because hard irradiation lasts considerably longer (approximately four times; the cells are at room temperature during irradiation) than soft irradiation for the same dose, and thus hard irradiation causes more undefined culture stress than soft irradiation. I have found no reference to the type of radiation used for the cell cultures discussed in the literature. Therefore, I wanted to test whether there are any differences in the impact of soft and hard irradiation on the cell culture resulting in the development of IR-induced premature cellular senescence, with the possible use of soft irradiation in mind.

To this end, diploid human fibroblasts (BJ) were seeded in culture media with high and low glucose concentration. On the next day, these cells were irradiated with either soft or hard radiation at a dose of 10 Gy, which was previously described to cause IR-induced premature cellular senescence in HCA2 and WI-38 human diploid fibroblasts (Rodier et al. 2009). After 6 days, the cells were analysed for the development of features typical of premature cellular senescence, namely the formation of DNA

damage foci and DDR activity, cell cycle arrest, morphological changes characteristic for cellular senescence, such as the increase of cellular surface, and the activity of SA-β-galactosidase (Dimri et al. 1995). In this experiment, two types of control cells were used. The first type of control cells was harvested on the first day and resembled nonirradiated proliferating cells (ctrl d0). The second type of control cells was harvested on the 6th day (ctrl d6); however, the cells were subcultured in the same regime as the irradiated cells, leading to a high density and thus the development of growth arrest (i.e. contact inhibition). In this way the 'ctrl d6' cells resembled nonproliferating cells which did not encounter external DNA damage.

X-radiation induces DNA DSBs which are recognized by DDR proteins triggering a massive signalling cascade. Due to the DDR, many proteins are attracted to the sites of DNA DSBs. This event leads to the formation of so-called DNA damage foci (Haaf et al. 1995). The presence of DNA damage foci is connected with IR-induced premature cellular senescence (Wallace 1998). My question was whether the different types of radiation filters and the glucose concentration in culture media would cause different number of DNA damage foci. Therefore, the cells were stained by indirect immunofluorescence with a marker of DNA DSBs (i.e. of DNA damage foci) γH2AX (Rogakou et al. 1998) and the number of γH2AX foci were quantified by high-throughput microscopy using the Olympus Scan^R (Fig. 2). All irradiated cells displayed about 1.6 DNA damage foci per nucleus at day six irrespective of glucose concentration; by contrast, 0.2 DNA damage foci per cell nucleus were found in the proliferating control cells. Both cells cultured in the medium with high or low glucose concentration irradiated with hard radiation displayed higher number of DNA damage foci (0.3-0.5 higher in comparison to the cells irradiated with soft radiation). However, this difference was not confirmed by the quantification of another DNA damage marker of DNA DSBs and DNA damage foci, the 53BP1 protein. Based on the assumption that both γH2AX and 53BP1 proteins mark the sites of DNA DSBs, there should be a change in both of them in parallel. Because I observed a higher number of DNA damage foci only after staining with the anti-γH2AX antibody and not after staining anti 53BP1, I did not consider the difference in the counts of γH2AX DNA damage foci as significant and attributed it instead to an experimental artefact. On the whole, X-radiation induced the formation of DNA damage foci. However, the DNA damage foci counts were not influenced by the type of filters used during irradiation or glucose concentration in the culture medium.

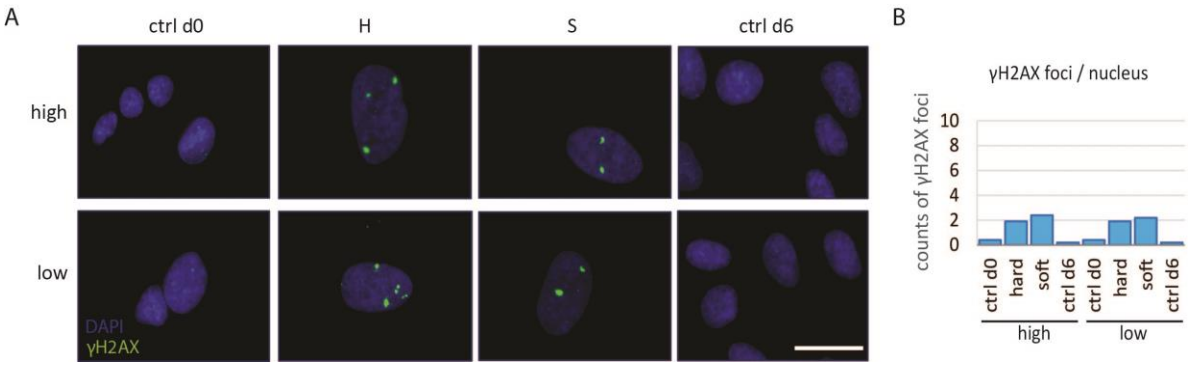


Figure 2. The effect of the spectrum of X-radiation and glucose concentration in the culture medium on the formation of γH2AX foci detected by indirect immunofluorescence. The detection of γH2AX (green) and DAPI (blue) (A). The graph shows γH2AX foci quantification (B). The normal human BJ fibroblasts were cultured in the culture medium with high (high) or low (low) glucose concentration and then irradiated (IR) with a dose of 10 Gy of hard (H) or soft (S) X-radiation. The irradiated cells were harvested on the 6th day after irradiation. The first batch of control cells were harvested immediately after irradiation (ctrl d0) resembling nonirradiated proliferating cells. The second batch of control cells (ctrl d6) were harvested on the 6th day but they were subcultured in the same regime as the irradiated cells, thus leading to the development of contact inhibition and growth arrest. The ctrl d6 cells thus resemble nonproliferating nonirradiated cells. The immunofluorescence staining represents the average of three independent experiments (A). The graph shows results from one of these experiments (B). Bar 50 µm.

To find whether the level of DDR activation correlates with the number of DNA damage foci, the level of the activated DDR was ascertained by the level of phosphorylated p53 on serine 15 (p53 pSer15) which is known to be phosphorylated by ATM kinase following DNA damage (Banin et al. 1998). The p53 pSer15 was measured also because p53 regulates the expression of the cyclin dependent kinase inhibitor p21$^{CIP1/WAF1}$ (Stein et al. 1999). Another assayed marker of active DDR signalling was Chk2 kinase. Chk2 is phosphorylated on threonine 68 (Chk2 pThr68), also by ATM, after encountering DNA damage (Ahn et al. 2000). The phosphorylated forms of p53 and Chk2 were detected by immunoblotting (Fig. 3). As expected, the p53 pSer15 and Chk2 pThr68 levels were elevated after irradiation. No significant differences in the level of activated p53 pSer15, Chk2 pThr68 after irradiation were found using the different types of radiation filters or glucose concentration in the culture medium.

DNA damage foci and DDR can trigger a signalling cascade leading to a cell cycle arrest. Premature cellular senescence is known to be related to the cell cycle arrest caused by the enhanced expression of CDKIs, in the IR-induced premature cellular senescence mostly by the level of p21$^{CIP1/WAF1}$ (Brown et al. 1997; Stein et al. 1999). Therefore, the level of p21$^{CIP1/WAF1}$ was measured by immunoblotting (Fig. 3). As expected, the p21$^{CIP1/WAF1}$ level was elevated after irradiation. No significant difference

in the $p21^{CIP1/WAF1}$ was seen by comparing the two types of radiation filters or of glucose concentration in the culture medium.

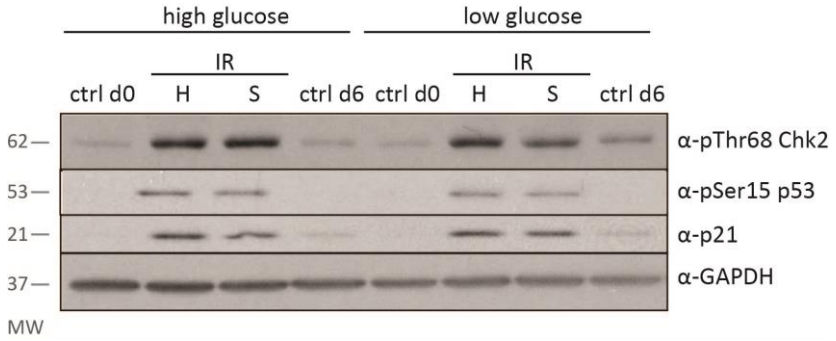


Figure 3. The effect of the spectrum of X-radiation and glucose concentration in medium on the level of Chk2 pThr68, p53 pSer15, and $p21^{CIP1/WAF1}$, measured by immunoblotting. The normal human BJ fibroblasts were cultured in the medium with high or low glucose concentration and then X-irradiated (IR) with a dose of 10 Gy of hard (H) or soft (S) radiation. The irradiated cells were harvested on the 6th day after irradiation. The first batch of control cells were harvested immediately after irradiation (ctrl d0) and resembled nonirradiated proliferating cells. The second batch of control cells (ctrl d6) were harvested on the 6th day but had been subcultured in the same regime as the irradiated cells, leading to a development of contact inhibition and growth arrest. The ctrl d6 cells thus resemble nonproliferating nonirradiated cells. The data represents the average of three independent experiments. Molecular weight (MW; in grey) of proteins is displayed in [kDa]. GAPDH was used as a loading control.

Altogether, the X-radiation induced the formation of DNA damage foci, activated DDR signalling (p53 pSer15, Chk2 pThr68), and activated cyclin-dependent kinase inhibitor ($p21^{CIP1/WAF1}$), but neither the formation of DNA damage foci, activity of DDR signalling, nor the level of $p21^{CIP1/WAF1}$ were influenced by the type of radiation filters used or by the glucose concentration in the culture medium.

To find whether the cell cycle arrest induced by DDR signalling was permanent and induced the development of IR-induced premature cellular senescence in irradiated cells in relation to the type of radiation filter and glucose concentration in the culture medium, characteristic features of cellular senescence (cellular morphology and SA-β-galactosidase activity) were assayed. Regarding morphology, the irradiated cells were enlarged, with increased cellular area and higher presence of the spindle-shaped phenotype compared to the proliferating cells. As for SA-β-galactosidase activity, this was measured by the blue colouring which arose from the conversion of added X-gal substrate to an indigo product by the SA-β-galactosidase enzyme (Fig. 4). The irradiated cells cultured in the medium with the high glucose concentration were positively stained for SA-β-galactosidase activity in the extent of 86% and 84% after hard and soft irradiation, respectively. Similarly,

the irradiated cells cultured in the medium with the low glucose concentration were positively stained in the extent of 86% and 85% after hard and soft irradiation, respectively. Only 1% of the proliferating control cells displayed a positive SA-β-galactosidase staining, irrespective of glucose concentration. However, because the proportion of positively stained cells in the hard- and soft-irradiated cells differed

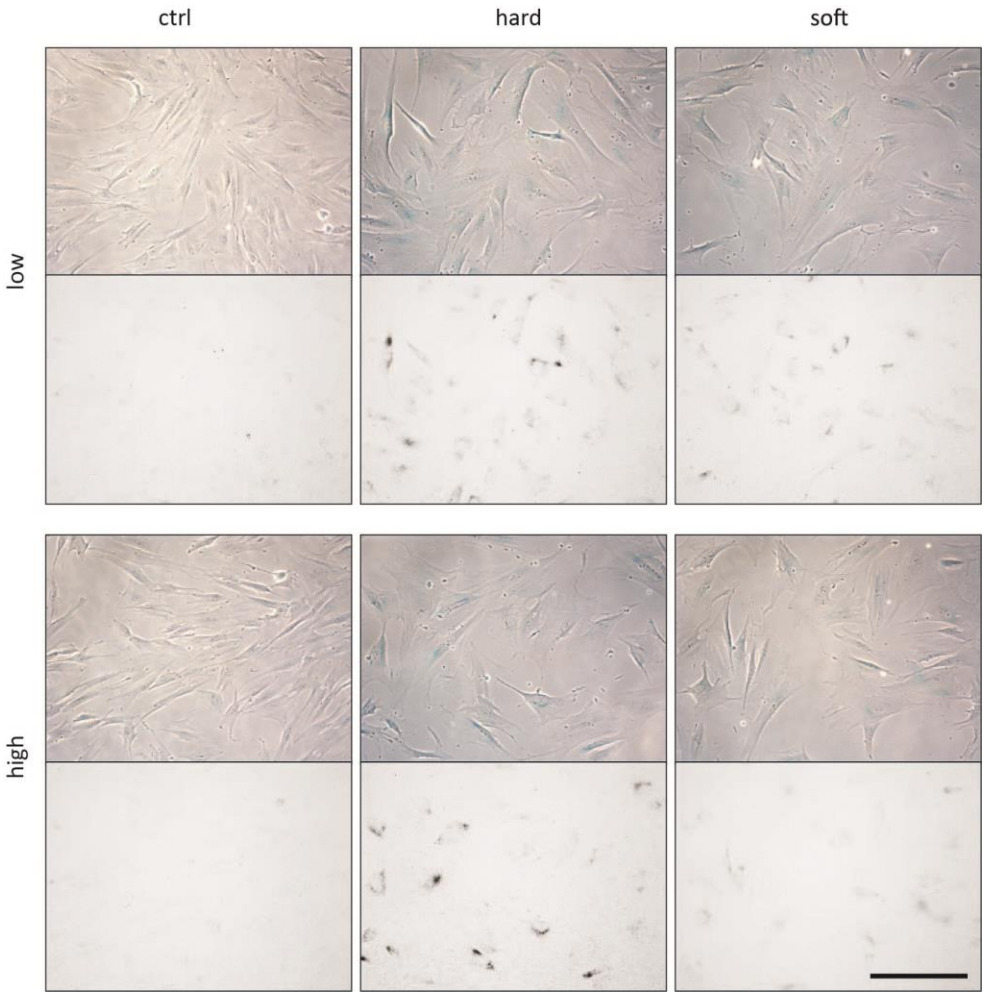


Figure 4. The effect of the spectrum of X-radiation and glucose concentration in the culture medium on senescent phenotype development and SA-β-galactosidase activity detected by microscopic imaging. The normal human BJ fibroblasts were cultured in culture media with high (high) or low (low) glucose concentration and then X-irradiated (IR) with a dose of 10 Gy of hard (H) or soft (S) radiation. The irradiated cells were harvested on the 6th day after irradiation. The control cells were harvested immediately after irradiation and resembled nonirradiated proliferating cells. Images of the same field were taken using phase contrast (upper) and bright field (lower) imaging. The staining for SA-β-galactosidase activity is shown in the lower images by black colouring. (The colouring was converted from blue to black in computer processing to improve contrast.) The data represents the average of three independent experiments. Bar 200 μm.

only by one or two percentage points, I did not consider the difference significant. In sum, X-radiation caused development of the senescent phenotype but neither the cellular morphology nor the SA-β-galactosidase activity was significantly influenced by the type of radiation filters used or by the glucose concentration in the culture medium.

In conclusion, X-radiation caused the formation of DNA damage foci, activation of DDR signalling (p53 pSer15, Chk2 pThr68), increase in the level of cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$, and induced senescent phenotype. However, the development of IR-induced premature cellular senescence was not significantly affected neither by glucose concentration in the medium nor by the type of radiation filter used. Therefore, the soft irradiation regime and low glucose concentration in the culture medium can be used for the culturing of BJ fibroblasts.

5.2 Optimization of radiation dose for the induction of IR-induced premature cellular senescence

The aim of my thesis was to study the role of PML in DDR and IR-induced premature cellular senescence after genotoxic stress, namely X-radiation. In the first part of my thesis (see Paragraph 5.1) I tested culture conditions of BJ fibroblasts which were intended to be used as the cell type model for the induction of IR-induced premature cellular senescence. However, during my first experiment, J. Kosla (Laboratory of Genome Integrity, IMG Prague) found that the BJ fibroblasts are incapable of clonal expansion and thus unsuitable for the intended knock out of PML gene by the CRISPR method. Therefore, another nontransformed cell type, RPE-1 hTERT cells, was chosen for subsequent experiments. Considering the results obtained from BJ fibroblasts, I did not test the culture conditions again but I used soft irradiation regime and culture medium with high glucose concentration in my subsequent experiments with RPE-1 hTERT cells.

The dose of IR necessary to induce IR-induced premature cellular senescence depends on cell type (Brown et al. 1997; Rodier et al. 2009; Noda et al. 2012; Münch et al. 2014; Rossiello et al. 2014). To find the most effective dose for the induction of IR-induced premature cellular senescence in RPE-1 hTERT cells, the effect of various X-radiation doses on the development of IR-induced premature cellular senescence was studied. The RPE-1 hTERT cells were irradiated with doses of 5, 10, 20, 50, or 100 Gy and the development of features of cellular senescence was followed as mentioned above (the activity of DDR, formation of DNA damage foci, expression of inhibitor of cyclin dependent kinases $p21^{CIP1/WAF1}$, morphological changes, and the activity of SA-β-galactosidase). The cells were harvested 6 days after irradiation.

As I mentioned above, X-radiation can cause DNA DSBs on whose sites DNA damage foci are formed (Haaf et al. 1995). DNA damage foci play a substantial role in IR-induced premature cellular senescence (Wallace 1998). My question was whether the different doses of X-radiation will induce different amounts of DNA damage foci. In order to examine the impact of different radiation doses on the formation of DNA damage foci, the number of the DNA damage foci was estimated. Again, the cells were stained by indirect immunofluorescence for γH2AX as a marker of DNA damage. The γH2AX foci were found in both proliferating control and the irradiated cells (Fig. 5). In the control cells, on average 2.9 foci per nucleus were observed. After irradiation with 5, 10, 20, 50, and 100 Gy, 4.7,

9.3, 10.5, 12.7, and 15.2 foci were observed, respectively. Altogether, the DNA damage foci counts rose with the increasing radiation dose, indicating a correlation between the dose and the extent of DNA damage.

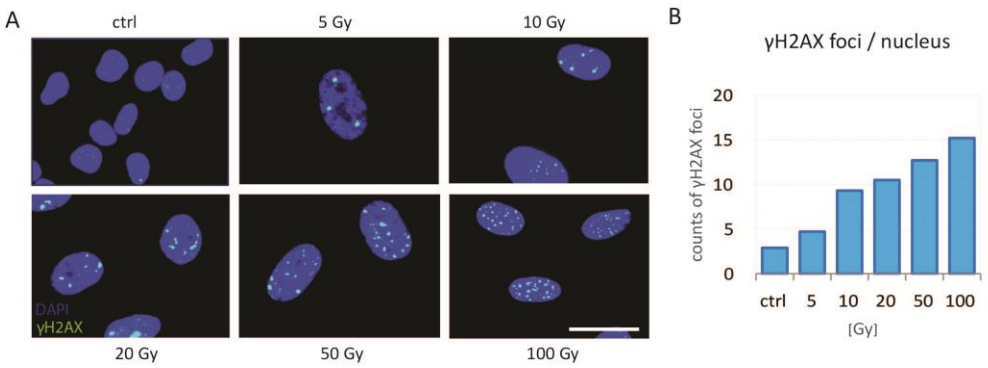


Figure 5. The effect of the increasing doses of X-radiation on the formation of γH2AX foci detected by indirect immunofluorescence. The detection of γH2AX (green) and DAPI (blue) (A). The graph shows γH2AX foci quantification (B). The RPE-1 hTERT cells were irradiated (IR) by different doses (5, 10, 20, 50 and 100 Gy). The irradiated cells were harvested on the 6th day after irradiation. The control cells were harvested immediately after irradiation. The immunofluorescence staining represents the average of two independent experiments (A). The graph shows results from one performed experiment (B). Bar 50 µm.

It is believed that premature cellular senescence is caused by permanent DDR activation, probably arising from unrepaired DNA damage. As already mentioned, DDR activation is routinely detected as increased levels of phosphorylated forms of p53 and Chk2 (p53 pSer15 and Chk2 pThr68) (Banin et al. 1998; Ahn et al. 2000). I wanted to find a radiation dose sufficient to activate permanent DDR signalling and premature cellular senescence. Again, the level of the activated DDR signalling was detected by the level of phosphorylated forms of p53 and Chk2 (p53 pSer15 and Chk2 pThr68) and the total form of p53 by immnunoblotting (Fig. 6) six days after irradiation. Chk2 pThr68 level was higher when the cells were irradiated with 20 and 50 Gy, compared to cells irradiated with 5 and 10 Gy. After 100 Gy, Chk2 pThr68 level was lower than after 50 Gy but higher than after 5 Gy. The level of p53 pSer15 was significantly higher after irradiation with 50 and 100 Gy compared to the lower radiation doses (5, 10, and 20 Gy). Interestingly, all radiation doses caused phosphorylation of p53 at almost the same level. In conclusion, persistent DDR signalling (p53 pSer15 and Chk2 pThr68) was activated by all radiation doses used.

DNA damage may trigger cell cycle arrest facilitated, for example, by the increased levels of CDKIs are responsible for cell cycle arrest during the development of premature cellular senescence. The cell cycle arrest in IR-induced premature cellular senescence is known to be caused mainly by p21$^{CIP1/WAF1}$, whose

expression is regulated by p53 (Brown et al. 1997; Stein et al. 1999). To induce cell cycle arrest, $p21^{CIP1/WAF1}$ influences phosphorylation of the Rb protein, which allows cell cycle progression in its hyperphosphorylated state (see chapter Introduction). To explore which radiation dose would cause sufficient DNA damage to induce efficient cell cycle arrest, the levels of cyclin-dependent inhibitor $p21^{CIP1/WAF1}$ and the Rb phosphorylated state were detected by immunoblotting (Fig. 6). The detection of Rb protein was administered by the immunostaining of the total Rb level, revealing two Rb forms on the immunoblot – hyperphosphorylated, ppRb, upper band and hypophosphorylated, pRb, lower band. The level of $p21^{CIP1/WAF1}$ induced by irradiation varied based on the dose used, and its level also correlated with the p53 pSer15 level. The ppRb (Fig. 6, upper band) was detected in the proliferating control RPE-1 hTERT cells and partly also after irradiation with a dose of 5 Gy, indicating some remaining proliferation activity after the 5 Gy irradiation. After irradiation with doses of 10 Gy and higher, only the pRb form (Fig. 6, lower band) was detected implying a predominantly nonproliferating state of the cells. From the point of view of activated cell cycle arrest (the Rb phosphorylation state and $p21^{CIP1/WAF1}$ activation), radiation doses of 10 Gy and higher can be used to induce IR-induced premature cellular senescence in RPE-1 hTERT cells.

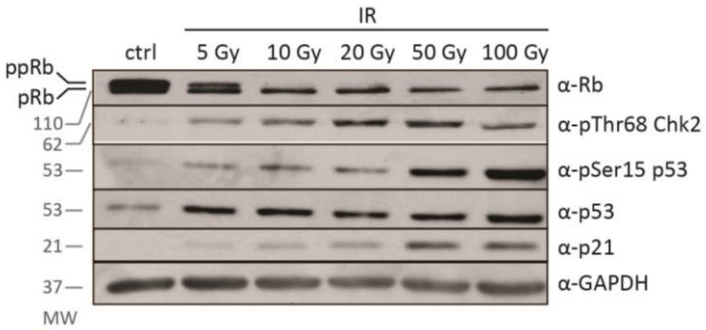


Figure 6. The effect of the X-radiation dose on the level of Rb, Chk2 pThr68, p53 pSer15, p53 and $p21^{CIP1/WAF1}$ detected by immunoblotting. The RPE-1 hTERT cells were irradiated (IR) by different doses (5, 10, 20, 50, and 100 Gy). The irradiated cells were harvested on the 6^{th} day after irradiation. The control cells were harvested immediately after irradiation. The data represents the average of two independent experiments. Molecular weight (MW; in grey) of proteins is displayed in [kDa]. GAPDH was used as a loading control. ppRb – hyperphosphorylated Rb, pRb – hypophosphorylated Rb, ctrl – proliferating control cells

Altogether, all doses of X-radiation used induced persistently active DDR signalling (p53 pSer15, Chk2 pThr68). Radiation doses of 10 Gy and higher (20, 50, 100 Gy) also induced activation of cell cycle arrest inductor cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$; no ppRb was detected.

To determine whether the cell cycle arrest led to the induction of premature cellular senescence, the development of the senescent phenotype of irradiated cells with respect to the radiation dose, cellular morphology, and SA-β-galactosidase activity were assessed. A flattened cellular morphology and an increased cellular area were observed after irradiation with all doses tested (Fig. 7). Interestingly, in cells irradiated with doses of 5, 10, or 20 Gy, the flattened phenotype dominated, in contrast to cells irradiated by doses of 50 and 100 Gy which displayed a more spindle-like phenotype. The irradiated cells were positively stained for SA-β-galactosidase activity after irradiation with all doses (Fig. 7). After irradiation with the doses of 5, 10, 20, 50, and 100 Gy, 47%, 54%, 69%, 72%, and 85% of cells were positively stained for SA-β-galactosidase activity, respectively, compared to the nonirradiated control (where no cells were stained positive for SA-β-galactosidase activity). Moreover, the intensity of the blue colouring correlated with the increasing dose, indicating a higher extent of metabolic changes accompanying the increasing doses of IR. Together, all irradiated cells showed a senescent phenotype irrespective of the dose used; however, the proportion of SA-β-galactosidase positively stained cells increased with the increasing dose of IR.

In conclusion, all radiation doses caused formation of DNA damage foci, activated DDR signalling (p53 pSer15, Chk2 pThr68), induced an increase in the level of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$, and induced the senescent phenotype. The 20 Gy dose was chosen as the most practical for the development of IR-induced premature cellular senescence in RPE-1 hTERT cells.

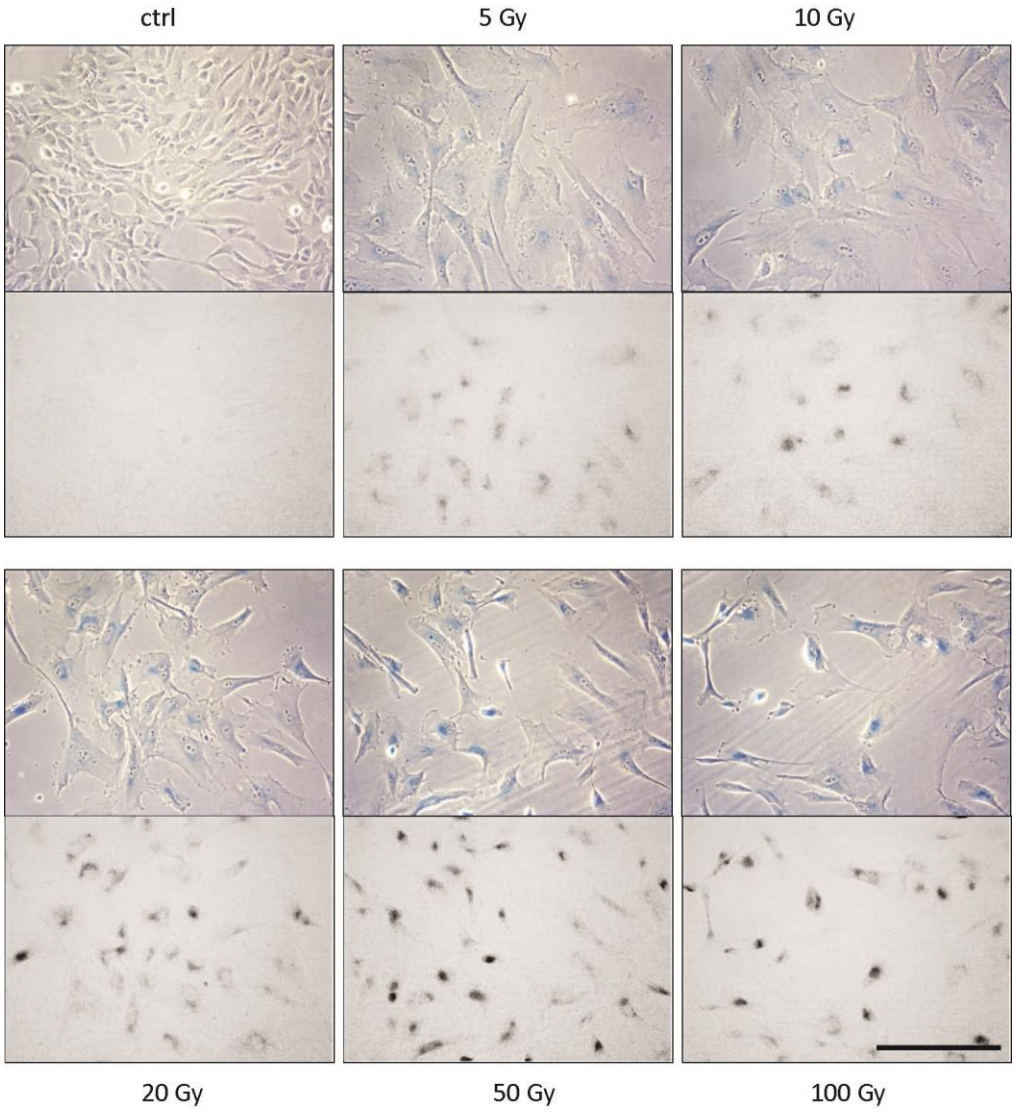


Figure 7. The effect of the different X-radiation doses on the senescent phenotype and SA-β-galactosidase activity detected by microscopic imaging. Images of the same field were taken using phase contrast (upper) and bright field (lower) imaging. The staining for SA-β-galactosidase activity is shown in the bottom line of images by black colouring. (The colouring was converted from blue to black by digital image processing to improve contrast.) RPE-1 hTERT were irradiated with the doses of 5, 10, 20, 50, and 100 Gy. The irradiated cells were harvested on the 6th day after irradiation. The control cells were harvested immediately after irradiation. The data represents the average of two independent experiments. Bar 200 μm.

5.3 The effect of PML knock down on the DNA damage response and the maintenance of IR-induced premature cellular senescence

The goal of my thesis was to study the PML interaction with DDR and the PML role in IR-induced premature cellular senescence on the grounds of following observation. The PML protein was found to participate in p53 acetylation, a posttranslational modification stabilizing the p53 protein and increasing its activity (Pearson et al. 2000). Also, the overexpression of the PML isoform IV was shown to induce premature cellular senescence (Bischof et al. 2002). Moreover, PML NBs serve as a localisation site for many proteins involved in DDR (Mirzoeva and Petrini 2001; Blander et al. 2002; Xu et al. 2003; Dellaire, Ching et al. 2006) and also PML NBs coassociate with DNA damage foci in senescent cells (Carbone et al. 2002; Hubackova et al. 2010; Rodier et al. 2011). Also, PML was found to be a direct p53 target (de Stanchina et al. 2004). These features raise the question whether the PML protein serves as a key mediator in IR-induced premature cellular senescence via the stabilization of p53. Therefore, I was interested in whether the presence of intact PML is necessary for the maintenance of IR-induced premature cellular senescence.

First, I focused on siRNA-mediated PML knock down (PML KD) in RPE-1 hTERT senescent cells. The transfection efficiency of both siPMLs was examined by immunoblotting (Fig. 9). To induce IR-induced premature cellular senescence, RPE-1 hTERT cells were X-irradiated with a dose of 20 Gy. After the development of premature cellular senescence (six days), the cells were transfected by two consecutive lipofections with corresponding siRNAs - a noncoding siRNA marked as siNC and serving as a control and two different siRNAs complementary to the mRNA of the PML protein termed siPML#3 and siPML#4 (indicated as #3 and #4, respectively, in the figures). As DNA damage foci were reported to coassociate with PML NBs in senescent cells (Carbone et al. 2002; Hubackova et al. 2010; Rodier et al. 2011), I wished to explore the functional involvement of PML and PML NBs in the DDR associated with the development of IR-induced premature cellular senescence. I first examined the number of DNA damage foci in PML KD cells. Both proliferating nonsenescent cells and transfected and nontransfected senescent cells were stained by indirect immunofluorescence for PML protein and the marker of DNA damage foci 53BP1 (Fig. 8). The 53BP1 foci were counted with high throughput microscopy using Olympus Scan^R. Both senescent cells transfected with siNC, siPML#3, and siPML#4 and nontransfected control senescent cells showed the same number of 53BP1 foci, about four foci per nucleus. Altogether,

the PML KD in RPE-1 hTERT cells had no impact on the number of DNA damage foci at eight days after X-irradiation.

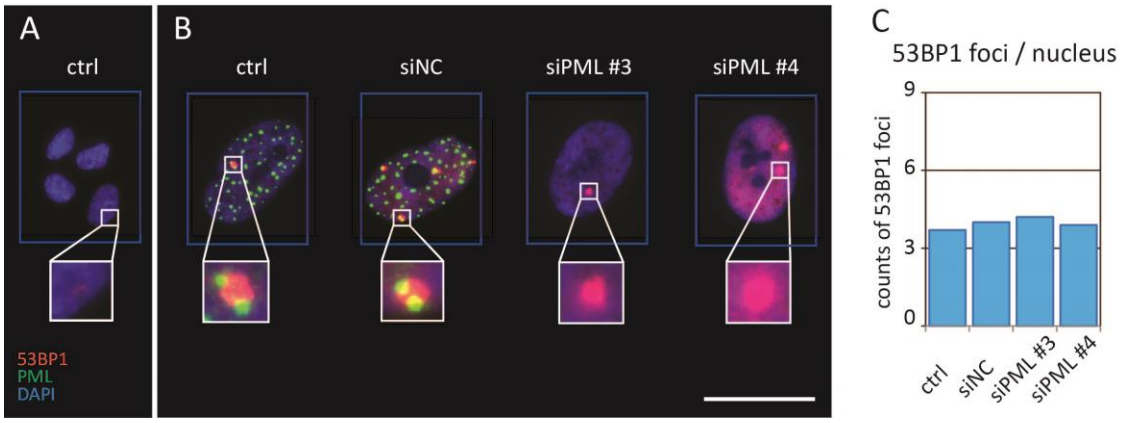


Figure 8. The effect of PML knock down on the number of DNA damage foci in IR-induced senescent RPE-1 TERT cells. Proliferating control RPE-1 hTERT cells (A) and senescent cells (B) nontransfected (ctrl) and transfected by two consecutive lipofections with noncoding control siRNA (siNC) and two siRNAs complementary to PML mRNA sequence (siPML#3, siPML#4). The cells were stained with indirect immunofluorescence for 53BP1 (red), PML (green), and DAPI (blue). The graph shows 53BP1 foci quantification with Olympus Scan^R automatic software (C). The proliferating control cells were harvested immediately after irradiation (A). The irradiated and transfected cells were harvested on the 13th day after irradiation (B, C). The immunofluorescence staining represents the average of two independent experiments (A, B). The graph shows results from one performed experiment (C). Bar 50 µm.

To further examine the impact of PML KD on the activation of DDR signalling in the IR-induced senescent cells, the changes in the phosphorylated forms of p53 (p53 pSer15) and Chk2 (Chk2 pThr68) were examined by immunoblotting (Fig. 9). As expected, the PML protein level was increased in the IR-induced senescent cells compared to the nonsenescent cells; increased levels of p53 pSer15 and Chk2 pThr68 were also detected in the IR-induced senescent cells. After the transfection with PML#3 and PML#4, the levels of both Chk2 pThr68 and p53 pSer15 were lower in comparison with the nontransfected senescent cells. Taken together, a decrease in Chk2 pThr68 and p53 pSer15 levels was observed after PML KD in RPE-1 hTERT IR-induced senescent cells.

Furthermore, due to the previously reported functional link between PML and p53 manifesting as cell cycle arrest (Pearson et al. 2000), the effect of PML KD on cyclin-dependent kinase inhibitor p21$^{CIP1/WAF1}$ and Rb protein levels was examined by immunoblotting (Fig. 9). As expected, the IR-induced senescent cells displayed an increased level of p21$^{CIP1/WAF1}$ compared to the proliferating control cells. Also, only pRb (Fig. 9, lower band) was detected in the IR-induced senescent cells (without any detected ppRb)

compared to the proliferating control cells. Interestingly, after PML KD with both siPMLs a decrease of p53 Ser15, $p21^{CIP1/WAF1}$ and ppRb levels and also the decrease of the pRb (i.e. the total) level were observed; note the destabilisation of Rb after a prolonged cell cycle arrest was shown also by (Lukas et al. 1999).

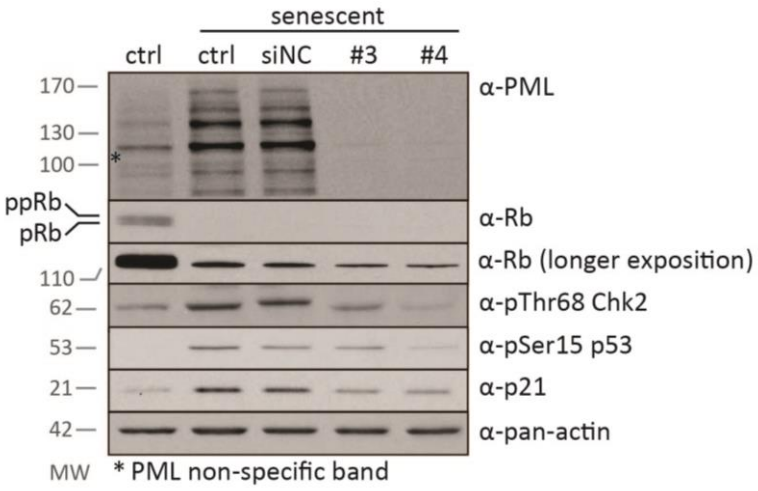


Figure 9. The effect of PML knock down on the level of Chk2 pThr68, p53 pSer15, $p21^{CIP1/WAF1}$ and Rb detected by immunoblotting. RPE-1 hTERT cells were irradiated with 20 Gy to induce premature cellular senescence. After 6 days, the senescent cells were transfected with siRNAs by two consecutive lipofections. A pan-actin antibody was used to control protein loading. The proliferating control cells were harvested immediately after irradiation. The irradiated and transfected cells were harvested on the 13^{th} day after irradiation. The data represents the average of three independent experiments. Molecular weight (MW; in grey) of proteins is displayed in [kDa]. ppRb – hyperphosphorylated Rb, pRb – hypophosphorylated Rb, ctrl – proliferating control cells, siNC - noncoding siRNA, #3 – siPML#3, #4 - siPML#4.

In conclusion, no change was seen in the number of DNA damage foci after X-irradiation after siRNA-mediated PML KD in senescent RPE-1 hTERT cells. After PML KD, a small decrease in p53 pSer15, pChk2 pThr68, as well as in $p21^{CIP1/WAF1}$ and pRb levels was observed in RPE-1 hTERT cells.

5.4 The effect of PML knock out on the establishment of IR-induced premature cellular senescence

In the chapter 5.3, the effect of PML KD was examined in the previously established IR-induced senescent cells. In this chapter, the emphasis was laid on the research of PML role in the development of IR-induced premature cellular senescence. To further verify the PML role in IR-induced premature cellular senescence, I utilized a complete PML KO prepared in human cells by CRISPR technology (see chapter Materials and methods). RPE-1 hTERT PML wt and PML KO cells were treated to induce premature cellular senescence either by X-radiation with the dose 20 Gy or by the treatment with 100 μM BrdU applied every 48 h. Note, BrdU is known inductor of premature cellular senescence (Michishita et al. 1998; Masterson and O'Dea 2007) and the BrdU-treated cells were used as a positive control for the induction of the premature cellular senescence. Irradiated and BrdU-treated RPE-1 hTERT PML wt a PML KO cells were harvested at various time points (i.e. 1.5 h, 1st day, 3rd day, 5th day and 7th day) and the development of selected features of cellular senescence was followed, namely the formation of DNA damage foci, the activity of DDR signalling, cyclin-dependent kinase inhibitor p21$^{CIP1/WAF1}$ level, senescent phenotype and SA-β-galactosidase activity.

5.4.1 The effect of PML knock out on the DNA damage foci formation, DDR activity and cell cycle arrest

As told above, PML NBs coassociate with the DNA damage foci in senescent cells (Carbone et al. 2002; Hubackova et al. 2010; Rodier et al. 2011). Again, the effect of complete PML KO on the induction of DNA damage foci was examined in RPE-1 hTERT PML wt and PML KO cells 7 days after the irradiation with 20 Gy or after BrdU-treatment (Fig. 10). The cells were stained with indirect immunofluorescence for γH2AX foci and analyzed by Olympus Scan^R software (Fig. 10). Note, the DNA damage foci were found also in control cells, i.e. 2.1 and 1.5 foci per nucleus in PML wt and PML KO cells, respectively. These counts are comparable to the BrdU-treated cells, where the foci counts reached 1.6 and 1.3 (PML wt and PML KO cells, respectively) foci per nucleus. The irradiation induced an increase in the formation of DNA damage foci in both PML wt and PML KO cells compared to the nonirradiated control cells, the cells displayed 7.3 and 5.6 foci per nucleus in PML wt and PML KO cells, respectively.

In all conditions tested (i.e. in the untreated control cells, after irradiation and after the BrdU treatment), the PML wt cells displayed ca 30% higher foci counts in average. However, if this 30% DNA damage foci decrease in PML KO cells was caused as an effect of the PML KO, the difference should be seen also in the foci counts in median. The median of DNA damage foci in nonproliferating control cells was 1 focus per nucleus in both PML wt and PML KO cells. The median of DNA damage foci in the irradiated cells was 5 and 4 foci per nucleus in PML wt and PML KO cells, respectively. The median of DNA damage foci in nonproliferating control cells was 0 foci per nucleus in both PML wt and PML KO cells. The DNA damage foci counts counted as median did not display a difference in 30% as the DNA damage foci counted as an average. Therefore, I did not consider the difference in DNA damage foci number counted in average as significant. However, the median showed an increased number of DNA damage foci after irradiation in PML wt cells in comparison to PML KO cells, as well as the average counts. This decrease of number of DNA damage foci in PML KO cells could indicate a functional connection of PML and the dynamics of formation of DNA damage foci.

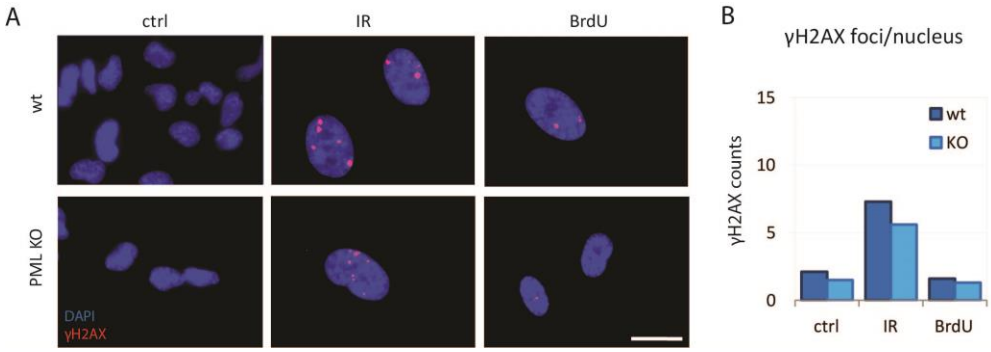


Figure 10. The formation of DNA damage foci in PML wt and PML KO cells after irradiation and BrdU-treatment detected by indirect immunofluorescence 7 days after IR. The DNA damage foci detected by γH2AX (red). Nuclei are stained with DAPI (blue) (A). The graph shows quantification of γH2AX-foci using Olympus Scan^R (B). The RPE-1 hTERT PML wt and PML KO cells were irradiated (IR) by the dose of 20 Gy or treated with 100 μM BrdU. All cells were harvested on the 7th day after respective treatment. The immunofluorescence staining represents the average of two independent experiments (A). The graph shows results from one performed experiment (B). Bar 25 μm.

To assess the impact of the PML KO on the activation of DDR signalling during the establishment of IR-induced premature cellular senescence in connection with the above mentioned interaction of PML with p53, both phosphorylated (p53 pSer15) and total form of p53 and phosphorylated form of Chk2 kinase (Chk2 pThr68) were detected by immunoblotting in control and irradiated RPE-1 hTERT PML wt and PML KO cells. The highest p53 pSer15 level was detected 1.5 h after irradiation and then

the p53 pSer15 level displayed a decreasing tendency within the following 7 days. Compared to the nonirradiated cells, the level of p53 pSer15 was elevated on day 7, indicating a persisting activity of DDR. The highest Chk2 pThr68 level was detected after 24 h. On the 3rd day, the Chk2 pThr68 level started to decrease but remained stable at lower level even at 5th and 7th day. Notably, no difference between the PML wt and PML KO cells was seen in the p53 pSer15 and Chk2 pThr68 levels after irradiation. However, a decrease of total p53 level in the PML KO cells was observed in comparison to the PML wt cells.

To explore the effect of PML KO on the development of cell cycle arrest during the establishment of IR-induced premature cellular senescence, the p21$^{CIP1/WAF1}$ and Rb level were examined by immunoblotting in RPE-1 hTERT PML wt and PML KO cells (Fig. 11 A, B). The cells were either irradiated with 20 Gy or treated with 100 µM BrdU and harvested within seven days (indicated above). Both treatments showed an increased level of p21$^{CIP1/WAF1}$ cyclin-dependent kinase inhibitor already after one day. While in the irradiated cells the p21$^{CIP1/WAF1}$ showed major increase after one day, it showed major increase after three days in the BrdU-treated cells. The ppRb (upper band, Fig. 11 A, B) disappeared after the first day after both irradiation and BrdU-treatment, and only the pRb (lower band, Fig. 11 A, B) was detected in both PML KO and PML wt cells. The level of ppRb was higher in the PML KO cells in comparison to the PML wt cells in the proliferating cells and after the irradiation after 1.5 h. After one day, both ppRb and pRb were detected in both PML wt and PML KO cells; however, the level of pRb was higher in the PML KO cells. Conversely, after three, five and seven days, a higher level of the pRb was detected in the PML wt cells than in the PML KO cells. Interestingly, the Rb showed higher increase in proliferating PML KO cells after both treatments in comparison to the PML wt cells (confirmed by relative optical densitometry of Rb/actin levels) (Fig. 11 C, D). Altogether, no difference in the p21$^{CIP1/WAF1}$ level was observed comparing the PML wt and PML KO cells. However, a higher Rb level was observed in the PML KO cells in comparison to the PML wt cells.

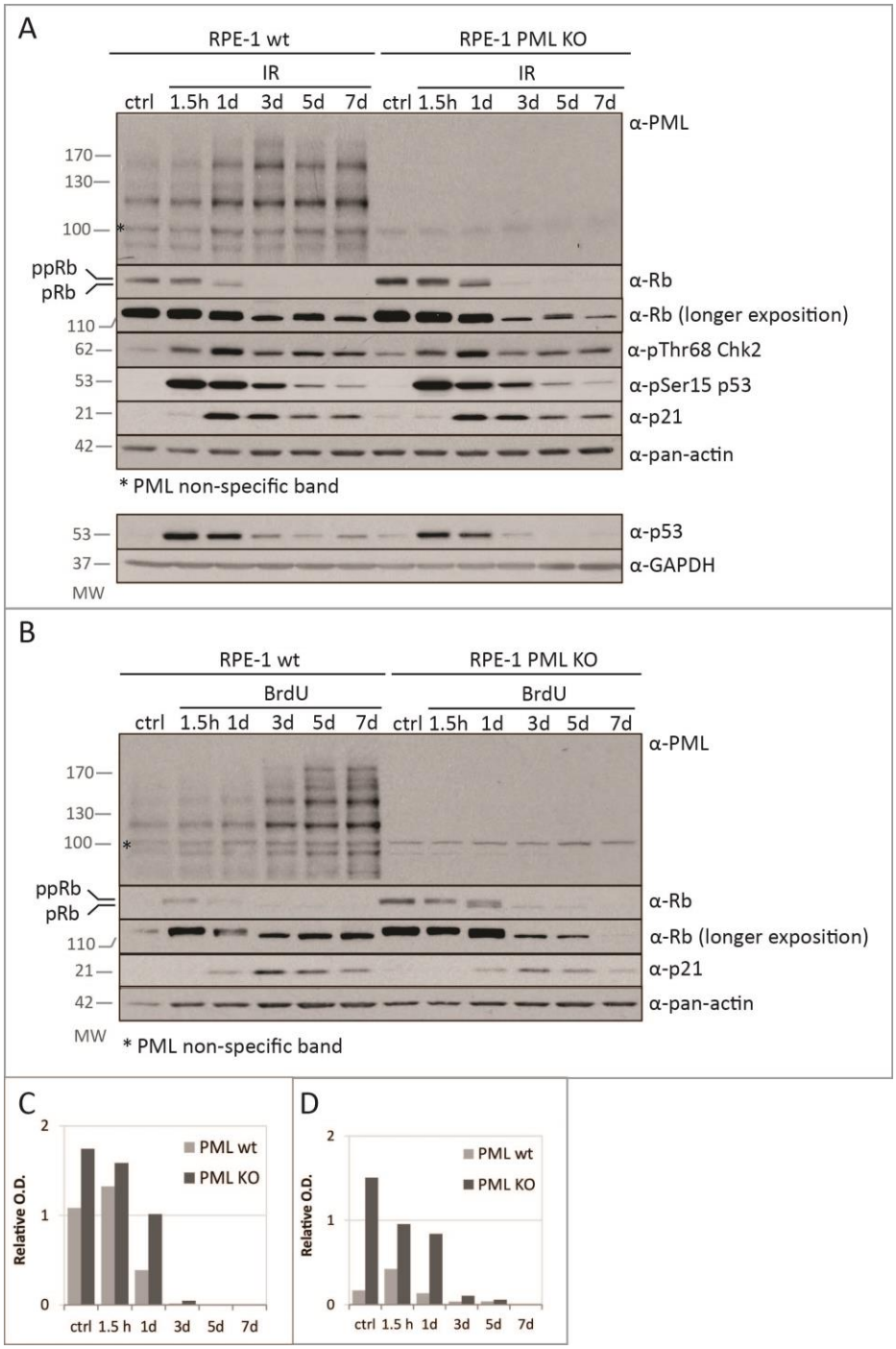


Figure 11. The effect of PML knock out on the level of Chk2 pThr68, p53 pSer15, p21$^{CIP1/WAF1}$ and Rb detected by immunoblotting. RPE-1 hTERT PML wt and PML KO cells were irradiated (IR) with 20 Gy (A) or BrdU-treated (B) to induce development of IR-induced premature cellular senescence. The cells were harvested after 1.5 h and after 1, 3, 5 and 7 days. Note inaccurate loading of the first line in (B). The data represents the average of two independent experiments. Molecular weight (MW; in grey) of proteins is displayed in [kDa]. Relative optical density (o.d.) of Rb/actin levels after irradiation (C) and BrdU-treatment (D) of respective bands from above. A pan-actin antibody was used to control protein loading. ppRb – hyperphosphorylated Rb, pRb - hypophosphorylated Rb

Taken together, the RPE-1 hTERT PML KO cells showed no difference in the level of p53 pSer15 and Chk2 pThr68. However, a small decrease of p53 level in the PML KO cells was shown in comparison to the PML wt cells. Although the activated DDR signalling (p53 pSer15 and Chk2 pThr68 levels) was on the same level in PML wt and PML KO cells, PML KO cells displayed lower DNA damage foci counts in comparison to PML wt cells. No difference in the $p21^{CIP1/WAF1}$ level was observed comparing the PML KO and PML wt cells. Bearing in mind the above mentioned indirect evidence for the PML role in DDR and premature cellular senescence, PML plays probably only indirect or circumstantial role in these processes. Interestingly, a higher Rb level in PML KO proliferating cells was detected in comparison to PML wt cells, indicating a possible PML interaction with Rb protein.

5.4.2 The effect of PML knock out on cellular proliferation

Based on the recent report of Münch et al. (2014), who showed that primary human foreskin fibroblasts with a stable shRNA-mediated PML KD proliferate with a lower rate, I studied the effect of PML KO on cell proliferation. Münch et al. (2014) reported that while primary human foreskin fibroblasts with a stable shRNA-mediated PML KD yielded 38 population doublings (PD), the wild type cells reached about 15 PD more. To confirm the observations also on a human PML KO cell type, both RPE-1 hTERT PML wt and PML KO cells were treated for 24 h with the nucleotide analogue EdU, stained by indirect immunofluorescence and the EdU-incorporation was measured in control and irradiated PML KO and PML wt cells during 7 days (Fig. 12). The EdU-incorporation in BrdU-treated cells was measured only on the 7^{th} day, based on the fact that the full development of senescent phenotype was reported already after 6 days and no further addition of BrdU was needed; this prevented artefacts of competition between BrdU and EdU incorporation (Masterson and O'Dea 2007). The proliferating PML wt and PML KO cells reached the incorporation rate 90 - 95% positive cells per sample. At all time points, the EdU-incorporation of proliferating control PML wt cells was always higher than of the PML KO cells ($p< 0.05$).

To underscore the findings of nucleotide incorporation, the proliferation rate of PML wt and PML KO cells was determined. After both conditions at all time points, the PML wt cells showed a higher cell counts than the PML KO cells (Fig. 13 A). Expressed preciously in percentage the 7^{th} day, the proliferating PML KO cells displayed the proliferation capacity of 65 ± 12% by the proliferating cells, 80 ± 14% by the irradiated cells and 73 ± 25% by the BrdU-treated cells in comparison to the PML wt cells

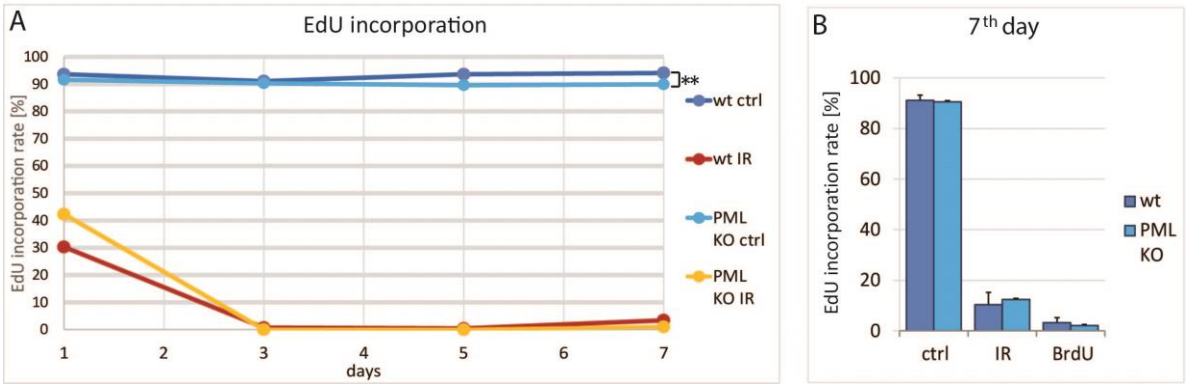


Figure 12. The effect of PML knock out on cellular proliferation measured by EdU incorporation. RPE-1 hTERT PML wt and PML KO cells were treated with 10 µM EdU for 24 h. The first graph (A) shows the percentage of the positively EdU-stained cells was counted on different time points within 7 days. The second graph (B) shows percentage of positively EdU-stained cells on the 7th day after 24 h of EdU incorporation (note, on the 7th was measured also the proliferation of BrdU-treated cells). The cells were harvested after 1.5 h and after 1, 3, 5 and 7 days. Asterisks (**) represent *p* value < 0.05 (Student's t-test). The graph (A) represents data from one performed experiment. The graph (B) shows results from two independent experiments.

(respective counts of PML wt cells were considered as 100%; Fig. 13 B). The computed cell cycle length was for PML KO cells 20 h 20 min and for PML wt cells 19 h 40 min, i.e. the cell cycle length is approximately 40 min longer by the PML KO cells than by PML wt cells. Briefly, the proliferative delay of PML KO is comparable to a delay of 1 PD in a period of 7 days. To summarize, the RPE-1 hTERT PML wt cells proliferated faster than the RPE-1 hTERT PML KO cells, indicating a PML role in the cell cycle progression.

A lower proliferation rate of PML KO cells was indicated by the EdU incorporation. Moreover, the difference between proliferation capacity of PML wt and PML KO cells was evident by comparison of the cell counts. In all conditions tested, the PML KO cells displayed lower cells counts. Thus, the difference in proliferation of the irradiated and BrdU-treated cells should be probably attributed rather to the primary difference in the proliferative capacity of PML KO cells than to the effect of the treatments themselves. In conclusion, a diminished proliferative capacity of RPE-1 hTERT PML KO cells was confirmed, indicating a possible PML role in the proliferation or in the cell cycle progression.

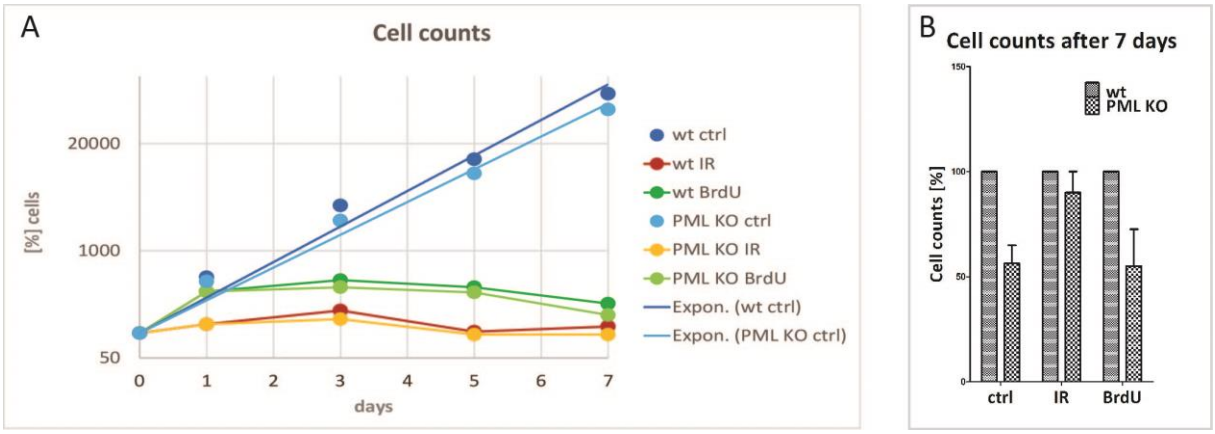
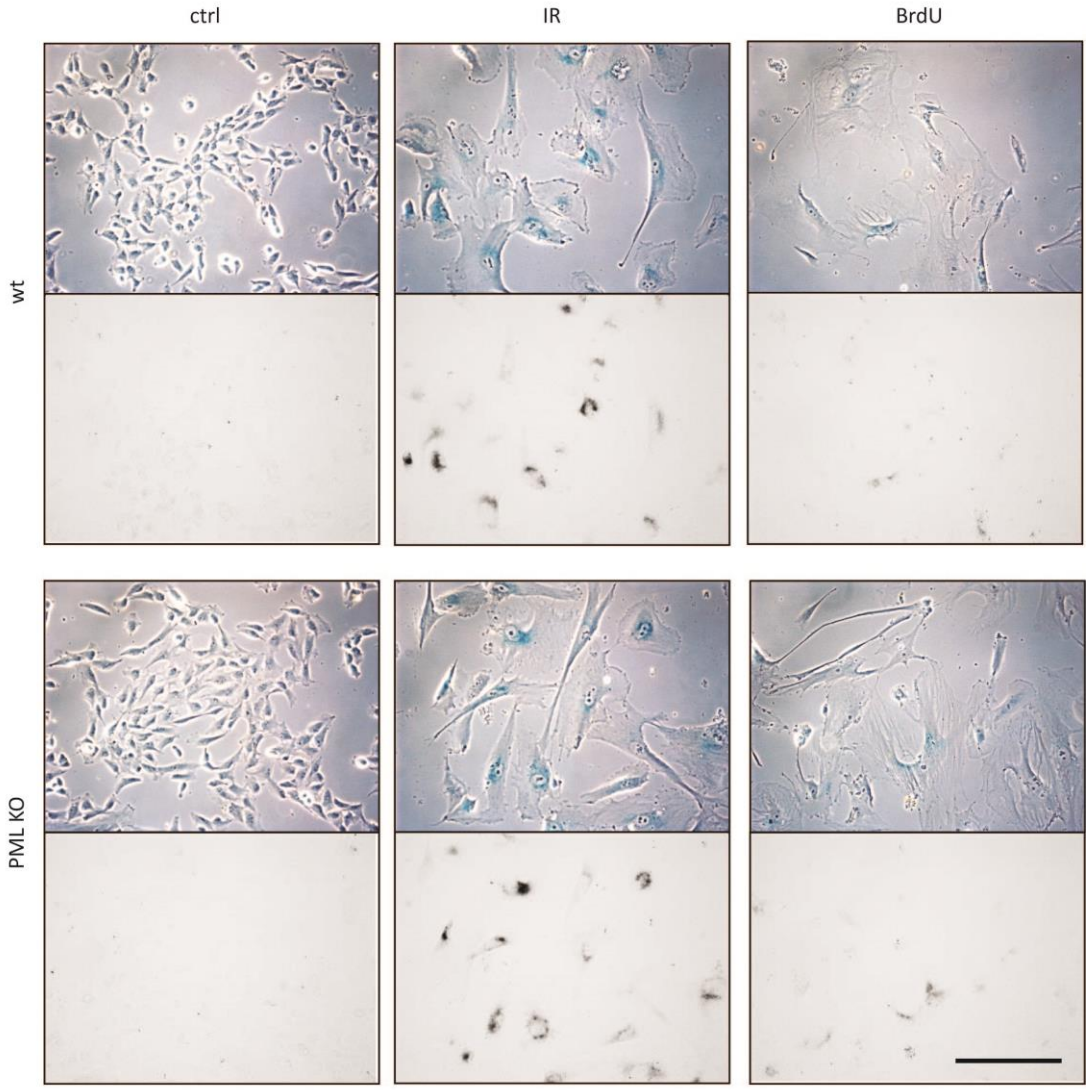


Figure 13. The effect of PML knock out on the cellular proliferation. RPE-1 hTERT PML wt and PML KO cells were counted on different time points within 7 days. In the graph A, the cell counts were recalculated to the seeding amount as 100% and the counts are displayed on a logarithmic scale (base 2). For a better visualisation of the difference in the proliferation rate on the 7th day, the PML KO cells counts were related to the PML wt cell counts; the PML wt cell counts were stipulated for each condition as 100% (B). (A - all time points; B – only 7th day). The cells were harvested after 1.5 h and after 1, 3, 5 and 7 days. The graph (A) represents data from one performed experiment. The graph (B) shows results from two independent experiments. ctrl – control untreated proliferating cells, IR – irradiated cells, BrdU – BrdU-treated cells

5.4.3 The effect of PML knock out on the establishment of senescent phenotype

The overexpression of PML was found to cause a premature cellular senescence (Pearson et al. 2000). To ascertain if the development of IR-induced premature cellular senescence will be influenced by the PML KO, the features of cellular senescence (i.e. the cellular morphology and the SA-β-galactosidase activity) after irradiation and BrdU-treatment after 7 days were assayed in RPE-1 hTERT PML wt and PML KO cells. Both irradiated and BrdU-treated PML wt and PML KO cells expressed senescent phenotype, i.e. flattened and partly spindle-shaped cellular morphology (Fig. 14). However, no differences in the morphology of the developed senescent phenotype after irradiation or BrdU-treatment between the PML wt and PML KO cells were observed. Subsequently, the SA-β-galactosidase activity was assayed (Fig. 14). As expected, no positive cells stained for the SA-β-galactosidase activity were found by both PML wt and PML KO proliferating control cells. 80% and 91% of the irradiated PML wt and PML KO cells, respectively, were positively stained for the SA-β-galactosidase activity. After BrdU-treatment, only 39% of both PML wt and PML KO cells were stained positively for the SA-β-galactosidase activity indicating a low concentration of BrdU for the full establishment of drug-induced premature cellular senescence in this cell type. Taken together, the PML KO had not an impact on the development of the senescent cellular morphology in RPE-1 hTERT cells.

A



B

[%] of cells positively stained for SA-β-galactosidase activity

	ctrl	IR	BrdU
PML wt	0%	80%	39%
PML KO	0%	91%	39%

Figure 14. The effect of PML KO on the development of senescent phenotype and SA-β-galactosidase activity. RPE-1 hTERT PML wt and PML KO cells were irradiated (IR) with 20 Gy or BrdU-treated every second day (BrdU) and harvested after 7 days. Images of the same field were taken using phase contrast (upper) and bright field (lower) imaging (A). The staining for SA-β-galactosidase activity is shown in lower line by black colouring (the blue colour was conversed to the black colour to visualize a better contrast). The graph shows a percentage of positively stained cells for SA-β-galactosidase activity (B). The cells were harvested on the 7th day after respective treatment. Bar 200 µm.

5.4.4 Summary

In conclusion, although the RPE-1 hTERT PML KO cells differed in some senescent features (formation of DNA damage foci, Rb level), the establishment of IR-induced premature cellular senescence was not significantly influenced by the absence of PML in RPE-1 hTERT PML KO cells after X-irradiation. The activated DDR (p53 pSer15 and Chk2 pThr68), activated $p21^{CIP1/WAF1}$, senescent phenotype, and SA-β-galactosidase activity exhibited a similar intensity in both RPE-1 hTERT PML wt and PML KO cells. Therefore, PML do not play a substantial role in the development of IR-induced premature cellular senescence, at least in RPE-1 hTERT cells.

However, a difference was seen in the Rb level (which was higher in RPE-1 hTERT PML KO cells) and in the proliferation rate, where RPE-1 hTERT PML KO cells showed diminished cell counts compared to the RPE-1 hTERT PML wt cells. The higher Rb level in the RPE-1 hTERT PML KO cells points to a possible PML interaction with Rb. Hypothetically, PML could function as a negative regulator of Rb. In consequence of the PML KO, the Rb protein would be more activated and would more effectively repress cell cycle progression, leading to a diminished proliferating capacity.

6. Discussion

Cellular senescence is a state of permanent growth arrest that serves as a tumorigenesis barrier by inhibiting the progression of premalignant lesions to invasive ones. Given the fact that overexpression of PML protein was found to induce cellular senescence and that PML was described to interact with p53 in several ways (Pearson et al. 2000), the aim of my thesis was to characterize the role of PML protein in the DDR and premature cellular senescence after genotoxic stress, namely X-radiation. In a recent work (Münch et al. 2014), the effect of permanent PML KD in human primary fibroblast was analysed. However, I focused on the effect of complete PML KO generated by CRISPR technology in human cells, which has not been examined yet. Based on previous reports, my hypothesis was that PML protein would support or increase the DDR signalling and the induction of IR-induced premature cellular senescence. Therefore, I analysed the development of selected features of cellular senescence, namely the formation of DNA damage foci, the activity of DDR signalling, levels of cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$, the senescent phenotype, and SA-β-galactosidase activity and examined the effectiveness of the establishment and maintenance of cellular senescence in cells with partial and complete PML depletion.

6.1 Optimisation of conditions for the development of IR-induced premature cellular senescence in cell culture model *in vitro*

High glucose concentration (hyperglycemia) in culture medium causes cellular stress. For instance, the K-562 human lymphoblasts cultured in hyperglycemic conditions in the short term, for 8 h, displayed an increased rate of DNA damage ascertained by comet assay (Hruda et al. 2010). Moreover, long term culturing in hyperglycemic conditions accelerated the onset of premature replicative cellular senescence in primary human fibroblasts (Blazer et al. 2002). It is clear that hyperglycemia also causes metabolic changes already in short term culturing, as increased activity of glycolytic enzymes was described in renal cells LLC-PK1 and OK after 24 h in hyperglycemic conditions (Gstraunthaler et al. 1999). However, whether the changes caused by hyperglycemia are also relevant for the establishment of IR-induced premature cellular senescence has not been clear. My results suggest that a glucose concentration in the culture medium of 5.5 versus 25 mM (normo- versus hyperglycemic conditions)

has no impact on the establishment of IR-induced premature cellular senescence during the short term culturing of normal human BJ fibroblasts. It is likely that the stress or DNA damage caused by the irradiation of 10 Gy is high enough in comparison to the damage caused by the hyperglycemia that the impact of the damage caused by hyperglycemia is not detectable. The observation that the effect of hyperglycemia in a long term culture is reversible only until a certain PD points to the conclusion that the damage caused by hyperglycemia is cumulative and, after exceeding a threshold, determines the cell's fate (Blazer et al. 2002). The underlying mechanism of the DNA damage caused by hyperglycemia is probably the generation of ROS because of the higher glycolysis rate which leads to a higher mitochondria activity and therefore to higher ROS generation in hyperglycemic conditions (Hruda et al. 2010). Intriguingly, it was reported that hyperglycemia did not accelerate the development of premature replicative senescence in normal BJ human fibroblasts immortalized by the ectopic hTERT expression (Blazer et al. 2002). However, it was suggested recently that hTERT may function by antagonizing ROS production in mitochondria and thus helping to decrease the amount of ROS-generated DNA damage (Singhapol et al. 2013). This finding could have profound implications for the higher stress resistance of cancerous cells. On the whole, the glucose concentration in culture medium had not an impact on the establishment of IR-induced premature cellular senescence in BJ fibroblasts.

To induce a DDR or to establish IR-induced premature cellular senescence, X-radiation is often the method of choice. For the irradiation of cell cultures, the hard radiation is often used. However, the hard radiation causes more undefined culture stress and thus possibly triggers different biological response in comparison to the soft radiation (see chapter 5.1). I studied the effect of the two irradiation regimes on the activation of DDR signalling and the development of IR-induced premature cellular senescence. My results suggest that there is no significant difference between the irradiation regimes in the impact on the activation of DDR signalling or on the development of IR-induced premature cellular senescence in normal human BJ fibroblasts. This finding could be useful by supporting the use of soft irradiation regime for cell cultures, as a soft irradiation regime means shorter irradiation time and thus lower cellular stress caused by the pH changes or the cooling of the culture medium when irradiated at room temperature. However, another aspect needs to be considered: Due to the lower penetration depth of soft radiation, its partial absorption by the material of the culture wells, and therefore a lower radiation dose ultimately reaching the cells, cannot be excluded. In line with this possibility, I have observed slightly decreased SA-β-galactosidase staining after soft irradiation. Nevertheless, no other

corresponding changes in DDR signalling or other features of IR-induced premature cellular senescence were observed. This implies that the soft irradiation regime might have influenced SA-β-galactosidase activity, but not the establishment of IR-induced premature cellular senescence or activation of DDR signalling per se. Therefore, the application of soft radiation regime for the establishment of IR-induced premature cellular senescence is acceptable.

Most of the cellular damage caused by X-radiation results from secondary damage caused by water ionisation; only a minimum of the damage is caused by a direct striking of the DNA by X-ray photons. Smaller radiation doses (ca 5 Gy and smaller) manifest mainly by DNA damage, whereas higher radiation doses (ca 20 Gy and more) manifest by considerable damage not only to the DNA but also to lipids, carbohydrates, or proteins. To establish IR-induced premature cellular senescence, various doses have been used, such as 6 Gy in HCA2 normal human fibroblasts (Noda et al. 2012), 10 Gy in HCA2 normal human fibroblasts (Rodier et al. 2009), or 20 Gy in human embryonic lung MRC5 fibroblasts (Hewitt et al. 2012). Although the development of drug-induced premature cellular senescence in RPE-1 hTERT cells has been described (Leontieva and Blagosklonny 2010; Lahtela et al. 2013), I have found no literature describing the establishment of IR-induced premature cellular senescence in this cell line. Therefore, various radiation doses were evaluated and the dose of 20 Gy was chosen for the development of IR-induced premature cellular senescence in RPE-1 hTERT cells as the most practical. The dose of 5 Gy was insufficient to induce IR-induced premature cellular senescence in the RPE-1 hTERT cells; this observation is in contrast to the observations of Noda et al. who reported an effective establishment of IR-induced premature cellular senescence after 6 Gy in HCA2 normal human fibroblasts (Noda et al. 2012). This discrepancy suggests that sensitivity to IR is cell type dependent. Moreover, Noda et al. also referred to a linear correlation between radiation doses and the amount of late DNA damage foci (Noda et al. 2012). However, I observed rather a nonlinear dependence of the number of DNA damage foci per cell nucleus on the radiation dose, indicating the results of the higher doses of 50 and 100 Gy approached saturation. However, Noda et al. (2012) only measured the dose interval of 2 to 10 Gy. As the cellular response to IR is probably cell type dependent, the most effective radiation dose for the development of IR-induced premature cellular senescence in RPE-1 hTERT cells is the dose of 20 Gy.

6.2 The effect of PML depletion on the establishment and maintenance of DDR signalling in IR-induced premature cellular senescence and on cell cycle progression

Several observations point to an interaction of PML with DDR signalling (Blander et al. 2002; Yang et al. 2002; Xu et al. 2003; Dellaire, Eskiw et al. 2006; Münch et al. 2014). For example, Münch et al. (2014) showed that a stable shRNA-mediated PML KD in primary human foreskin fibroblasts did not significantly impair the DDR signalling 24 h after irradiation. This can be explained by an inefficient knock down and thus remaining function of residual PML. To overcome this potential obstacle, complete knock out of PML can be advantageous. However, in concert with the observation of Münch et al. (2014), I showed that the DDR signalling was not impaired at any time point during the establishment of IR induced premature cellular senescence in human RPE-1 hTERT PML KO cells. The results of experiments exploring the effect of a stable PML KD (Münch et al. 2014) and my results from the RPE-1 hTERT PML KO cells point to the same conclusion – that the role of PML in the analyzed components of DDR is probably nonessential. This is very intriguing, because PML was shown to act as a DNA damage sensor in normal human diploid fibroblasts (Dellaire, Ching et al. 2006) and PML NBs also coassociate with DNA damage foci in normal human fibroblasts (Carbone et al. 2002; Rodier et al. 2011), most importantly also in senescent cells. However, the nature of this coassociation event remains still unclear. The PML role in DDR signalling was deduced mostly from the spatial and temporal colocalization of PML NBs with DDR factors at DNA damage sites. In contrast to the unaffected DDR signalling in RPE-1 hTERT PML KO cells, I saw a decrease in the activity of DDR signalling (p53 pSer15 and Chk2 pThr68 levels) in RPE-1 hTERT IR-induced senescent cells with a siRNA-mediated PML KD. However, considering my negative results investigating the PML role in the DDR and IR-induced premature cellular senescence in RPE-1 hTERT PML KO cells (see chapter 5.4), the most probable explanation for the decrease in p53 pSer15, Chk2 pThr68, $p21^{CIP1/WAF1}$ and pRb levels is that of an experimental artefact or of an unknown agent. On the grounds of my observations in RPE-1 hTERT PML KO cells, I would propose that PML plays only a negligible or replaceable role in DDR signalling and that cells easily adapt to the absence of PML. Therefore, another explanation for the PML NBs' colocalization with DDR factors should be sought. Since PML NBs are often found at DNA DSBs sites, as are most of the known DDR factors, perhaps the PML protein is not directly involved in DDR signalling but instead facilitates some steps of

DNA repair. This notion is consistent with the published data, where the PML's role in homologous recombination (HR) repair was shown (Boichuk et al. 2011; Münch et al. 2014).

The tumour suppressor p53 is known as the main "genome guardian"; it collects a broad range of information relating to cellular fitness and regulates the final outcomes of cells encountering several types of stress. The direct interaction of PML protein with p53 was described in the development of OIS, where PML protein facilitates p53 acetylation and thus increases p53 stability (Pearson et al. 2000). PML was also reported to help stabilize p53 after DNA damage caused by treatment with the topoisomerase inhibitor doxorubicin in normal human fibroblast WI-38 where PML sequestrates the p53 negative regulator E3 ubiquitin ligase Mdm2 into the nucleolus (Bernardi et al. 2004). However, the effect of PML on p53 level with respect to IR has not yet been described. I demonstrated that the induced p53 level was lower in the RPE-1 hTERT PML KO in comparison to the RPE-1 hTERT PML wt cells after irradiation with 20 Gy at all time points (up to and including day 7). This result correlates with the observation that PML helps stabilize p53 after stress (implying lower p53 stability without PML). In contrast, my analysis shows that the DDR signalling was in no way impaired in the RPE-1 hTERT PML KO cells. This is a remarkable observation, bearing in mind that p53 plays an important role in DDR signalling. However, neither the p53 pSer15 level (a DDR component) nor the $p21^{CIP1/WAF1}$ level (as a p53 transcriptional target) were changed, implying that the slight decrease in the p53 level in RPE-1 hTERT PML KO cells did not influence the $p53/p21^{CIP1/WAF1}$ signalling pathway. As p53 protein is subject to many posttranslational modifications, I cannot exclude that other functions of p53 were affected by the PML KO.

As mentioned, PML has been shown to coassociate with DNA damage foci in both human and murine fibroblasts (Carbone et al. 2002; Rodier et al. 2011; Münch et al. 2014). Noda et al. (2012) showed that DNA damage foci play a central role in IR-induced premature cellular senescence. However, the fate of the DNA damage foci in senescent cells after siRNA-mediated PML KD was not described. My hypothesis was that the PML NBs would affect the structure of the DNA damage foci, in that for instance the DNA damage foci may disappear or disintegrate when PML is depleted. However, no such effect was observed. The formation of DNA damage foci in normal human fibroblasts was shown to not be impaired in the stable shRNA-mediated PML KD (Münch et al. 2014). Next, I tested the same in RPE-1 hTERT PML KO cells. I saw a decrease in the number of DNA damage foci in RPE-1 hTERT PML KO cells after irradiation with 20 Gy in comparison to the PML wt cells, although

the level of the activated DDR signalling (p53 pSer15 and Chk2 pThr68 levels) did not differ. Hypothetically, which possible explanations could clarify the observation of higher counts of DNA damage foci in PML wt cells after irradiation? Firstly, if there is really more DNA damage foci in PML wt cells, PML could stimulate their formation, possibly through facilitating the DDR signalling. Secondly, if the DNA damage foci counts do not differ in RPE-1 hTERT PML wt and PML KO cells, but the detection of DNA damage foci is impaired instead (not the formation) in PML KO cells, the possible reason could be that PML helps in the early phase of DDR signalling by facilitating the spreading of DDR signalling through the flanking chromatin, e.g. γH2AX phosphorylation. This is in notion with the finding that PML can behave as a DNA damage sensor helping triggering the DDR (Dellaire, Ching et al. 2006). Thirdly, the measured higher DNA damage foci counts in PML wt cells could be only an artefact. After the impaired DNA damage foci formation, the level of DDR activation should correspondingly be decreased also. Based on the fact that immunoblotting data did not show any differences in the activity of DDR signalling, I am inclined to prefer the third explanation. However, the number of DNA damage foci in PML KO cells after irradiation was measured using on only a single replicate. To exclude the possibility of error, the experiment should be repeated.

Altogether, the PML protein does not play an important role in the formation of DNA damage foci in RPE-1 hTERT cells after irradiation with 20 Gy and BrdU-treatment. Thus the reason why PML NBs colocalize with DNA damage foci should be investigated further. Based on various observations of other groups, Münch et al. (2014) suggested that the PML protein might be important for the DNA repair (HR concretely), rather than for DDR signalling.

The permanent DNA damage foci belong to the features of senescent cells. The reason why DNA damage foci persist in senescent cells remains elusive. Noda et al. (2012) concluded that the heterochromatin structure formed at the DNA damage foci may inhibit the access of DNA repair factors. There is also the possibility that the persistent DNA damage foci evolved at sites which already contained a higher amount of heterochromatin proteins, which inhibited the access of repair factors and thus the repair of any potential DNA double strand breaks – leading to the formation of persistent DNA damage foci (Noda et al. 2012). However, it was reported that the DNA damage foci caused by small doses of radiation (100 mGy to 1 Gy) were all repaired (Grudzenski et al. 2010). This is in contrast to the hypothesis that DNA DSBs are irreparable if they are formed in the heterochromatin sections of DNA. If this were the case then some DNA damage foci would have

to be permanent also when caused by small doses of radiation, because irradiation inflicts DNA damage in a stochastic manner in which DNA sections with heterochromatin proteins cannot be excluded. Nevertheless, the coassociation of persistent DNA damage foci with a telomeric signal was reported, suggesting that the irreparable DNA damage foci remain in the telomeric regions because of repressed nonhomologous end joining (NHEJ) (Hewitt et al. 2012) or HR-directed repair (Zimmermann et al. 2013). However, Noda et al. (2012) did not see colocalization of DNA damage foci with a telomeric signal (Noda et al. 2012). They also showed that senescent cells are able to repair DNA damage, as was shown by their repeated irradiation (Mayer et al. 1986; Noda et al. 2012). Noda et al. (2012) conclude that the irreparable DNA DSBs might be protected by a special molecular structure which also prevents access of repair enzymes. The prevention of repair access seems a plausible explanation for the irreparable character of late DNA damage foci. Another explanation of the irreparable character of some DNA damage foci could lie in the fact that Brca1 (which recruits repair factors in HR-directed repair) is dissociated from chromatin in senescent cells (Tu et al. 2011). Or thirdly, the irreparable DNA damage foci could be constituted by a certain type of DNA damage (e.g. by cross-linked DNA or others).

The presence DNA damage foci was reported among others after one month (Noda et al. 2012). However, the presence of these DNA damage foci need not to mean that the DNA damage foci do not change. However probable the unchanging and persistent character of DNA damage foci after longer timer periods could seem, there is no evidence for this hypothesis. The DNA damage foci could evolve or be repaired during longer periods (e.g. weeks) but it would not be discovered until the sum of the DNA damage foci remains on the same level. To overcome this uncertainty, single cell time-laps microscopy imaging should be applied to track the dynamics of specific DNA damage foci in senescent cell by long-term imaging. However, such experiment would be nowadays very time- and money-consuming, apart from the photothermal damage caused by image acquisition to the cells. On the other hand, the time-lapse microscopy was used for the imaging of DDR in the time interval of minutes and hours, suggesting that in the future perhaps there will be equipment to track DNA DSBs after IR not only in the early phases of DNA damage foci formation but also in senescent cells (Lisby et al. 2003; Mortusewicz et al. 2007). Interestingly, following fluorescently labelled DNA DSBs after IR in yeast, DSBs were shown as highly mobile structures which migrate to the sites of NHEJ or HR repair in the time interval of hours, where some of them fuse creating larger DNA damage foci (Lisby et al. 2003).

It also remains unclear if the foci which are described as DNA damage foci genuinely contain unrepaired DBSs. The presence of DNA DSBs is often indirectly ascertained through γH2AX detection, which is thought to be a common marker of DNA DSBs (Rogakou et al. 1998). However, histone H2AX is also phosphorylated in an ATR-dependent manner in the presence of DNA single strand breaks, mostly at the sites of stalled replication forks (Ward and Chen 2001). Noda et al. (2012) presented various pieces of indirect evidence to support the idea of the presence of DNA DSBs. However, the authors did not performed more direct tests for the presence of DNA DSBs, such as the comet assay (Ostling and Johanson 1984) or pulsed-field gel electrophoresis (Schwartz et al. 1983). Nevertheless, neither a comet assay nor pulse-field gel electrophoresis are sensitive enough to detect isolated DNA DSBs; the detection limit is 50 strand breaks per diploid mammalian cell for the comet assay (Olive and Banáth 2006), and PFGE is able to fractionate DNA molecules only down to 10 Mb in length (Herschleb et al. 2007). Thus, the presence of DNA DSBs in DNA damage foci remains still elusive.

PML is involved in the OIS regulation (Pearson et al. 2000). However, Münch et al. (2014) followed the development of IR-induced premature cellular senescence over 6 days in PML wt human fibroblasts and in human fibroblasts with a stable PML KD. They concluded that PML protein is nonessential for the induction of IR-induced premature cellular senescence in primary human fibroblasts. Based on findings of my and other studies, I confirmed the nonessentiality of the PML protein in the establishment of IR-induced premature cellular senescence even in cells bearing a PML KO. It cannot be excluded that the signalling pathways which regulate IR- and oncogene-induced premature cellular senescence are diverse and that perhaps PML is not necessary for IR-induced premature cellular senescence. To explore this possibility, it would be interesting to investigate the development of OIS in PML wt and PML KO cells.

PML interaction with Rb was shown in murine and human fibroblasts forced to premature cellular senescence by overexpression of PML (Ferbeyre et al. 2000; Mallette et al. 2004). Furthermore, PML protein cooperates with Rb in the silencing of E2F-responsive genes during the establishment of OIS in MEFs (Vernier et al. 2011; Talluri and Dick 2014). My results show that total Rb level is higher in proliferating RPE-1 hTERT PML KO in comparison with RPE-1 hTERT PML wt cells. A possible explanation could be that PML negatively regulates Rb expression or stability, e.g. that PML protein cooperates with other proteins to repress transcription of Rb. In this way, PML could influence the Rb level and thus indirectly also cell cycle progression. However, the exact mechanism through

which PML influences Rb level has yet to be revealed. Bearing in mind that Rb is a repressor of cell cycle progression into the S phase, a higher level of Rb could result in a higher threshold for the transition from G1 to S phase. This would result in a prolonged G1 phase and consequently lower proliferation rate. However, neither I nor Münch et al. (2014) analyzed which cell cycle phases are delayed. The hypothesis of a prolonged G1 phase indeed corresponds to my observations that PML KO cells proliferate more slowly compared to PML wt cells. The estimated difference in cell cycle length is approximately 40 min. This is in agreement with the study of Münch et al. (2014), who show that primary human foreskin fibroblasts with a stable siRNA-mediated PML KD proliferated with a lower efficiency then PML wt cells (PML KD human foreskin fibroblasts reached 10 PD fewer than the PML wt cells, which reached 38 PD). The final proliferation rate of the cell culture is the sum of the growth and death rates. Having this in mind, the lower proliferation of cells with PML KO could be caused not only by a cell cycle delay but also by a higher apoptosis rate. However, this explanation can be excluded as higher apoptosis was not observed in proliferating PML KO compared to proliferating PML wt cells.

The inability to detect activated DDR signalling and the low number of DNA damage foci after BrdU treatment in both PML wt and PML KO cells correlates with the low rate of positively stained cells for SA-β-galactosidase activity, raising questions regarding the efficiency of the used BrdU dose, which was likely insufficient to cause a major activation of DDR signalling. On the other hand, the administered BrdU dose was sufficient to cause a permanent cell cycle arrest, manifested through the $p21^{CIP1/WAF1}$ increased level, the absence of ppRb and the presence of pRb

Nonetheless, a substantial shortcoming of my results is the fact that only one clone of the RPE-1 hTERT cells (derived from a single cell) bearing a knock out of the PML gene was analyzed. To exclude the possibility that all my results and the differences in Rb level are clone-specific, other PML KO cell clones should be analysed in the future.

7. Conclusions

1. I optimized the conditions for the induction of IR-induced premature cellular senescence as follows: I showed that the glucose concentration in the culture medium had no effect on the development of IR-induced premature cellular senescence in short-term culturing of human BJ fibroblasts. I found that the two types of irradiation (hard and soft) have almost the same impact on the establishment of IR-induced premature cellular senescence in human BJ fibroblasts. Bearing that in mind, I used the soft irradiation regime for the development of IR-induced premature cellular senescence in subsequent experiments. I chose the dose of 20 Gy as the most effective for the induction of IR-induced premature cellular senescence in the human epithelial RPE-1 hTERT cells.

2. I saw no changes in the DNA damage foci in RPE-1 hTERT IR-induced senescent cells after PML knock down. I found a slight decrease of the Chk2 phosphorylation on Thr68, hypophosphorylated Rb and of $p21^{CIP1/WAF1}$ levels after PML knock down in RPE-1 hTERT IR-induced senescent cells. However, considering results from point 3 in these conclusions (see below), the most plausible explanation for this decrease is an experimental artefact.

3. I observed no significant difference in the DDR and IR-induced premature cellular senescence in the RPE-1 hTERT cells bearing a knock out in the PML gene (PML KO) in comparison to the RPE-1 PML wt cells after seven days. Concerning the indirect evidence of PML connection with DDR and IR-induced premature cellular senescence, PML is probably replaceable or circumstantial in these processes.

4. However, I found a higher level of Rb protein in RPE-1 hTERT PML KO cells comparing to the RPE-1 hTERT PML wt cells. I also described that RPE-1 hTERT PML KO cells proliferated with a lesser efficiency compared to the PML wt cells. Because Rb is a repressor of cell cycle progression, the last finding reveals a possible influence of PML protein on the Rb level, cell cycle progression and also, indirectly, on cellular proliferation.

8. References

Adamson, a L. & Kenney, S. (2001): Epstein-Barr Virus Immediate-Early Protein BZLF1 Is SUMO-1 Modified and Disrupts Promyelocytic Leukemia Bodies. Journal of Virology 75: 2388–99.

Ahn, J.H. & Hayward, G.S. (2000): Disruption of PML-Associated Nuclear Bodies by IE1 Correlates with Efficient Early Stages of Viral Gene Expression and DNA Replication in Human Cytomegalovirus Infection. Virology 274: 39–55.

Ahn, J.Y., Schwarz, J.K., Piwnica-Worms, H., & Canman, C.E. (2000): Threonine 68 Phosphorylation by Ataxia Telangiectasia Mutated Is Required for Efficient Activation of Chk2 in Response to Ionizing Radiation. Cancer Research 60: 5934–36.

Alcalay, M., Tomassoni, L., Colombo, E., Stoldt, S., Grignani, F., Fagioli, M., Szekely, L., Helin, K., & Pelicci, P.G. (1998): The Promyelocytic Leukemia Gene Product (PML) Forms Stable Complexes with the Retinoblastoma Protein. Molecular and Cellular Biology 18: 1084–93.

Alcorta, D. a, Xiong, Y., Phelps, D., Hannon, G., Beach, D., & Barrett, J.C. (1996): Involvement of the Cyclin-Dependent Kinase Inhibitor p16 (INK4a) in Replicative Senescence of Normal Human Fibroblasts. Proceedings of the National Academy of Sciences of the United States of America 93: 13742–47.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y., & Ziv, Y. (1998): Enhanced Phosphorylation of p53 by ATM in Response to DNA Damage. Science (New York, N.Y.) 281: 1674–77.

Bartkova, J. et al. (2006): Oncogene-Induced Senescence Is Part of the Tumorigenesis Barrier Imposed by DNA Damage Checkpoints. Nature 444: 633–37.

Bernardi, R. & Pandolfi, P.P. (2007): Structure, Dynamics and Functions of Promyelocytic Leukaemia Nuclear Bodies. Nature Reviews. Molecular Cell Biology 8: 1006–16.

Bernardi, R., Scaglioni, P.P., Bergmann, S., Horn, H.F., Vousden, K.H., & Pandolfi, P.P. (2004): PML Regulates p53 Stability by Sequestering Mdm2 to the Nucleolus. Nature Cell Biology 6: 665–72.

Bick, M.D. & Devine, E.A. (1977): Interaction of Chromosomal Proteins with BrdU Substituated DNA as Determined by Chromatin-DNA Competition. Nucleic Acids Research 4: 3687–3700.

Bischof, O., Kirsh, O., Pearson, M., Itahana, K., Pelicci, P.G., & Dejean, A. (2002): Deconstructing PML-Induced Premature Senescence. EMBO Journal 21: 3358–69.

Blander, G., Zalle, N., Daniely, Y., Taplick, J., Gray, M.D., & Oren, M. (2002): DNA Damage-Induced Translocation of the Werner Helicase Is Regulated by Acetylation. The Journal of Biological Chemistry 277: 50934–40.

Blazer, S., Khankin, E., Segev, Y., Ofir, R., Yalon-Hacohen, M., Kra-Oz, Z., Gottfried, Y., Larisch, S., & Skorecki, K.L. (2002): High Glucose-Induced Replicative Senescence: Point of No Return and Effect of Telomerase. Biochemical and Biophysical Research Communications 296: 93–101.

Boichuk, S., Hu, L., Makielski, K., Pandolfi, P.P., & Gjoerup, O. V. (2011): Functional Connection between Rad51 and PML in Homology-Directed Repair. PLoS ONE 6: 1–13.

Boisvert, F.M., Hendzel, M.J., & Bazett-Jones, D.P. (2000): Promyelocytic Leukemia (PML) Nuclear Bodies Are Protein Structures That Do Not Accumulate RNA. Journal of Cell Biology 148: 283–92.

Branzei, D. & Foiani, M. (2008): Regulation of DNA Repair throughout the Cell Cycle. Nature Reviews. Molecular Cell Biology 9: 297–308.

Brown, J.P., Wei, W., & Sedivy, J.M. (1997): Bypass of Senescence after Disruption of p21CIP1/WAF1 Gene in Normal Diploid Human Fibroblasts. Science (New York, N.Y.) 277: 831–34.

Campisi, J. & d'Adda di Fagagna, F. (2007): Cellular Senescence: When Bad Things Happen to Good Cells. Nature reviews. Molecular cell biology 8: 729–40.

Carbone, R., Pearson, M., Minucci, S., & Pelicci, P.G. (2002): PML NBs Associate with the hMre11 Complex and p53 at Sites of Irradiation Induced DNA Damage. Oncogene 21: 1633–40.

Chan, J.Y., Li, L., Fan, Y.H., Mu, Z.M., Zhang, W.W., & Chang, K.S. (1997): Cell-Cycle Regulation of DNA Damage-Induced Expression of the Suppressor Gene PML. Biochemical and Biophysical Research Communications 240: 640–46.

Chang, B.-D., Swift, M.E., Shen, M., Fang, J., Broude, E. V, & Roninson, I.B. (2002): Molecular Determinants of Terminal Growth Arrest Induced in Tumor Cells by a Chemotherapeutic Agent. Proceedings of the National Academy of Sciences of the United States of America 99: 389–94.

Condemine, W., Takahashi, Y., Le Bras, M., & de Thé, H. (2007): A Nucleolar Targeting Signal in PML-I Addresses PML to Nucleolar Caps in Stressed or Senescent Cells. Journal of cell science 120: 3219–27.

Condemine, W., Takahashi, Y., Zhu, J., Puvion-Dutilleul, F., Guegan, S., Janin, A., & De Thé, H. (2006): Characterization of Endogenous Human Promyelocytic Leukemia Isoforms. Cancer Research 66: 6192–98.

Coppé, J.P., Rodier, F., Patil, C.K., Freund, A., Desprez, P.Y., & Campisi, J. (2011): Tumor Suppressor and Aging Biomarker p16 INK4a Induces Cellular Senescence without the Associated Inflammatory Secretory Phenotype. Journal of Biological Chemistry 286: 36396–403.

d'Adda di Fagagna, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P., & Jackson, S.P. (2003): A DNA Damage Checkpoint Response in Telomere-Initiated Senescence. Nature 426: 194–98.

D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., Piaggio, G., Fanciulli, M., Appella, E., & Soddu, S. (2002): Homeodomain-Interacting Protein Kinase-2 Phosphorylates p53 at Ser 46 and Mediates Apoptosis. Nature Cell Biology 4: 11–19.

De Stanchina, E., Querido, E., Narita, M., Davuluri, R. V, Pandolfi, P.P., Ferbeyre, G., & Lowe, S.W. (2004): PML Is a Direct p53 Target That Modulates p53 Effector Functions. Molecular Cell 13: 523–35.

De The, H., Chomienne, C., Lanotte, M., Degos, L., & Dejean, A. (1990): The t(15;17) Translocation of Acute Promyelocytic Leukaemia Fuses the Retinoic Acid Receptor [alpha] Gene to a Novel Transcribed Locus. Nature 347: 558–61.

Dedon, P.C. & Goldberg, I.H. (1992): Free-Radical Mechanisms Involved in the Formation of Sequence-Dependent Bistranded DNA Lesions by the Antitumor Antibiotics Bleomycin, Neocarzinostatin, and Calicheamicin. Chemical Research in Toxicology 5: 311–32.

Dellaire, G., Ching, R.W., Ahmed, K., Jalali, F., Tse, K.C.K., Bristow, R.G., & Bazett-Jones, D.P. (2006): Promyelocytic Leukemia Nuclear Bodies Behave as DNA Damage Sensors Whose Response to DNA Double-Strand Breaks Is Regulated by NBS1 and the Kinases ATM, Chk2, and ATR. Journal of Cell Biology 175: 55–66.

Dellaire, G., Ching, R.W., Dehghani, H., Ren, Y., & Bazett-Jones, D.P. (2006): The Number of PML Nuclear Bodies Increases in Early S Phase by a Fission Mechanism. Journal of cell science 119: 1026–33.

Dellaire, G., Eskiw, C.H., Dehghani, H., Ching, R.W., & Bazett-Jones, D.P. (2006): Mitotic Accumulations of PML Protein Contribute to the Re-Establishment of PML Nuclear Bodies in G1. Journal of cell science 119: 1034–42.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre', M., Nuciforo, P.G., Bensimon, A., Maestro, R., Pelicci, P.G., & d'Adda di Fagagna, F. (2006): Oncogene-Induced Senescence Is a DNA Damage Response Triggered by DNA Hyper-Replication. Nature 444: 638–42.

Di Micco, R., Sulli, G., Dobreva, M., Liontos, M., Botrugno, O. a, Gargiulo, G., dal Zuffo, R., Matti, V., D'Ario, G., Montani, E., Mercurio, C., Hahn, W.C., Gorgoulis, V., Minucci, S., & d'Adda di Fagagna, F. (2011): Interplay between Oncogene-Induced DNA Damage Response and Heterochromatin in Senescence and Cancer. Nature Cell Biology 13: 292–302.

Dierick, J.-F., Eliaers, F., Remacle, J., Raes, M., Fey, S.J., Larsen, P.M., & Toussaint, O. (2002): Stress-Induced Premature Senescence and Replicative Senescence Are Different Phenotypes, Proteomic Evidence. Biochemical Pharmacology 64: 1011—1017.

Dimri, G.P., Leet, X., Basile, G., Acosta, M., Scorrt, G., Roskelley, C., Medrano, E.E., Linskensi, M., Rubeljii, I., Pereira-smithii, O., Peacocket, M., & Campisi, J. (1995): A Biomarker That Identifies Senescent Human Cells in Culture and in Aging Skin in Vivo. Proceedings of the National Academy of Sciences of the United States of America 92: 9363–67.

Duprez, E., Saurin, a J., Desterro, J.M., Lallemand-Breitenbach, V., Howe, K., Boddy, M.N., Solomon, E., de Thé, H., Hay, R.T., & Freemont, P.S. (1999): SUMO-1 Modification of the Acute Promyelocytic Leukaemia Protein PML: Implications for Nuclear Localisation. Journal of Cell Science 112: 381–93.

Dyck, J. a., Maul, G.G., Miller, W.H., Chen, J.D., Kakizuka, A., & Evans, R.M. (1994): A Novel Macromolecular Structure Is a Target of the Promyelocyte-Retinoic Acid Receptor Oncoprotein. Cell 76: 333–43.

Eskiw, C.H., Dellaire, G., & Bazett-Jones, D.P. (2004): Chromatin Contributes to Structural Integrity of Promyelocytic Leukemia Bodies through a SUMO-1-Independent Mechanism. Journal of Biological Chemistry 279: 9577–85.

Everett, R.D. & Chelbi-Alix, M.K. (2007): PML and PML Nuclear Bodies: Implications in Antiviral Defence. Biochimie 89: 819–30.

Fagioli, M., Alcalay, M., Pandolfi, P.P., Venturini, L., Mencarelli, A., Simeone, A., Acampora, D., Grignani, F., & Pelicci, P.G. (1992): Alternative Splicing of PML Transcripts Predicts Coexpression of Several Carboxy-Terminally Different Protein Isoforms. Oncogene 7: 1083—1091.

Ferbeyre, G., De Stanchina, E., Querido, E., Baptiste, N., Prives, C., & Lowe, S.W. (2000): PML Is Induced by Oncogenic Ras and Promotes Premature Senescence. Genes and Development 14: 2015–27.

Flenghi, L., Fagioli, M., Tomassoni, L., Pileri, S., Gambacorta, M., Pacini, R., Grignani, F., Casini, T., Ferrucci, P.F., & Martelli, M.F. (1995): Characterization of a New Monoclonal Antibody (PG-M3) Directed against the Aminoterminal Portion of the PML Gene Product: Immunocytochemical Evidence for High Expression of PML Proteins on Activated Macrophages, Endothelial Cells, and Epithelia. Blood 85: 1871–80.

Fogal, V., Gostissa, M., Sandy, P., Zacchi, P., Sternsdorf, T., Jensen, K., Pandolfi, P.P., Will, H., Schneider, C., & Del Sal, G. (2000): Regulation of p53 Activity in Nuclear Bodies by a Specific PML Isoform. The EMBO Journal 19: 6185–95.

Fumagalli, M., Rossiello, F., Clerici, M., Barozzi, S., Cittaro, D., Kaplunov, J.M., Bucci, G., Dobreva, M., Matti, V., Beausejour, C.M., Herbig, U., Longhese, M.P., & d'Adda di Fagagna, F. (2012): Telomeric DNA Damage Is Irreparable and Causes Persistent DNA-Damage-Response Activation. Nature Cell Biology 14: 355–65.

Fuxe, J., Goike, H.M., Collins, V.P., & Pettersson, R.F. (2000): Adenovirus-Mediated Overexpression of p15 INK4B Inhibits Human Glioma Cell Growth , Induces Replicative Senescence , and Inhibits Telomerase Activity Similarly to p16 INK4A. Cell Growth & Differentiation 11: 373–84.

Grande, M. a., Van Der Kraan, I., Van Steensel, B., Schul, W., De Thé, H., Van Der Voort, H.T.M., De Jong, L., & Van Driel, R. (1996): PML-Containing Nuclear Bodies: Their Spatial Distribution in Relation to Other Nuclear Components. Journal of Cellular Biochemistry 63: 280–91.

Grudzenski, S., Raths, A., Conrad, S., Rübe, C.E., & Löbrich, M. (2010): Inducible Response Required for Repair of Low-Dose Radiation Damage in Human Fibroblasts. Proceedings of the National Academy of Sciences of the United States of America 107: 14205–10.

Gstraunthaler, G., Seppi, T., & Pfaller, W. (1999): Impact of Culture Conditions, Culture Media Volumes, and Glucose Content on Metabolic Properties of Renal Epithelial Cell Cultures. Cellular Physiology and Biochemistry 9: 150–72.

Guo, A., Salomoni, P., Luo, J., Shih, A., Zhong, S., Gu, W., & Paolo Pandolfi, P. (2000): The Function of PML in p53-Dependent Apoptosis. Nature Cell Biology 2: 730–36.

Gurrieri, C., Capodieci, P., Bernardi, R., Scaglioni, P.P., Nafa, K., Rush, L.J., Verbel, D. a, Cordon-Cardo, C., & Pandolfi, P.P. (2004): Loss of the Tumor Suppressor PML in Human Cancers of Multiple Histologic Origins. Journal of the National Cancer Institute 96: 269–79.

Haaf, T., Golub, E.I., Reddy, G., Radding, C.M., & Ward, D.C. (1995): Nuclear Foci of Mammalian Rad51 Recombination Protein in Somatic Cells after DNA Damage and Its Localization in Synaptonemal Complexes. Proceedings of the National Academy of Sciences of the United States of America 92: 2298–2302.

Hardy, K., Mansfield, L., Mackay, A., Benvenuti, S., Ismail, S., Arora, P., O'Hare, M.J., & Jat, P.S. (2005): Transcriptional Networks and Cellular Senescence in Human Mammary Fibroblasts. Molecular Biology of the Cell 16: 943–53.

Hayflick, L. & Moorhead, P.S. (1961): The Serial Cultivation of Human Diploid Cell Strains. Experimental Cell Research 25: 585–621.

He, D., Mu, Z., Le, X., Hsieh, J., Pong, R., Chung, L.W.K., & Chang, K. (1997): Adenovirus-Mediated Expression of PML Suppresses Growth and Tumorigenicity of Prostate Cancer Cells. Cancer Research: 1868–72.

Herschleb, J., Ananiev, G., & Schwartz, D.C. (2007): Pulsed-Field Gel Electrophoresis. Nature Protocols 2: 677–84.

Hewitt, G., Jurk, D., Marques, F.D.M., Correia-Melo, C., Hardy, T., Gackowska, A., Anderson, R., Taschuk, M., Mann, J., & Passos, J.F. (2012): Telomeres Are Favoured Targets of a Persistent DNA Damage Response in Ageing and Stress-Induced Senescence. Nature Communications 3: 1–9.

Hirao, a, Kong, Y.Y., Matsuoka, S., Wakeham, a, Ruland, J., Yoshida, H., Liu, D., Elledge, S.J., & Mak, T.W. (2000): DNA Damage-Induced Activation of p53 by the Checkpoint Kinase Chk2. Science (New York, N.Y.) 287: 1824–27.

Hofmann, T.G., Möller, A., Sirma, H., Zentgraf, H., Taya, Y., Dröge, W., Will, H., & Schmitz, M.L. (2002): Regulation of p53 Activity by Its Interaction with Homeodomain-Interacting Protein Kinase-2. Nature Cell Biology 4: 1–10.

Hruda, J., Sramek, V., & Leverve, X. (2010): High Glucose Increases Susceptibility to Oxidative-Stress-Induced Apoptosis and DNA Damage in K-562 Cells. Biomedical Papers 154: 315–20.

Hubackova, S., Krejcikova, K., Bartek, J., & Hodny, Z. (2012): Interleukin 6 Signaling Regulates Promyelocytic Leukemia Protein Gene Expression in Human Normal and Cancer Cells. Journal of Biological Chemistry 287: 26702–14.

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: 3085–99.

Ishov, A.M., Sotnikov, A.G., Negorev, D., Vladimirova, O. V., Neff, N., Kamitani, T., Yeh, E.T.H., Strauss, J.F., & Maul, G.G. (1999): PML Is Critical for ND10 Formation and Recruits the PML-Interacting Protein Daxx to This Nuclear Structure When Modified by SUMO-1. Journal of Cell Biology 147: 221–33.

Itahana, K., Campisi, J., & Dimri, G.P. (2004): Mechanisms of Cellular Senescence in Human and Mouse Cells. Biogerontology 5: 1–10.

Janderova-Rossmeislova, L., Novakova, Z., Vlasakova, J., Philimonenko, V., Hozak, P., & Hodny, Z. (2007): PML Protein Association with Specific Nucleolar Structures Differs in Normal, Tumor and Senescent Human Cells. Journal of Structural Biology 159: 56–70.

Jensen, K., Shiels, C., & Freemont, P.S. (2001): PML Protein Isoforms and the RBCC/TRIM Motif. Oncogene 20: 7223–33.

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G., & Sherr, C.J. (1997): Tumor Suppression at the Mouse INK4a Locus Mediated by the Alternative Reading Frame Product p19ARF. Cell 91: 649—659.

Kamitani, T., Nguyen, H.P., Kito, K., Fukuda-Kamitani, T., & Yeh, E.T.H. (1998): Covalent Modification of PML by the Sentrin Family of Ubiquitin-like Proteins. Journal of Biological Chemistry 273: 3117–20.

Koken, M.H., Linares-Cruz, G., Quignon, F., Viron, A., Chelbi-Alix, M.K., Sobczak-Thépot, J., Juhlin, L., Degos, L., Calvo, F., & de Thé, H. (1995): The PML Growth-Suppressor Has an Altered Expression in Human Oncogenesis. Oncogene 10: 1315—1324.

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 p16ink4a. Cell Cycle 10: 457–68.

Kubbutat, M.H., Jones, S.N., & Vousden, K.H. (1997): Regulation of p53 Stability by Mdm2. Nature 387: 299—303.

Kulju, K.S. & Lehman, J.M. (1995): Increased p53 Protein Associated with Aging in Human Diploid Fibroblasts. Experimental Cell Research.

Kurz, D.J., Decary, S., Hong, Y., & Erusalimsky, J.D. (2000): Senescence-Associated (beta)-Galactosidase Reflects an Increase in Lysosomal Mass during Replicative Ageing of Human Endothelial Cells. Journal of Cell Science 113: 3613–22.

Lahtela, J., Corson, L.B., Hemmes, A., Brauer, M.J., Koopal, S., Lee, J., Hunsaker, T.L., Jackson, P.K., & Verschuren, E.W. (2013): A High-Content Cellular Senescence Screen Identifies Candidate Tumor Suppressors, Including EPHA3. Cell Cycle 12: 625–34.

Lee, B.Y., Han, J. a., Im, J.S., Morrone, A., Johung, K., Goodwin, E.C., Kleijer, W.J., DiMaio, D., & Hwang, E.S. (2006): Senescence-Associated B-Galactosidase Is Lysosomal B-Galactosidase. Aging Cell 5: 187–95.

Lee & Paull. (2005): ATM Activation by DNA Double-Strand Breaks through the Mre11-Rad50-Nbs1 Complex. Science (New York, N.Y.) 308: 551–54.

Leontieva, O. V. & Blagosklonny, M. V. (2010): DNA Damaging Agents and p53 Do Not Cause Senescence in Quiescent Cells, While Consecutive Re-Activation of mTOR Is Associated with Conversion to Senescence. Aging 2: 924–35.

Lisby, M., Mortensen, U.H., & Rothstein, R. (2003): Colocalization of Multiple DNA Double-Strand Breaks at a Single Rad52 Repair Centre. Nature Cell Biology 5: 572–77.

Lukas, J., Lukas, C., & Bartek, J. (2011): More than Just a Focus: The Chromatin Response to DNA Damage and Its Role in Genome Integrity Maintenance. Nature Cell Biology 13: 1161–69.

Lukas, J., Sørensen, C.S., Lukas, C., Santoni-Rugiu, E., & Bartek, J. (1999): p16INK4a, but Not Constitutively Active pRb, Can Impose a Sustained G1 Arrest: Molecular Mechanisms and Implications for Oncogenesis. Oncogene 18: 3930–35.

Lupa, M.W., Kalfalah, F., Florea, A.M., Sass, S., Kruse, F., Rieder, V., Tigges, J., Fritsche, E., Krutmann, J., Busch, H., Meyer, H.E., Boege, F., Theis, F., & Reifenberger, G. (2014): Proteome Wide Analysis Reveals an Age Associated Cellular Phenotype of in Situ Aged Human Fibroblasts. Aging 6: 856–78.

Mailand, N., Bekker-Jensen, S., Faustrup, H., Melander, F., Bartek, J., Lukas, C., & Lukas, J. (2007): RNF8 Ubiquitylates Histones at DNA Double-Strand Breaks and Promotes Assembly of Repair Proteins. Cell 131: 887–900.

Mallette, F. a, Goumard, S., Gaumont-Leclerc, M.-F., Moiseeva, O., & Ferbeyre, G. (2004): Human Fibroblasts Require the Rb Family of Tumor Suppressors, but Not p53, for PML-Induced Senescence. Oncogene 23: 91–99.

Maser, R.S., Monsen, K.J., Nelms, B.E., & Petrini, J.H. (1997): hMre11 and hRad50 Nuclear Foci Are Induced during the Normal Cellular Response to DNA Double-Strand Breaks. Molecular and Cellular Biology 17: 6087–96.

Masterson, J.C. & O'Dea, S. (2007): 5-Bromo-2-Deoxyuridine Activates DNA Damage Signalling Responses and Induces a Senescence-like Phenotype in p16-Null Lung Cancer Cells. Anti-Cancer Drugs 18: 1053–68.

Maul, G.G., Yu, E., Ishov, A.M., & Epstein, A.L. (1995): Nuclear Domain 10 (ND10) Associated Proteins Are Also Present in Nuclear Bodies and Redistribute to Hundreds of Nuclear Sites after Stress. Journal of Cellular Biochemistry 59: 498—513.

Mayer, P.J., Bradley, M.O., & Nichols, W.W. (1986): No Change in DNA Damage or Repair of Single- and Double-Strand Breaks as Human Diploid Fibroblasts Age in Vitro. Experimental Cell Research 166: 497–509.

McConnell, B.B., Starborg, M., Brookes, S., & Peters, G. (1998): Inhibitors of Cyclin-Dependent Kinases Induce Features of Replicative Senescence in Early Passage Human Diploid Fibroblasts. Current biology 8: 351–54.

Michishita, E., Nakabayashi, K., Ogino, H., Suzuki, T., Fujii, M., & Ayusawa, D. (1998): DNA Topoisomerase Inhibitors Induce Reversible Senescence in Normal Human Fibroblasts. Biochemical and Biophysical Research Communications 253: 667–71.

Mirzoeva, O.K. & Petrini, J.H. (2001): DNA Damage-Dependent Nuclear Dynamics of the Mre11 Complex. Molecular and Cellular Biology 21: 281–88.

Mortusewicz, O., Ame, J.-C., Schreiber, V., & Leonhardt, H. (2007): Feedback-Regulated poly(ADP-Ribosyl)ation by PARP-1 Is Required for Rapid Response to DNA Damage in Living Cells. Nucleic Acids Research 35: 7665–75.

Mukhopadhyay, D., Ayaydin, F., Kolli, N., Tan, S.H., Anan, T., Kametaka, A., Azuma, Y., Wilkinson, K.D., & Dasso, M. (2006): SUSP1 Antagonizes Formation of Highly SUMO2/3-Conjugated Species. Journal of Cell Biology 174: 939–49.

Münch, S., Weidtkamp-Peters, S., Klement, K., Grigaravicius, P., Monajembashi, S., Salomoni, P., Pandolfi, P.P., Weißhart, K., & Hemmerich, P. (2014): The Tumor Suppressor PML Specifically Accumulates at RPA/Rad51-Containing DNA Damage Repair Foci but Is Nonessential for DNA Damage-Induced Fibroblast Senescence. Molecular and Cellular Biology 34: 1733–46.

Muñoz-Espín, D., Cañamero, M., Maraver, A., Gómez-López, G., Contreras, J., Murillo-Cuesta, S., Rodríguez-Baeza, A., Varela-Nieto, I., Ruberte, J., Collado, M., & Serrano, M. (2013): Programmed Cell Senescence during Mammalian Embryonic Development. Cell 155: 1104—1118.

Muratani, M., Gerlich, D., Janicki, S.M., Gebhard, M., Eils, R., & Spector, D.L. (2002): Metabolic-Energy-Dependent Movement of PML Bodies within the Mammalian Cell Nucleus. Nature Cell Biology 4: 106–10.

Narita, M., Nun, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., Lowe, S.W., Brook, S., & York, N. (2003): Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence. Cell 113: 703–16.

Noda, A., Hirai, Y., Hamasaki, K., Mitani, H., Nakamura, N., & Kodama, Y. (2012): Unrepairable DNA Double-Strand Breaks That Are Generated by Ionising Radiation Determine the Cell Fate of Normal Human Cells. Journal of Cell Science: 5280–87.

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: 273–84.

Olive, P.L. & Banáth, J.P. (2006): The Comet Assay: A Method to Measure DNA Damage in Individual Cells. Nature Protocols 1: 23–29.

Ostling, O. & Johanson, K.J. (1984): Microelectrophoretic Study of Radiation-Induced DNA Damages in Individual Mammalian Cells. Biochemical and Biophysical Research Communications 123: 291—298.

Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P.P., & Pelicci, P.G. (2000): PML Regulates p53 Acetylation and Premature Senescence Induced by Oncogenic Ras. Nature 406: 207—210.

Pearson, M. & Pelicci, P.G. (2001): PML Interaction with p53 and Its Role in Apoptosis and Replicative Senescence. Oncogene 20: 7250–56.

Robles, S.J. & Adami, G.R. (1998): Agents That Cause DNA Double Strand Breaks Lead to p16INK4a Enrichment and the Premature Senescence of Normal Fibroblasts. Oncogene 16: 1113–23.

Rodier, F. & Campisi, J. (2011): Four Faces of Cellular Senescence. The Journal of Cell Biology 192: 547–56.

Rodier, F., Coppé, J., Patil, C.K., Wieteke, a M., Muñoz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R., Campisi, J., Berkeley, L., & Road, O.C. (2009): Persistent DNA Damage Signaling Triggers Senescence-Associated Inflammatory Cytokine Secretion. Nature Cell Biology 11: 973–79.

Rodier, F., Muñoz, D.P., Teachenor, R., Chu, V., Le, O., Bhaumik, D., Coppé, J.-P., Campeau, E., Beauséjour, C.M., Kim, S.-H., Davalos, A.R., & Campisi, J. (2011): DNA-SCARS: Distinct Nuclear Structures That Sustain Damage-Induced Senescence Growth Arrest and Inflammatory Cytokine Secretion. Journal of Cell Science 124: 68–81.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S., & Bonner, W.M. (1998): Double-Stranded Brekas Induce Histone H2AX Phosphorylation on Serine 139. The Journal of Biological Chemistry 273: 5858–68.

Rossiello, F., Herbig, U., Longhese, M.P., Fumagalli, M., & d'Adda di Fagagna, F. (2014): Irreparable Telomeric DNA Damage and Persistent DDR Signalling as a Shared Causative Mechanism of Cellular Senescence and Ageing. Current Opinion in Genetics and Development 26: 89–95.

Sachs, R.K., Chen, P.L., Hahnfeldt, P.J., & Hlatky, L.R. (1992): DNA Damage Caused by Ionizing Radiation. Mathematical biosciences 112: 271–303.

Scaglioni, P.P., Yung, T.M., Cai, L.F., Erdjument-Bromage, H., Kaufman, A.J., Singh, B., Teruya-Feldstein, J., Tempst, P., & Pandolfi, P.P. (2006): A CK2-Dependent Mechanism for Degradation of the PML Tumor Suppressor. Cell 126: 269–83.

Schwartz, D.C., Saffran, W., Welsh, J., Haas, R., Goldenberg, M., & Cantor, C.R. (1983): New Techniques for Purifying Large DNAs and Studying Their Properties and Packaging. Cold Spring Harbor Symposia on Quantitative Biology 47 Pt 1: 189—195.

Seeler, J.S., Marchio, a, Sitterlin, D., Transy, C., & Dejean, a. (1998): Interaction of SP100 with HP1 Proteins: A Link between the Promyelocytic Leukemia-Associated Nuclear Bodies and the Chromatin Compartment. Proceedings of the National Academy of Sciences of the United States of America 95: 7316–21.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., & Lowe, S.W. (1997): Oncogenic Ras Provokes Premature Cell Senescence Associated with Accumulation of p53 and p16(INK4a). Cell 88: 593–602.

Shen, Lin, Scaglioni, Yung, & Pandolfi. (2006): The Mechanisms of PML-Nuclear Body Formation. Molecular Cell 24: 1–14.

Shieh, S.Y., Ikeda, M., Taya, Y., & Prives, C. (1997): DNA Damage-Induced Phosphorylation of p53 Alleviates Inhibition by MDM2. Cell 91: 325–34.

Shiels, C., Islam, S. a, Vatcheva, R., Sasieni, P., Sternberg, M.J., Freemont, P.S., & Sheer, D. (2001): PML Bodies Associate Specifically with the MHC Gene Cluster in Interphase Nuclei. Journal of Cell Science 114: 3705–16.

Singhapol, C., Pal, D., Czapiewski, R., Porika, M., Nelson, G., & Saretzki, G.C. (2013): Mitochondrial Telomerase Protects Cancer Cells from Nuclear DNA Damage and Apoptosis. PLoS One 8: 1–11.

Smith, B.L., Bauer, G.B., & Povirk, L.F. (1994): DNA Damage Induced by Bleomycin, Neocarzinostatin, and Melphalan in a Precisely Positioned Nucleosome. Asymmetry in Protection at the Periphery of Nucleosome-Bound DNA. Journal of Biological Chemistry 269: 30587–94.

Stadler, M., Chelbi-Alix, M.K., Koken, M.H., Venturini, L., Lee, C., Saïb, A., Quignon, F., Pelicano, L., Guillemin, M.C., & Schindler, C. (1995): Transcriptional Induction of the PML Growth Suppressor Gene by Interferons Is Mediated through an ISRE and a GAS Element. Oncogene 11: 2565—2573.

Stein, G.H., Drullinger, L.F., Soulard, A., & Dulić, V. (1999): Differential Roles for Cyclin-Dependent Kinase Inhibitors p21 and p16 in the Mechanisms of Senescence and Differentiation in Human Fibroblasts. Molecular and Cellular Biology 19: 2109–17.

Stewart, G.S. et al. (2009): The RIDDLE Syndrome Protein Mediates a Ubiquitin-Dependent Signaling Cascade at Sites of DNA Damage. Cell 136: 420–34.

Stuurman, N., de Graaf, A., Floore, A., Josso, A., Humbel, B., de Jong, L., & van Driel, R. (1992): A Monoclonal Antibody Recognizing Nuclear Matrix-Associated Nuclear Bodies. Journal of Cell Science 101: 773–84.

Sulli, G., Di Micco, R., & di Fagagna, F. d'Adda. (2012): Crosstalk between Chromatin State and DNA Damage Response in Cellular Senescence and Cancer. Nature Reviews Cancer 12: 709–20.

Takahashi, Y., Lallemand-Breitenbach, V., Zhu, J., & de Thé, H. (2004): PML Nuclear Bodies and Apoptosis. Oncogene 23: 2819–24.

Takai, H., Smogorzewska, A., & de Lange, T. (2003): DNA Damage Foci at Dysfunctional Telomeres. Current Biology 13: 1549–56.

Talluri, S. & Dick, F. a. (2014): The Retinoblastoma Protein and PML Collaborate to Organize Heterochromatin and Silence E2F-Responsive Genes during Senescence. Cell Cycle 13: 641–51.

Terris, B., Baldin, V., Dubois, S., Terris, B., Degott, C., Hã, D., & Dejean, A. (1995): PML Nuclear Bodies Are General Targets for Inflammation and Cell Proliferation PML Nuclear Bodies Are General Targets for Inflammation and Cell Proliferation. Cancer Research 55: 1590–97.

Tresini, M., Mawal-Dewan, M., Cristofalo, V.J., & Sell, C. (1998): A Phosphatidylinositol 3-Kinase Inhibitor Induces a Senescent-like Growth Arrest in Human Diploid Fibroblasts. Cancer Research 58: 1–4.

Trotman, L.C., Alimonti, A., Scaglioni, P.P., Koutcher, J.A., Cordon-Cardo, C., & Pandolfi, P.P. (2006): Identification of a Tumour Suppressor Network Opposing Nuclear Akt Function. Nature 441: 523–27.

Tu, Z., Aird, K.M., Bitler, B.G., Nicodemus, J.P., Beeharry, N., Xia, B., Yen, T.J., & Zhang, R. (2011): Oncogenic Ras Regulates BRIP1 Expression to Induce Dissociation of BRCA1 from Chromatin, Inhibit DNA Repair, and Promote Senescence. Developmental Cell 21: 1077–91.

Valent, L. & Strasser, A. (2013): Distinct Target Genes and Effector Processes Appear to Be Critical for p53-Activated Responses to Acute DNA Damage versus p53-Mediated Tumour Suppression. BioDiscovery 3: 1–16.

Vermeulen, K., Van Bockstaele, D.R., & Berneman, Z.N. (2003): The Cell Cycle: A Review of Regulation, Deregulation and Therapeutic Targets in Cancer. Cell Proliferation 36: 131–49.

Vernier, M., Bourdeau, V., Gaumont-Leclerc, M.F., Moiseeva, O., Bégin, V., Saad, F., Mes-Masson, A.M., & Ferbeyre, G. (2011): Regulation of E2Fs and Senescence by PML Nuclear Bodies. Genes and Development 25: 41–50.

Wallace, S.S. (1998): Enzymatic Processing of Radiation-Induced Free Radical Damage in DNA. Radiation Research 150: S60–79.

Wang, B., Matsuoka, S., Carpenter, P.B., & Elledge, S.J. (2002): 53BP1, a Mediator of the DNA Damage Checkpoint. Science (New York, N.Y.) 298: 1435–38.

Wang, J., Shiels, C., Sasieni, P., Wu, P.J., Islam, S. a., Freemont, P.S., & Sheer, D. (2004): Promyelocytic Leukemia Nuclear Bodies Associate with Transcriptionally Active Genomic Regions. Journal of Cell Biology 164: 515–26.

Wang, Z.G., Delva, L., Gaboli, M., Rivi, R., Giorgio, M., Cordon-Cardo, C., Grosveld, F., & Pandolfi, P.P. (1998): Role of PML in Cell Growth and the Retinoic Acid Pathway. Science (New York, N.Y.) 279: 1547–51.

Ward, I.M. & Chen, J. (2001): Histone H2AX Is Phosphorylated in an ATR-Dependent Manner in Response to Replicational Stress. Journal of Biological Chemistry 276: 47759–62.

Ward, J.F. (1985): Biochemistry of DNA Lesions. Radiation Research 104: S103–11.

Weidtkamp-Peters, S., Lenser, T., Negorev, D., Gerstner, N., Hofmann, T.G., Schwanitz, G., Hoischen, C., Maul, G., Dittrich, P., & Hemmerich, P. (2008): Dynamics of Component Exchange at PML Nuclear Bodies. Journal of Cell Science 121: 2731–43.

Weintraub, S.J., Prater, C.A., & Dean, D.C. (1992): Retinoblastoma Protein Switches the E2F Site from Positive to Negative Element. Nature 358: 259—261.

Xu, Z.-X., Timanova-Atanasova, A., Zhao, R.-X., & Chang, K.-S. (2003): PML Colocalizes with and Stabilizes the DNA Damage Response Protein TopBP1. Molecular and Cellular Biology 23: 4247–56.

Yang, S., Jeong, J.H., Brown, A.L., Lee, C.H., Pandolfi, P.P., Chung, J.H., & Kim, M.K. (2006): Promyelocytic Leukemia Activates Chk2 by Mediating Chk2 Autophosphorylation. Journal of Biological Chemistry 281: 26645–54.

Yang, S., Kuo, C., Bisi, J.E., & Kim, M.K. (2002): PML-Dependent Apoptosis after DNA Damage Is Regulated by the Checkpoint Kinase hCds1/Chk2. Nature Cell Biology 4: 865–70.

Zhang, R., Chen, W., & Adams, P.D. (2007): Molecular Dissection of Formation of Senescence-Associated Heterochromatin Foci. Molecular and Cellular Biology 27: 2343–58.

Zhong, S., Delva, L., Rachez, C., Cenciarelli, C., Gandini, D., Zhang, H., Kalantry, S., Freedman, L.P., & Pandolfi, P.P. (1999): A RA-Dependent, Tumour-Growth Suppressive Transcription Complex Is the Target of the PML-RARalpha and T18 Oncoproteins. Nature Genetics 23: 287–95.

Zhong, S., Müller, S., Ronchetti, S., Freemont, P.S., Dejean, a, & Pandolfi, P.P. (2000): Role of SUMO-1-Modified PML in Nuclear Body Formation. Blood 95: 2748–52.

Zimmermann, M., Lottersberger, F., Buonomo, S.B., Sfeir, A., & de Lange, T. (2013): 53BP1 Regulates DSB Repair Using Rif1 to Control 5’ End Resection. Science (New York, N.Y.) 339: 700–704.