Regulation of promyelocytic leukemia protein expression

Jana Dobrovolná

PhD Thesis

Charles University
Faculty of Science

Academy of Sciences of the Czech Republic
Institute of Experimental Medicine
Institute of Molecular Genetics

Supervisor: Zdeněk Hodný, MD, PhD

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CONTENTS

ABBREVIATIONS

AAF, α-interferon activated factor

AML, acute myeloid leukemia

APL, acute promyelocytic leukemia

APNC, actinomycin- induced PML nucleolar cap

ARF, alternative reading frame protein

ATM, ataxia telangiectasia mutated kinase

ATP, adenosine triphosphate

ATR, ATM and Rad3 related kinase

BLM, Bloom syndrome helicase

BRCA1, breast cancer type 1 susceptibility protein

BrdU, 5-bromo-2'-deoxyuridine

Brg1, Brahma-related gene 1

BrU, 5-bromouridine

BrUTP, 5-bromouridine triphosphate

C/EBP, CCAAT/enhancer binding protein

CBP, CREB (cAMP response element-binding protein) binding protein

Chk1, check kinase 1

Chk2, check kinase 2

CK2, casein kinase 2

DFC, dense fibrillar center

DMA, distamycin A

ERK, extracellular signal-regulated kinase

FC, fibrillar center

GAF, γ-interferon activated factor

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GAS, γ-interferon activated site

GC, granular component

HAT, histone acetyltransferase

HAUSP, herpesvirus associated ubiquitin specific protease

HDAC, histone deacetylase

HeLa, human cervical carcinoma cells

HIPK2, homeodomain interacting protein kinase 2

HIRA, histone repressor A

HMG, high mobility group protein

hMSCs, human mesenchymal stem cells

HP1, heterochromatin protein 1

HR, homologous recombination

HSF, human skin fibroblasts

IFNα, interferon-α

IFN, interferon

IL, interleukin

IRF, interferon regulatory factor

ISG, interferon-stimulated genes

ISGF3, interferon stimulated gene factor 3

ISRE, interferon-stimulated response element

JAK, Janus kinase

MAPK, mitogen-activated protein kinase (ERK1/2)

MDM2, mouse double minute 2 protein

MEF, mouse embryonic fibroblasts

MRE11, meiotic recombination 11 homolog

mRNA, messenger ribonucleic acid

MS-275, N-(2-aminophenyl)-4-[N-(pyridine-3-ylmethoxycarbonyl)-aminomethyl]benzamide

NAD, nicotinamide adenine dinucleotide

NBS1, Nijmegen breakage syndrome protein 1

N-CoR, nuclear corepressor

NER, nucleotide excision repair

NES, nuclear export signal

NHEJ, non-homologous end joining

NLS, nuclear localization signal

PCNA, proliferating cell nuclear antigen

PML NBs, PML nuclear bodies

PML, promyelocytic leukemia protein

PML-NDS, PML-nucleolus-derived structure

pRB, retinoblastoma susceptibility protein

PRD, positive regulatory domain

RA, retinoic acid

RAR, retinoic acid receptor

rDNA, ribosomal deoxyribonucleic acid

RNase, ribonuclease

RPA, replication protein A

rRNA, ribosomal ribonucleic acid

RT-PCR, reverse transcription polymerase chain reaction

S/MARs, scaffold/nuclear matrix attachment regions

SAHA, suberoylanilide hydroxamic acid

SAHF, senescence-associated heterochromatin foci

SIRT, sir2-like protein

SMRT, silencing mediator of retinoic acid and thyroid hormone receptor

STAT, signal transducer and activator of transcription

SUMO, small ubiquitin-like modifier

TF, transcription factor

TGFß, transforming growth factor ß

TIF, transcription initiation factor

TNFα, tumor necrosis factor α

TRAIL, tumor-necrosis factor-related apoptosis inducing ligand

TSA, trichostatin A

UBF, upstream binding factor

WB, Western blotting

WRN, Werner helicase

1. ABSTRACT

Promyelocytic leukemia protein (PML), a tumor suppressor, is a marker of and a crucial protein for formation of multiprotein nuclear structures called PML nuclear bodies (PML NBs) that dynamically change its number, size and content in response to a variety of internal and external stimuli. PML and PML NBs are implicated in many processes of vital importance for survival of cell and maintenance of its genomic integrity like regulation of proliferation, stress response (DNA damage, heat shock, viral infection), apoptosis, and senescence. Despite of participation in these processes, a specific function of PML and PML NBs remains obscure. Also very little is known about mechanisms driving expression of PML. Up-regulation of PML was described in inflammatory tissues and after viral infection as a result of activated interferon (IFN) signaling pathways. Moreover, PML expression was increased in cells prematurely senesced by oncogene overexpression. The presented PhD thesis is concentrated on revealing mechanisms that regulate PML gene expression in response to IFNs and during an onset of specific cellular phenotype – drug-induced premature senescence.

The main part of the PhD thesis addresses the mechanism of PML induction by IFNs with especial interest on the role of acetylation in this signaling (Research Paper I). We demonstrated in several human cell lines and in skin fibroblasts that the deacetylation step is essential for IFNα-induced expression of PML gene. All tested inhibitors of histone deacetylases (HDACIs), causing general protein hyperacetylation, suppressed IFNα-induced accumulation of both PML mRNA and PML protein. We suggested that a deacetylation target(s) lies downstream of ISGF3, a main transcriptional factor mediating IFNα signaling, as HDACIs did not block its translocation between cytoplasm and cell nucleus and binding to PML promoter. In addition to PML, IFNα-mediated activation of Sp100 (another structural component of PML NBs) and IRF1 was also negatively influenced by HDACIs, supporting an assumption that this phenomenon is common for all interferon-stimulated genes (ISGs). In broader context, these findings can help to understand mechanisms of HDACIs action, which could be of especial importance as HDACIs are currently tested as potential anticancer drugs.

Next part of the PhD thesis is dedicated to the analysis of expression and localization of PML in growing and senescent human mesenchymal stem cells (hMSC;

Research Paper II). We reported that PML is readily expressed in hMSC, and importantly, number of PML NBs increases with their proliferative age. This indicates a role of PML in senescence (in this case replicative senescence). Drug-induced premature senescence was in more detail studied in the following part of presented PhD thesis. Moreover, we discovered and characterized novel forms of nuclear PML compartment associated with nucleoli in hMSC and in skin fibroblasts under normal growth-permitting conditions. In addition, a part of PML pool translocated to surface of actinomycin D-inactivated nucleoli. Of note, specific PML compartments associating with nucleoli were not found in several immortal cell lines. However, in HeLa cells, PML affinity to either active or inactivated nucleoli was restored by a treatment causing premature senescence (i.e. simultaneous administration of 5-bromo-2'-deoxyuridine and distamycin A, see below). These findings indicate that PML may be involved in nucleolar functions of normal non-transformed or senescent cells and that PML association with the nucleolus might be important for cell cycle regulation.

To study the role of PML and its expression in senescent cells in more detail, we established and optimized a model of premature senescence induced by 5-bromo-2'-deoxyuridine and/or distamycin A (Research Paper III). This treatment induced senescence and elevated both PML mRNA and PML protein levels resulting in observed increase in number of PML bodies in several human cell lines. Interestingly, persistent activation of JAK-STAT signaling pathway, expression of IFNβ and induction of interferon regulatory factors (IRFs) and ISGs was found in cells prematurely senescent by chemical drug administration. These results indicate that strong positive feedback loop operates in senescent cells, comprising IFNβ expression, its secretion and activation of JAK-STAT pathway via paracrine stimulation, induction of tumor suppressors (PML and STAT1) and transcription factors from IRF family (IRF1 and IRF7), which are responsible for activation of IFNβ gene. We hypothesize that such sustained interferon response could contribute to initiation and maintenance of senescence program and to regulation of PML expression in senescent cells.

In summary, this work revealed new features of cancer cell lines and broaden our understanding of signaling pathways triggered during the response to DNA damage, the regulatory mechanisms of the expression of interferon stimulated genes including several potent tumor suppressors and the establishment of cellular senescence.

2. GENERAL INTRODUCTION

2.1. PROTEIN ACETYLATION IN GENE EXPRESSION

Changes in gene expression pattern underlie cell differentiation into distinct cellular types and reflect dynamics of actual metabolic state of the individual in response to the external and internal stimuli. Besides other mechanisms like control of RNA stability or regulation of translation, gene expression is in the first place regulated at the transcription level[1]. Transcription of all protein-coding genes in eukaryotes is provided by RNA polymerase II, which needs cooperation of other proteins for initiation of transcription. Mandatory transcription components are general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and THIIH), which are necessary for gene promoter recognition and loading of polymerase into promoters of all genes (reviewed in[2, 3]). Turning on or off individual genes is established by orchestrated action of a set of regulatory proteins – gene specific transcription factors and mediators. Gene specific transcription factors (activators or repressors) bind to regulatory sequences that can be located at long distances, i.e. thousands of base pairs, in both directions from transcription start and positively or negatively influence transcription initiation. Mediators (also called adaptors, cofactors, or coactivators and corepressors), do not bind directly to DNA but mediate interaction between gene specific and general transcription factors and polymerase (reviewed in[4]). Action of regulatory protein strongly depends on the promoter context; the same regulatory protein can influence transcription positively or negatively under different conditions; an effect of one regulatory protein could be strongly synergized by other(s) and the initiation of transcription often comprises from sequence of regulatory proteins' binding, promoter structure reorganization and consequent recruitment of other regulatory proteins causing additional changes[3, 5].

Spatial compaction of eukaryotic DNA is achieved by its organization in chromatin. A basic structural element of chromatin is a nucleosome, which consists of DNA wrapped almost twice around histone octamer[6]. Tight DNA packaging limits the access of the transcriptional machinery to the DNA template and represents one of the major hurdles in activation of transcription *in vivo*[7, 8]. Therefore, the main role of

transcription regulatory proteins, in addition to direct effects on assembly of initiation complex, is to cause local changes in chromatin structure by recruitment of ATP-dependent chromatin remodeling complexes and enzymes changing posttranslational modifications of chromatin components[9]. Posttranslational modifications of histones appear to be a key event in the regulation of gene expression as they mediate chromatin remodeling into transcription active or inactive state. Probably the most well studied modifications are acetylation and deacetylation of histones. It is widely accepted that histones at transcriptionally active sites are highly acetylated, which allows loosening of chromatin structure, nucleosome reposition in promoter region (demonstrated by increased sensitivity of nucleosome-"free" DNA of activated promoter to DNase I) and loading of specific transcription factors and the general transcription machinery. Histone acetyltransferases (HATs), enzymes responsible for histone acetylation, are present at active promoters, where they act as coactivators. On the contrary, histone deacetylases (HDACs) are believed to function as corepressors, to cause chromatin compaction. They are associated with transcriptionally silent promoters[10] (**Figure 1**). Since mid 1990s, when first HATs and HDACs were identified and histone acetylation was directly linked to regulation of gene expression thanks to extensive research interest, two original notions of these enzymes had to be corrected. Firstly, there is an accumulating evidence that deacetylation is not exclusively connected with transcriptional repression. Different research groups reported - depending on model system - from 2 up to 22% of all genes to be affected by HDACs inhibitors (HDACIs), i.e. by general protein hyperacetylation[11-14]. Surprisingly, the ratio of up- and down-regulated genes was almost 1:1[15]. This findings change the overall presumption that hyperacetylation in connected predominantly with activation of transcription. Our own investigation (Research Paper I) describes the case when HDACIs suppress the transcription of several interferon-responsible genes. In concordance with others, we assume that HDACIs possess general negative effect on transcription of interferon stimulated genes. Second correction in our view of HATs and HDACs function regards their targets. Although HAT and HDAC proteins were named for their abilities to modify histones, they have many non-histone substrates and work both in the nucleus and the cytoplasm of the cell[16-20]. Indeed, extensive phylogenetic analysis revealed that HDAC enzymes are found from bacteria to human, thus it is thought that they evolved before histone proteins and caused deacetylation of various substrates before histones become their most abundant target in eukaryotes[21]. Among HAT and HDAC non-

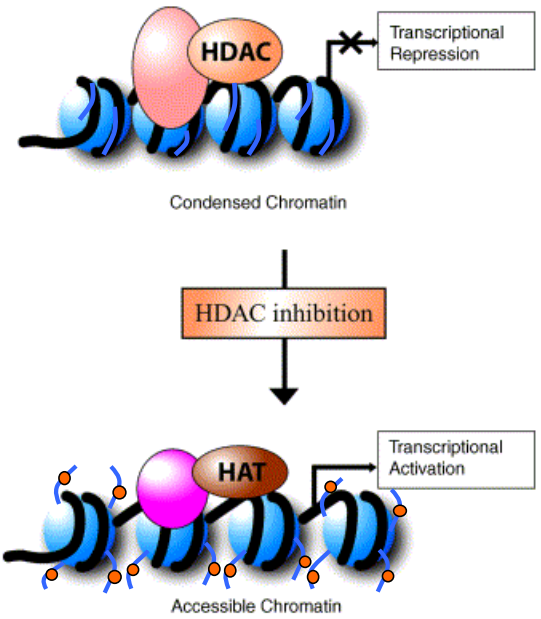

Figure 1: Effect of HATs and HDACs on chromatin organization
HATs add acetyl groups (orange spots) on lysines of histone tails and thus mask positive charge of lysines that interact with negatively charged DNA molecule. Histone acetylation results in chromatin loosening and increased accessibility of DNA for transcription factors. The conversely acting HDACs remove the acetyl groups from nucleosomes, which leads to condensation of chromatin. HDAC inhibitors block HDAC activity, leading to increased levels of histone acetylation and chromatin decondensation. Both HDACs and HATs are components of multicomponent protein complexes that interact with transcription factors.
(adopted from McLaughlin et al. 2004[22])

histone targets are transcription factors (over 60 of them); many regulators of DNA repair, recombination and replication; viral proteins; metabolic enzymes; nuclear import proteins; kinases, phosphatases and other signaling regulators (**Figure 2**). The fact, that besides histones also transcription factors are subjects of acetylation, makes the regulation of gene transcription even more complicated. The posttranslational modification can change factors' DNA- or regulatory proteins-binding properties and in turn their ability to alter gene transcription.

Epigenetic changes, including deacetylation, were described in several types of cancer and aberrant recruitment of HDACs has important role in leukemogenesis, thus inhibitors of HDACs (HDACIs) are intensively studied as potent anti-cancer drugs in clinical trials. Considering the pleiotropic cellular effects and rapidly increasing number of newly identified targets of acetylation and deacetylation, it becomes critically important to understand the ramifications of these modifications on protein function and mechanisms of HDACI effects to foresee potential off-target effects.

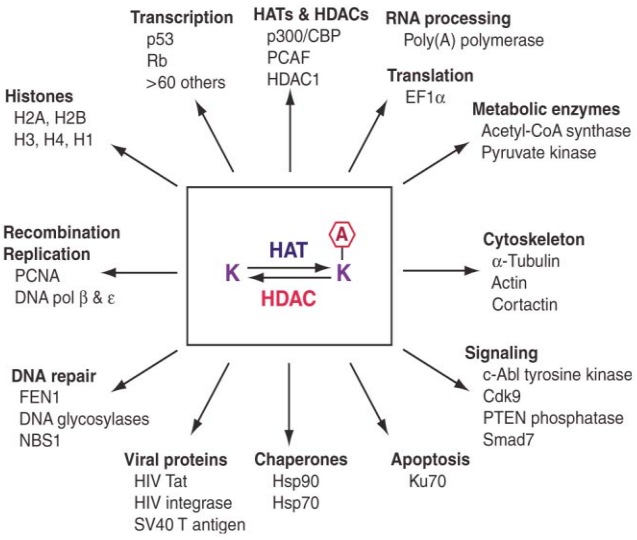

Figure 2: Non-histone targets of HATs and HDACs
Representative proteins are listed for each process. Note that HATs/HDACs can be also modified by acetylation.
(from Yang et al. 2007[23])

2.1.1. HATs

HATs catalyze acetylation of ε-amino groups on lysine residues in histones' N-terminal domains and thus disrupt interaction of these amino groups with negatively charged DNA, loosen chromatin structure and make DNA accessible for transcription factors[10] (**Figure 1**). Because HATs themselves cannot directly bind to DNA, most of them take part in huge multiprotein complexes that are responsible for their locus targeting. HATs function therefore as cofactors that are dependent on other DNA-binding proteins[24]. It has been demonstrated that HATs are evolutionary conserved from yeast to human. There are three major families of HATs: GNAT, p300/CBP, and MYST. **GNAT family** (Gcn5-related N-acetyl transferases; reviewed in[25]) includes GCN5 (general control non-inducible 5) and PCAF (p300/CBP-associated factor). **p300/CBP family** (reviewed in[26]) has two members: p300 and CBP (CREB-binding protein). Originally, p300 was found to be associated with adenoviral E1A protein and CBP with transcription factor CREB (cyclic AMP response element binding protein). Today p300/CBP are regarded as general transcription coactivators that are ubiquitously expressed and mediate acetylation of many transcription factors including well known tumor suppressor p53. **MYST family** (reviewed in[25]) is named after its founding

members (MOZ, Ybf2/Sas3, Sas2, Tip60), which are grouped together on basis of their close sequence similarities, particularly in highly conserved MYST domain that uses different catalytic mechanism than other HATs families.

Beside these three families of HATs, also some other proteins where described to have acetyltransferase activity, for example TAFII250 (TATA binding protein (TBP)-associated factor, a subunit of TFIID)[27] or nuclear receptor coactivator ACTR[28]. TAFII250 has beside of intrinsic HAT activity also tandem pair of about 120-residue motif known as a bromodomain[29]. Bromodomain recognizes and binds to acetylated lysines. It is assumed that bromodomain of TAFII250 serves for recognition of nucleosome-bound promoter, which contains histones acetylated by some upstream-binding coactivator, and mediates binding of TFIID to promoter resulting in recruitment of other chromatin remodeling factors and transcription machinery[29]. Bromodomain motif is present in a variety of proteins including nuclear histone acetyltransferases (p300, CBP, GCN5, PCAF), kinases, and chromatin remodeling factors[30, 31]. Recognition of acetylated histones by bromodomain-containg HATs is important for maintainance of opened chromatin structure[32].

2.1.2. HDACs

HDACs counteract action of HATs and catalyze removal of acetyl groups from lysine residues of histones and other proteins. By histone deacetylation, HDACs create a non-permissive chromatin conformation that prevents the gene transcription. Similarly as HATs, HDACs function as co-factors and act on multiprotein complexes. Several HDACs even from different classes can co-exist in the same complex. During the last decade, more than a dozen histone deacetylases have been identified in mammalian cells. Based on sequence similarities, HDACs are divided into four functional classes (for a review, see[33]). **Class I** deacetylases (HDAC 1, 2, 3, and 8) are related to yeast Rpd3 HDAC and share homology in catalytic site. These proteins were found to be associated with classical transcriptional corepressors including Sin3, NCoR, SMRT and methyl CpG binding proteins[34]. HDAC class I is ubiquitously expressed, whereas class II and IV show tissue specificity (**Table 1**). With exception of HDAC3, class I and IV are constitutively nuclear proteins (**Table 1**). **Class II** (HDAC 4, 5, 6, 7, 9, and 10) are related to yeast Hda1 HDAC. They share homology in two regions, N-terminal regulatory domain and C-terminal catalytic site. HDACs of class II shuttle between

cytoplasm and nucleus, which suggests their role in deacetylation of cytoplasmic substrates or mechanism of regulation of their action. **Class III** deacetylases, SIRT1 to SIRT7, are also called sirtuins according to their sequence similarity with yeast transcriptional repressor Sir2 (for a review of Sir2 protein family, see[35]). They are NAD^+-dependent enzymes and are structurally unrelated to other classes of HDACs (sometimes reported as classical HDACs). As SIRTs have not been extensively studied in mammalian systems and HDACIs used in our study have no effect on their activity, they are not described in the thesis and HDACs abbreviation is used in meaning of classical HDACs in following text. The recently described **class IV** of HDAC11-related proteins[21, 36] contains conserved residues in catalytic core regions shared by both class I and II HDACs. All classical HDACs (Class I, II and IV) possess catalytic domain formed by a region of about 390 amino acids, which contains a set of in eukaryotic cells conserved residues. The active site of enzyme consists of a tubular pocket with hydrophobic walls and a Zn^{2+} cation at the bottom. An acetylated lysine residue fits in the pocket, where Zn^{2+} catalyzes the hydrolysis of the acetyl group. Many HDACIs are designed to fit into the pocket, to chelate the cation and, with a rest of molecule, to block the pocket entry[37].

Enzyme	Subcelullar localization	Expression in human tissues
Class I		
HDAC1	Nuclear	Ubiquitous
HDAC2	Nuclear	Ubiquitous
HDAC3	Mostly nuclear	Ubiquitous
HDAC8	Nuclear	Ubiquitous
Class II		
HDAC4	Both nuclear and cytoplasmatic	Brain, heart, skeletal muscle
HDAC5	Both nuclear and cytoplasmatic	Brain, heart, skeletal muscle
HDAC7	Both nuclear and cytoplasmatic	Thymocytes, heart, skeletal muscle
HDAC9	Both nuclear and cytoplasmatic	Brain, heart, skeletal muscle
HDAC10	Both nuclear and cytoplasmatic	Liver, spleen, kidney
HDAC6	Mostly cytoplasmatic	Testis, others
Class IV		
HDAC11	Mostly nuclear	Kidney, heart, brain ,testis, skeletal muscle

Table 1: Human classical HDACs
(modified from Villar-Garea 2004[37])

2.1.3. Non-histone targets of HDACs/HATs

List of non-histone proteins modified by acetylation is rapidly growing. Acetylation has many different effects on protein properties, like protein stability, protein-protein and protein-DNA interactions, and cellular localization. The functional effect of acetylation can differ from protein to protein. It is usual that modification acts at once on several levels (e.g. acetylation of p53 influence its stability, its DNA binding and transcriptional activity[38]). Examples of HATs/HDACs action on protein properties and the role of modified proteins in various biological processes is described further (**Figure 3**).

Acetylation and protein stability

The ε-amino group of lysine residue is, in addition to acetylation, subject to other modifications, including methylation, ubiquitination, sumoylation, propioylation, or butyrylation. Acetylation could preclude other modifications, and vice versa. Thus, HATs and HDACs control availability of lysine residue for other potential covalent modifications. As acetylation and ubiquitylation often occur on the same lysine[39], HDACs can make the way ubiquitylation and thus decrease the half-time of several substrates: e.g. p53[38], E2F1[40], α-tubulin[41], Smad7[42], and others[43-45].

Acetylation and protein-protein interactions

Similarly to phosphorylation, acetylation can mediate or prevent protein-protein interactions. For instance, cytosolic acetylation triggers STAT3 dimerization and subsequent nuclear translocation[18, 46]. Acetylation of hypoxia-inducible factor 1 stabilize its interaction with von Hippel-Lindau protein[47] (described in detail in 2.1.4). Deacetylated Ku70 protein interacts with Bax and thus blocks Bax translocation into mitochondria and induction of apoptosis. Under cellular stress, Ku70 is acetylated that results in Bax releasing, its translocation to mitochondria and activation of apoptosis[48].

Acetylation and DNA-protein interactions

The acetylation can both increase DNA-binding activity of several proteins and their subsequent transcriptional activity (e.g. p53[16], E2F1[40]) or impair protein-DNA interactions in other cases (IRF7[49]). Resulting effect of acetylation might be dependent on localization of modified lysine, whether it is direct constituent of DNA binding

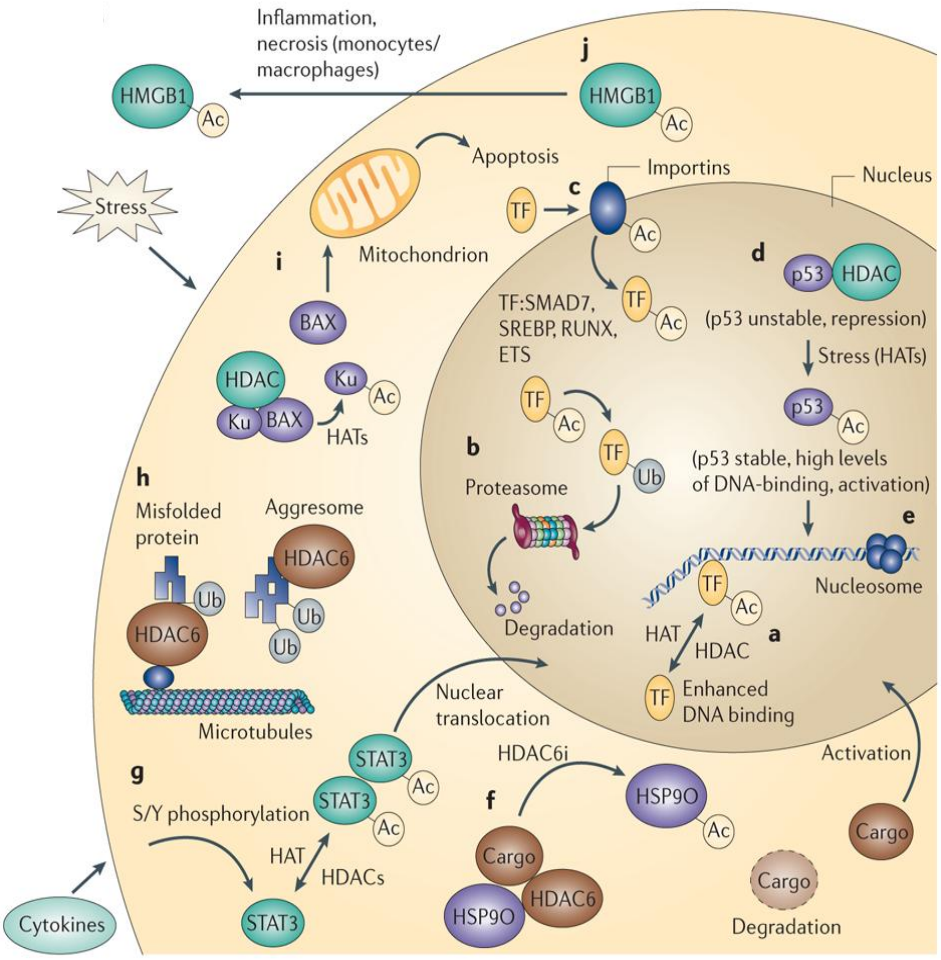

Figure 3: Effects of HATs and HDACs

A partial list of biological processes that are regulated by acetylation is sketched in a-j.

(a) Acetylation (Ac) may regulate an association of transcription factor (TF) with DNA.

(b) Protein stability is influenced by HATs/HDACs, the acetylation can protect lysine from ubiquitylation (Ub) and consequent degradation in proteasome.

(c) The nuclear import and export of proteins is regulated by acetylation of importins.

(d) Acetylation of p53 results in its increased stability, DNA-binding and transcriptional activity.

(e) Acetylation of histone tails cause chromatin decondenzation and nucleosome repositioning. In addition, acetyl group on histone lysines can be recognized by bromodomain of some regulatory proteins.

(f) Acetylation of heat shock protein HSP90 (in absence of HDAC6 or under treatment with HDACIs) prevents its interaction with target protein and thus negatively influences its chaperon functions. The proteins (including some oncoproteins) dependent on HSP90 function are then improperly folded and directed for degradation.

(g) Acetylation of STAT3 induces its dimerization and nuclear translocation.

(h) HDAC6 is required for proper recruitment of ubiquitylated proteins to aggresomes (organelles responsible for efficient clearance of misfolded or toxic proteins), inhibition of HDAC6 leads to accumulation of polyubiquitylated proteins and subsequent cell death.

(i) DNA-damage associated protein Ku70 binds in its deacetylated form pro-apoptotic protein Bax, prevents its localization to mitochondria and thus protects cells from apoptosis.

(j) High mobility group HMGB1 protein, normally bound in chromatin, is secreted by special types of cells to induce inflammation. Acetylation causes nuclear export and cytosolic accumulation of HMGB1 before its secretion.

(adopted from Minucci 2006[53])

domain or is localized in close proximity of this domain. Positive charge of lysine in DNA binding domain can mediate interaction of transcription factor with negatively charged DNA; the acetylation of such lysine can disrupt this interaction.

Acetylation and protein localization

Protein–protein interactions can be dependent on acetylation status of one of interacting proteins and as such can lead to distinct cellular localization (i.e. above mentioned STAT3 dimerization[18, 46], Ku70 and Bax interaction[48], p53 and MDM2 interaction[38]). Moreover, the acetylation of importins and other proteins responsible for nuclear transport was reported. It suggests that acetylation can lead to changes in their localization and play a role in general export and import into the nucleus[50, 51].

2.1.4. The role of HATs/HDACs in cancer development

There is a strict balance how HATs and HDACs action is maintained. The shift in this balance may have dramatic consequences on the cell phenotype, like cancer onset. In addition to influencing the expression of tumorigenesis-related genes by deacetylating histones in their promoters, HDACs/HATs modify also many non-histone proteins and thus affect their properties (described in 2.1.3.). These substrates are directly or indirectly involved in various biological processes, such as gene expression, DNA repair and regulation of proliferation, differentiation and cell death pathways, therefore also imbalance in their modifications could contribute to cancer development.

Expression of HATs/HDACs in tumors

Several lines of evidence showed that HATs are important for normal cell proliferation, growth and differentiation and that loss or misregulation of HATs' functions may lead to cancer (for a review, see[52]). Function of p300 and CBP HATs is impaired by interaction with viral oncoproteins (adenoviral E1A, human papilloma virus E6 and simian virus 40, SV40, T large antigen). Rubinstein-Taybi syndrome is associated with monoallelic mutation of either p300 or CBP and patients exhibit developmental defects and are prone to cancer (solid tumors, leukemia and lymphoma). Although the chromosomal translocations associated with certain leukemias indicate that a few gain-of-function mutations in HAT are also oncogenic, there is prevailing opinion that HATs act as tumor suppressors and that overall loss of acetylation is

connected with tumor development. In concordance with this presumption, mutations disrupting function of HDACs have not been reported[53], but overexpression of HDACs is observed in many cancer types and is often associated with p21 repression (HDAC1 - prostate and gastric cancer, HDAC2 - gastric and colorectal carcinomas, cervical dysplasias, endometrial stromal sarcomas, HDAC3 - colon carcinoma; for a review, see[54]). Also aberrant targeting of HDACs is well described in leukemias (see below). Most of convincing evidence that HDACs behave differently in cancer cells and normal cells was brought from pharmacological manipulations of HDACs activity through the use of HDAC inhibitors.

HDACs in differentiation

The importance of balance of HDACs action is well illustrated on pathogenesis of leukemias – APL and AML. In these leukemias the fusion proteins, PML-RAR and AML1-ETO, respectively, are responsible for aberrant recruitment of HDACs that results in repression of the transcription of genes essential for myeloid differentiation (for a detailed mechanism, see APL section 2.4.8.). According to multiple-hit model, more than one mutation are required for cancer development, considerable effort was made to find second hit – mutation in some key regulator of cell growth. Although mutation in p53 gene is extremely rare in APL patients[55], intriguingly, it was found recently that p53 function in APL is compromised by other mechanism. In APL, PML-RAR fusion protein associates with class I HDACs and also retains ability to bind p53. HDACs cause p53 deacetylation that enables binding of MDM2 protein acting as ubiquitin ligase and directing p53 to proteasome degradation. This is the first evidence that alternations in p53 acetylation are connected with tumorigenesis, which strengthen the importance to study posttranslational modifications of non-histone proteins in cancer[56, 57].

Other examples of HDACs' influence on cell differentiation are transcription factors of GATA family, which are important for differentiation of hematopoietic and epithelial cells, and mucin Muc2 that plays a role in gastrointestinal cell differentiation. In some, but not all, types of cancers associated with transcriptional repression of GATA[58] and Muc2[59] genes, HDACIs increase histone acetylation in their promoters and restore their expression.

HDACs in prolifferation

The impediment of inappropriate cell growth is critical to prevent cancer. Abnormal action of HDACs often targets proliferation-restraining genes. One of the best studied targets of HDACs, which is transcriptionally silenced (repressed) in many solid tumors, is cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ (p21), a tumor suppressor blocking entry into S-phase of cell cycle. Treatment of many cancer cell lines with any of several HDACIs causes hyperacetylation of H3 and H4 histones in p21 promoter and the transcriptional upregulation of this antiproliferative gene independently on p53[60, 61]. Multiple HDACs repress expression of p21 in different cell types. HDAC1 was revealed to be the most important for normal development because its targeted disruption leads to embryonic lethality at early stages of development, mainly owing to arrest of cell growth that was associated with upregulation of cyclin-dependent kinase inhibitors p21 and $p27^{Kip}$ (p27). Although the expression of HDAC2 and HDAC3 is increased in HDAC1-/- embryos, it is not sufficient to compensate the loss of HDAC1. It indicates that HDAC1 has a crucial, non-redundant role in regulating cell proliferation[62].

Next, transforming growth factor β (TGFβ) signaling pathway is impaired by HDACs. In contrast to normal cells, where TGFβ inhibits cell growth, many cancer cells are resistant to TGFβ inhibitory effect, owing to loss of TGFβ receptors (TGFβR). HDACs repress expression of both receptors: TGFβRII (that binds TGFβ)[63] and TGFβRI (which transduces the signal by activation of Smad family members)[64] but their expression was restored after HDACIs treatment.

HDACs and cancer progression

In addition to regulation of genes involved in the genesis of cancer, histone acetylation and deacetylation modulate also genes involved in cancer progression. This includes the regulation of angiogenesis that supplies tumor with oxygen and nutrients and thus permits increased tumor growth as well as regulation of adhesion, cell migration and invasion required for metastasis.

Hypoxia-inducible factor 1 (HIF-1) plays a central role in cellular adaptation to changes in oxygen availability. HIF-1 stimulates transcription of erythropoietin and vascular endothelial growth factor (VEGF) genes, which are responsible for erythropoiesis and angiogenesis. Under normal conditions, α subunit of HIF-1 (HIF-1α) associates with von Hippel-Lindau (VHL), which mediates ubiquitylation of HIF-1α and its quick

degradation in proteasome. Acetylation of HIF-1α stabilizes its interaction with VHL, therefore enhances its degradation and in consequence blocks angiogenesis[47]. Hypoxia induces HDAC expression and activity. Overexpression of HDAC1 represses the tumor suppressors p53 and VHL but induces the hypoxia-responsive genes: HIF-1α and VEGF and increases angiogenesis[65, 66]. HDACIs prevent new vessel formation and tumor growth[67-69].

Cancer cells that lose the ability to interact properly with neighboring cells can migrate into other tissues and set metastasis. Class I HDACs were shown to regulate expression of E-cadherin that is important for cell-cell adhesion. HDAC1 and HDAC2 are recruited by repressor Snail to E-cadherin promoter and repress its expression. Trichostatin A, a HDAC inhibitor, abolished Snail-mediated repression[70]. Moreover, HDAC6 is responsible for deacetylation of tubulin, which promotes independent cell movement as occurs in metastasis. Tubacin, a specific HDAC6 inhibitor, decrease cell motility and thus could be potentially used as anti-metastasic drug[71].

2.1.5. HDAC inhibitors

Several HDACIs from natural sources, like sodium butyrate or trichostatin A (TSA), were tested as chemical compounds with antiproliferative effects far before the discovery of HDACs and their biochemical functions. In fact, these inhibitors were used as tools for purification and cloning of mammalian HDACs. In addition to natural HDACIs, many synthetic ones were prepared. Based on their chemical structure, HDACIs are divided into several groups: short chain fatty acids (butyrate derivates and valproic acid), hydroxamic acids/hydroxamates (SAHA, TSA), cyclic tetrapeptides (trapoxin, apicidin, depsipeptide), and benzamides (MS-275).

HDACs inhibitors have been shown to cause differentiation, growth arrest and/or apoptosis of cancer cells both *in vitro* and *in vivo*[72-74]. Some of them efficient at nanomolar concentrations are well-tolerated by normal cells and are currently intensively tested as anti-tumor drugs in clinical trials[33, 75](**Table 2, Box 1**). For a wide range of cell lines derived from solid tumors (bladder, breast, ovaria, prostate, colon, lung, brain) and from lymphomas, leukemias and multiple myelomas, the growth inhibitory activity of HDACIs was reported. Treatment of normal and tumor cells with HDACIs causes an accumulation of acetylated histones H4, H3, H2A and H2B. In clinical trials, the accumulation of acetylated histones in cells, such as peripheral

Drug	Structural class	HDAC target	Stage of developnet	Company
Zolinza (vorinostat, SAHA)	hydroxamate	Class I and II	FDA approval for cutaneous T-cell lymphoma	Merck
Romidepsin (depsipeptide, FK-228)	bicyclic peptide	Class I	phase II for cutaneous and peripheral T-cell lymphoma	Gloucester Pharmaceuticals
MS-275	benzamide	Class I (HDAC1)	phase II for refractory solid tumors, leukemias and lymphomas	Schering AG
Valproic acid	short chain fatty acid	Class I and II	phase I/II for leukemias, myelodysplasias and cervical cancer	Abbott
LAQ-824	hydroxamate derivate	Class I and II	phase I for refractory solid tumors, leukemias and lymphomas	Novartis
PXD-101	hydroxamate derivate	Class I and II	phase II for advanced solid tumors	CuraGen, TopoTarget

Table 2: HDAC inhibitors in clinical trials
(adopted from Kelly 2002[33], Minucci 2006[53], Garber 2007[83])

mononuclear cells, is useful marker of biological HDACIs activity[75]. Histone hyperacetylation is believed to cause chromatin remodeling and reactivation of tumor suppressor genes inactivated in cancer cells. An exemplary case is p21 gene, which transcription is silenced in variety of cancers by epigenetic changes. Inhibition of deacetylase activity by SAHA, a HDACs inhibitor, led to hyperacetylation in p21 loci, reactivation of this gene and to growth arrest of transformed cells[61]. Strikingly, normal cells are almost always considerably more resistant than tumor cells to HDACIs[76]. This resistance could be explained, at least partially, by the fact, that in leukemias[77, 78] and also various solid tumors[79-82] but in normal cells, HDACIs induce the expression of members of the TRAIL and FAS death receptor pathways. This induction is responsible for the tumor-specific pro-apoptotic effects of HDACIs.

Today, there is no doubt that HDACIs are powerful anticancer drugs in cell cultures, however, in clinical tests they appeared to have little effect due to low stability and fast degradation in human bloodstream. Therefore, considerable effort is made to prepare synthetic inhibitors with better properties and more selective effect. With few exceptions, HDAC inhibitors are nonselective or poorly selective drugs affecting all or most of class I and class II enzymes[76]. Only selective HDACIs that has been reported up today are: MS-275, which preferentially inhibits HDAC1 (IC_{50} at 0.3 μM) and at much higher concentration also HDAC3 (IC_{50} at 8 μM) and has little or no inhibitory effect

against HDAC6 and HDAC8[84]. SK7041, SK7068 and depsipeptide preferentially target HDAC1 and HDAC2[85, 86]. Tubacin selectively inhibits HDAC6 activity, causes accumulation of acetylated α-tubulin, and does not influence histone acetylation[71]. As individual HDACs seem to have specific functions like HDAC1 in cell growth and HDAC2 in apoptosis [87], finding of selective HDAC inhibitor would be of great benefit for more targeted treatment of patients and for better understanding of acetylation/deacetylation events in leukemogenesis and cancer.

Box 1: Clinical trials

The clinical testing of the experimental drugs is mostly designated into three phases defined by the Food and Drug Administration (FDA).

Phase I studies are primarily concerned with assessing the drug's safety. This initial phase of testing in humans is done in a small number of healthy volunteers (20-100). The safe dosage range and side effects are determined.
Phase II is concentrated on drug effectiveness in safe dosage range. Health conditions of several hundred patients receiving an experimental drug or placebo are monitored.
Phase III is large-scale testing (includes several hundred to several thousand patients). The drug effectiveness is studied more thoroughly, is compared to commonly used treatments and range of possible adverse reactions determined. Once a phase III study is successfully completed, a pharmaceutical company can request FDA approval for marketing the drug.
www.fda.gov

2.2. INTERFERON SIGNALING PATHWAYS

One of the vitally important tasks of the cell is to defend to bacterial and viral infection and to signal the presence of such infection to surrounding cells. To cope with this demand, a complex network of signaling pathways was evolved (for reviews, see[88, 89]). Innate immune response starts by recognition of specific structures of invading pathogens by host pattern recognition receptors (PRRs) located at the plasma membrane or inside the cell. Pathogen recognition leads to activation of Toll-like receptor (TLR)-dependent or -independent (e.g. through cytosolic receptors: retinoic acid-inducible gene I, RIG-I, and melanoma differentiation-associated gene 5, MDA5) signaling pathways and subsequent activation of transcription factors (NFκB, IRF3, IRF7, and AP-1). These transcription factors can directly activate genes of antiviral response or can do it indirectly through prior transcriptional induction of a large number of cytokines (**Figure 4**), including a key players of innate immune response – type I interferons (especially IFNβ). Expressed IFN proteins are secreted into the intercellular space through autocrine and paracrine mechanisms, which evoke antiviral state in infected and surrounding cells, and thus hinder spreading of the infection. IFNs operate mainly through so called Jak-STAT signaling pathway that transmits signal from the receptor on plasmatic membrane into nucleus, where interferon responsible genes are activated[90]. After IFN binding to specific receptor on plasmatic membrane, receptor-associated Janus kinase (Jak) family members are activated and phosphorylate latent cytoplasmic signal transducer and activator of transcription (STAT) transcription factors, which in turn form homo-, hetero-dimers or tripartite complex with IRF9, translocate to nucleus, bind to specific DNA sequence and induce transcription of number of interferon stimulated genes (for a review, see[91]). This part represents fast primary response to IFNs (early response). Among genes activated in early response are also interferon regulatory factors (IRFs). IRFs bind to promoters of IFN genes (and other ISGs), induce their expression and thereby start late response, when the effect of IFN is sustained and amplified by this positive loop[88]. On the other hand, some IRFs (IRF2, IRF4) act as inhibitors and can suppress transcription of ISGs in negative loop (reviewed in [92]).

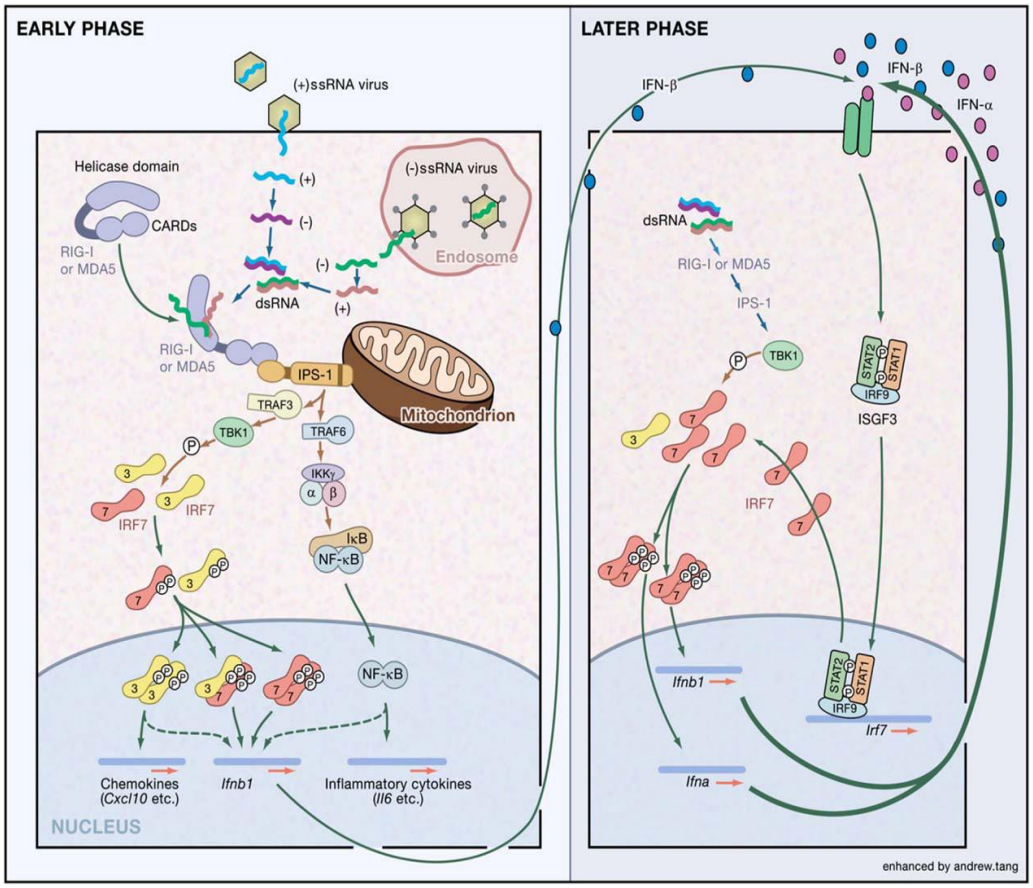

Figure 4: Cytokine activation in innate antiviral response and positive feedback mechanism

The presence of dsRNA in the cytosol triggers host responses via a specific cytosolic pattern-recognition system. The interaction of dsRNA - a replication intermediate of positive (+) and negative (-) RNA viruses - with the helicase domain of RIG-I and MDA5 induces the unwinding of dsRNA and, at the same time, induces the conformational changes of RIG-I and MDA5. The conformational changes promote the interaction between the RIG-I and adaptor protein IPS-1, which is located on the mitochondrial membrane, resulting in the activation of TBK1 as well as IKK. Activated TBK1 induces the phosphorylation of IRF3 and IRF7, resulting in their homo- or heterodimerization. These dimers then translocate to the nucleus and induce small amounts of IFNβ as well as other inflammatory cytokines and chemokines. Secreted IFNβ then stimulates type I IFN receptor in an autocrine and a paracrine fashion, leading to activation of ISGF3 (heterotrimer of STAT1, STAT2, and IRF9) and the transcription of IRF7 gene and other ISGs that participate on the onset of antiviral state. Activation of the newly synthesized IRF7 results in further amplification of transcription for IFNβ and IFNα genes, and thereby a positive-feedback loop is established.
(adopted from Honda 2006[88])

2.2.1. Interferons

Interferons belong to the family of cytokines that regulate cellular antiviral, antiproliferative and immunological responses (for a review, see[93]). There are two classes of IFNs: type I and type II. **Type I IFNs** (IFNα, IFNβ, IFNε, IFNκ, and IFNω) are a group of structurally related proteins produced by most types of virally infected cells. IFNα and IFNβ are most well studied type I IFNs. In humans, 13 genes encoding subtypes of IFNα have been found, whereas only one gene of IFNβ exists. Although IFNα and β share the same signaling pathway, IFNα subtypes do not compensate for the loss of IFNβ[94]. This suggests that IFNβ has a unique role and is essential for a fully effective antiviral response. **Type II IFN** class is represented by only one protein - IFNγ. For its antiviral activities, IFNγ was classified as interferon, although it has no structural homology to type I IFNs (for a review, see[95]). Whereas IFNα and IFNβ are synthesized and secreted by leukocytes and fibroblasts, respectively, in response to viral infection or dsRNA treatment, IFNγ is expressed only in specialized immune cells (T-lymphocytes and natural killer cells) upon induction by antigens and mitogens.

Antiviral action of IFNs is mediated by expression of their target genes (ISGs) that are responsible for production of a broadly effective cellular antiviral state refractory to virus replication (for a review, see[96]). At least 300 ISGs which mediate various biological responses, were found induced by IFN type I and type II using oligonucleotide microarray assay[97]. Some of these genes are regulated by both types of IFNs, whereas others are selectively regulated by distinct IFNs. Between main ISGs implicated in antiviral action of IFNs are: RNA-dependent protein kinase (PKR, which catalyzes phosphorylation of translation initiation factor eIF-2α leading to inhibition of translation), 2',5'-oligoadenylate synthetase (OAS, synthesizes oligoadenylates that are necessary for activation of RNase L), RNase L (has endoribonuclease activity and cleaves both viral and cellular RNA, including cellular rRNA), RNA-specific adenosine deaminase (ADAR, changes functional activity of viral RNA by deamination of its adenines to yield inosine), and Mx protein GTPases (block either transport of viral nucleocapsid into nucleus or viral RNA synthesis)[96]. Notably, tumor suppressors like p53[98] or PML[99] were also found between genes induced by IFNs.

Originally, IFNs were believed to be selective antiviral agents with no effects on uninfected cells and therefore they were considered as promising therapeutic agents. Later their pleiotropic effects on uninfected cells were discovered and adverse effects

limiting their dosage were reported in clinical studies (for a review, see[100]). Today IFNs are used in treatment of several cancers (**Box 2**). Although the exact mechanism of IFNs action remains unknown, their therapeutic effects are attributed to general ability to slow down cellular growth, an implicated role in induction of apoptosis and senescence, modulation of differentiation, and antiangiogenic activity (for review, see[101]; the role of IFNβ in senescence is discussed in section 2.3.5. and in Research paper III).

BOX 2: Clinical uses of interferons[91]
IFNα - *heamatological malignancies:* chronic myeloid leukemia, cutaneous T-cell lymphoma, hairy-cell leukemia, multiple myeloma; *solid tumors:* malignant melanoma, renal-cell carcinoma, AIDS-related Kaposi's sarcoma; *viral syndromes:* hepatitis C, hepatitis B, severe acute respiratory syndrome
IFNβ - multiple sclerosis
IFNγ - chronic granulomatous disease, severe malignant osteopetrosis

2.2.2. Type I IFN signaling pathway

All type I IFNs bind to the same receptor located on the cell surface. The receptor consists from two subunits: IFNAR1 associated with tyrosine kinase 2 (Tyk2) and IFNAR2 associated with Janus activated kinase 1 (Jak1). Ligand binding causes conformational rearrangement and dimerization of the receptor subunits, followed by autophosphorylation and activation of associated kinases. Janus kinases phosphorylate latent cytoplasmic STAT proteins on tyrosine residues. Activated STAT proteins than form dimers through intermolecular reciprocal interactions of their Src homology 2 (SH2)–domains with phosphotyrosines. In response to type I IFNs, two complexes are formed: IFNα-activated factor (AAF) and interferon stimulated gene factor 3 (ISGF3). AAF, a homodimer of activated STAT1 (phosphorylated on tyrosine Y701), is identical with GAF factor stimulated by IFNγ and binds to the same responsive elements. ISGF3 is a trimeric complex consisting of STAT1, STAT2 and IRF9 (ISGFγ, p48). After translocation into nucleus, ISGF3 binds to IFN-stimulated response element (ISRE) in promoters of target genes (**Figure 5**).

2.2.3. Type II IFN signaling pathway

IFNγ binding to receptor subunits IFNGR1 and IFNGR2 induces activation of associated kinases Jak1 and Jak2, respectively, and they in turn activate cytoplasmic STAT1 protein. Y701 phosporylated STAT1 proteins form homodimers called IFNγ-activated factor (GAF) and rapidly translocate into cell nucleus, where they recognize specific consensus DNA sequence (IFNγ-activated site, GAS) and initiate transcription activation (reviewed in[102]) (**Figure 5**). Although IFNγ does not activate STAT2 and thus does not cause ISGF3 complex formation, it could induce transcription of some

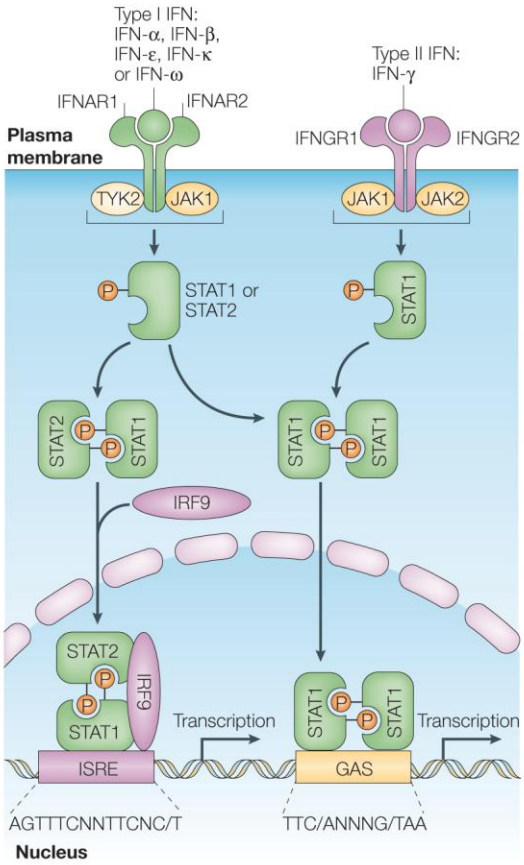

Figure 5: Interferon activated classical JAK-STAT signaling pathways
All type I interferons (IFNs) induce the formation of STAT1–STAT2–IRF9 complexes, known as ISGF3 complexes. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA to initiate gene transcription. Both type I and type II IFNs also induce the formation of STAT1–STAT1 homodimers, called AAF in case of type I stimulation and GAF in type II stimulation. These homodimers translocate to the nucleus and bind GAS (IFNγ-activated site) elements that are present in the promoters of certain ISGs, thereby initiating the transcription of these genes. The consensus sequences of ISRE and GAS element are shown. N, any nucleotide.
(adopted from Platanias 2005[91])

genes with ISRE containing promoters indirectly through IRF1. IFNγ is potent inducer of IRF1, which can bind to ISRE elements of several ISGs and activates their transcription[103].

2.2.4. Other IFN-induced signaling pathways

In addition to classical Jak-STAT pathway mediated by ISGF3 and AAF/GAF complexes, other IFN-induced signaling cascades are necessary for generation of all divergent biological activites of IFNs (reviewed in[91]). IFN-induced cascades engaging STAT1, STAT2, STAT3, STAT4, STAT5, and STAT6 homo- or hetero-dimers that bind to GAS element of ISGs were described (for a review, see[104]). Moreover, cooperation with mitogen-activated protein kinase (MAPK)[105, 106], phosphatidylinositol 3-kinase (PI3K)[107], or RNA-dependent protein kinase (PKR)[108] pathways was reported.

2.2.5. Transcriptional activation of ISGs

It was described first for the type II IFN[109, 110] and later also for type I IFNs[111] that STAT1 needs to be phosphorylated, in addition to Y701, also at serine S727, which is located in PMSP motif of C-terminal transactivation domain. Only the presence of both modifications provides full transcriptional activation of target genes[111]. With exception of STAT2 and STAT6, serine phosphorylation of remaining STATs was described and occurs in response to different stimuli (cellular stress – UV irradiation, inflammatory signals) through distinct signal transduction pathways[112] (reviewed in[113]). Posttranslational modifications of STATs can be crucial for recruitment of transcriptional cofactors. Although the exact mechanism of transcriptional activation of ISGs is still under investigation, several coactivators have been found to associate with STATs during IFN stimulation. It was shown that both STAT1 and STAT2 interact with HATs enzymes (CBP/p300, GCN5) and recruit them to ISG promoters[114-116]. Unexpectedly, HDACs were found to act as coactivators on ISGs promoters (detailed information about the role of acetylation in IFN pathway is given in section 2.2.8)[117-119]. In addition, STAT2 recruits Brahma-related gene 1 (Brg1), the ATP-binding subunit of switching defective/sucrose nonfermenting (SWI/SNF) chromatin remodeling complex, to some but not all ISG promoters[120]. Brg1 destabilizes nucleosomes, makes DNA accessible for transcription factors and cofactors and thus its recruitment leads to

enhanced expression of ISGs. For mediating interaction of distal transcription activator and the RNA polymerase II complex that is positioned at the transcription initiation site, eukaryotes use multimeric coactivator complex called generally mediator (for a review, see[121]). It was demonstrated that STAT2 associates with DRIP150, a subunit of mediator, and that this interaction is essential for ISGF3-mediated transcription[122].

2.2.6. Interferon regulatory factors

Interferon regulatory factors are growing family of transcription factors that have been implicated in antiviral defense, cell growth, and immune regulation (for a review, see[123]). Nine members of IRF family were identified in mammals so far: IRF1, IRF2, IRF3, IRF4 (Pip, ISCAT), IRF5, IRF6, IRF7, IRF8 (ICSBP), IRF9 (p48, ISGFγ)[92, 124]. They are characterized by a well-conserved N-terminal DNA binding domain that recognizes DNA consensus sequence 5'-AANNGAAA-3'. With exception of IRF1 and IRF2, they have an IRF association domain (IAD) on C-terminus mediating homo- or hetero-dimeric interactions between IRF family members or with other transcription factors. Among IRFs, IRF1, IRF3, IRF5, and IRF7, have been implicated as positive regulators of type I IFN gene transcription. Although IRF1 overexpression induces the expression of type I IFN genes, gene-targeting studies revealed that IRF1[125] and IRF5[126] are dispensable for type I IFN induction. IRF2 has been generally described as a transcriptional repressor competing for binding site with the transcriptional activator IRF1[127]. However, IRF2 also can act as a positive regulator for ISRE-like sequences of some genes (e.g. histone H4[128]). IRF3[129, 130] and IRF7[131, 132] have been identified as direct transducers of virus-mediated signaling and they are key regulators of type I IFN gene expression. Both IRF3 and IRF7 reside in cytoplasm in latent form. After viral infection, they are activated by phospohorylation, dimerize in homodimers or IRF3-IRF7 heterodimers, translocate to nucleus and bind to target promoters inducing their transcription. IRF3 is more potent activator of IFNβ gene than IFNα genes; IRF7 activates both efficiently (reviewed in[88]). In contrast to IRF3, which is constitutively expressed, expression of IRF7 is very low under normal conditions but dramatically increases in response to type I IFN signaling. Boosted IRF7 expression is responsible for later phase of virus-induced response, prolonged or delayed expression of ISGs and for the onset and maintenance of potent antiviral state. Very recent study on IRF7- and IRF3-deficient mice has shown that homodimer of IRF7 or heterodimer IRF7-IRF3,

rather than IRF3 homodimer, are critical for induction of type I IFN expression[133]. Therefore IRF7 is critical also for the early response to viral infection.

Besides playing a role in innate immune response, IRFs are also implicated in the response to stress caused by DNA damage. For instance, in response to genotoxic stress, IRF1 is stabilized and induces transcription of p21 gene[134]. IRF3 and IRF7 are phosphorylated by the sensor of DNA double stranded breaks - DNA-dependent protein kinase (DNA-PK) and translocate to the nucleus[135, 136].

2.2.7. Regulation of type I IFN genes transcription

Expression of the type I IFN genes is efficiently induced by viruses at transcriptional level. Transcriptional switch of IFNβ gene have been extensively studied and is well characterized (for a review, see[137]). IFNβ promoter contains four regulatory elements designated positive regulatory domains (PRDI - PRDIV)[138]. IRFs family members bind to PRD I and PRD III, whereas PRDII and PRD IV are recognized by NFκB and AP-1 (a heterodimer of activating transcription factor 2, ATF2, and c-Jun), respectively. Promoters of IFNα genes contain PRDI- and PRDII-like elements (PRD-LE)[139](**Figure 6**). Individual transcription factors bind to promoter with low affinity and are not able to induce transcription efficiently. For switching on the IFNβ gene, a

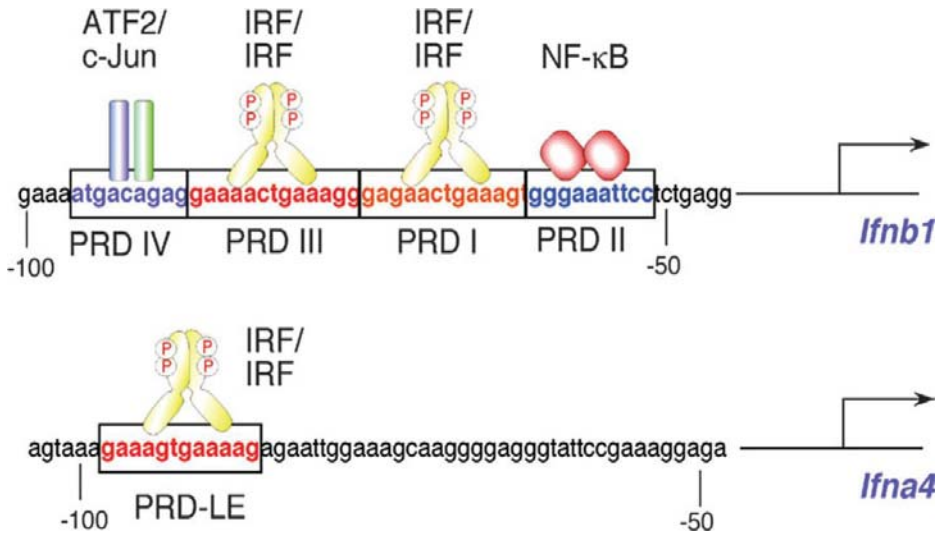

Figure 6: Structure of IFNβ (Ifnb1) and IFNα4 (Ifna4) promoter
(from Honda 2005[137])

formation of a high-order nucleoprotein complex called enhanceosome is essential[140]. In enhaceosome, transcription factors (NF-κB; AP-1; and IRF3, IRF7 or IRF1) and an architectural protein HMG I(Y) (high mobility group) are held together and bind cooperatively to the nucleosome-free enhancer DNA. DNA- and protein-interactions of HMG I(Y) cause bending of DNA that facilitates binding of transcription factors. Simultaneously bound factors activate transcription in synergistic manner[141]. Enhanceosome represents also a platform for recruitment of transcriptional coactivators such as GCN5/PCAF or BRG1 that participate in chromatin remodeling of nucleosome, which masks TATA box and start site of transcription[5, 138]. A set of negative regulatory factors is responsible for postinduction shutting off of the IFNβ promoter. Among these factors belong PRD-BF (PRD-binding factor) that binds to PRDI and PRDII elements, and IRF2 binding to PRDI[142].

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2.2.8. Role of acetylation in innate immune response pathways

As was depicted in section 2.1., the acetylation is an important posttranslational modification modulating function/activity of various proteins and has an impact on many cellular processes. Also in innate immune response pathway, precise balance of and rapid switch between HATs and HDACs is required. Several members of IFN and other cytokine signaling pathways (e.g. IRF1[143], IRF2[143, 144], IRF3[145], IRF7[49], STAT1[146], STAT3[18, 46], STAT6[20]) were found to be associated with HATs/HDACs and/or to be modified transiently by acetylation. There is accumulating evidence suggesting that activation of interferon-responsible genes demands not only acetylation but also deacetylation step. This hypothesis rises from several observations that HDACIs suppress induced expression of IFNβ gene and several ISGs.

Role of acetylation in IFN pathway

In 2003, Genin et al.[147] found that TSA was able to suppress virus-induced expression of IRF-7 gene and IFNα-induced expression of ISG54, ISG56, ISG15 and IFI6-16 genes in murine L929 cells. At the same time, Nusinzon and Horvath[118] reported a suppressive effect of TSA on IFNα- and IFNγ-induction of several interferon-stimulated genes (ISGs) in various human cell lines, and Sakamoto et al.[119] demonstrated the suppressive effect of TSA on IFNβ-induction of a similar set of ISGs in human foreskin fibroblasts. In addition, we have reported negative effect of TSA and

other HDACIs on IFNα-induced expression of PML at protein and mRNA level in several human cell lines, and TSA-mediated suppression of IFNα-induced Sp100 and IRF1 protein levels (Paper I). Microarray analysis confirmed general requirement of deacetylase activity in IFNα-induced ISGs expression[117]. The expression of all tested ISGs in response to IFNα treatment was suppressed by TSA, but basal levels of ISGs were unaffected[117]. Since the block of de novo protein synthesis by cycloheximide did not influence suppressing effect of TSA, it seems that HDACIs directly interfere with IFN signaling and their effect is not caused by lack of some regulatory protein[117, 118]. HDACIs suppressed also the expression of majority of IFNγ-inducible ISGs, although exceptions were described (e.g. IFNγ-induced transcription of major histocompatibility complex II genes was enhanced by cotreatment with TSA[148, 149]). Moreover, IL3- and IL2-induced activation of target genes mediated by STAT5 also need deacetylation step[150, 151]. Microarray analysis further showed that deacetylase activity is required for activation of all STAT5 target genes[152]. These results raise a hypothesis that HDAC activity is essential for general cytokine-induced STAT-dependent transcriptional activation.

To uncover the mechanism of HDACI interference with IFN stimulation of target genes, Jak-STAT signaling pathway was scrutinized, but obtained data are highly controversial. In mouse cells, Genin et at.[147] observed TSA caused blockage of virus-induced nuclear accumulation of STAT2 and impairment in ISGF3 promoter binding. Klampfer et al.[153] described that butyrate is a strong inhibitor of signaling by IFNγ in human colorectal carcinoma cells and impairs IFNγ-induced phosphorylation of Jak2 and STAT1, nuclear translocation of STAT1 and its DNA binding activity. Recently, Guo et al.[146] described that TSA and valproic acid block IFNγ-induced expression of inducible nitrate oxide synthetase (iNOS) in RAW 264.7 macrophages. The authors correlate this effect with increased acetylation of STAT1 that was accompanied by decreased STAT1 binding to GAS-like element in iNOS promoter, despite of fact that STAT1 was activated by tyrosine phosphorylation. However, they did not comment that STAT1 was acetylated also in only IFNγ treated cells. On the contrary to above mentioned data (discrepancy could be caused by prolonged HDACI treatments used in the above studies), but in concordance with our results (Research Paper I), Nusinzon et al.[118] and Chang et al.[117] reported no impairment in Jak-STAT-ISGF3 signaling pathway induced by IFNα in human HeLa and 2fTGH cells (even in presence of TSA, IFNα

induced phosphorylation of STAT1 and STAT2, their translocation to the nucleus, formation of ISGF3 complex together with IRF9, and binding to promoters of ISGs). They assumed that essential deacetylation event of histones or some regulatory factor occurs between ISGF3 binding and RNA polymerase II loading to promoter. Two opposing actions in histone modifications on ISG54 promoter were reported in response to IFNα: histone H3 was hyperacetylated by histone acetyltransferase GCN5 recruited to promoter by STAT2[116] and basal acetylation of histone H4 was locally reduced[118]. Importantly, this H4 deacetylation was prevented by TSA treatment implicating it as a necessary event for ISGs activation. In addition, transactivation domain of STAT2 can also interact with HDAC1 (but not with HDAC4 and HDAC5), inhibition of HDAC1 by siRNA decreases and, conversely, the expression of HDAC1 augments IFNα-induced transcription[118]. However, HDAC1 siRNA had only partial effect when compared to TSA that inhibits IFNα-stimulated transcription more efficiently. In concordance, we found that HDAC inhibitor MS-275, used in concentrations referred as selective for HDAC1[84], has only slight suppressive effect on IFNα-stimulated transcription (Research Paper I). This suggests that HDAC1 is not the only one of deacetylase enzymes responsible for full IFN responsiveness.

Nusinzon and Horvath[154] created a simplified model of the current view of HATs/HDACs role in IFNα/β stimulation of ISGs (**Figure 7**). Although the importance of deacetylation for IFN response was proved, specific HDAC(s) and their substrate(s) need to be identified and thus the mechanism underlying HDACIs suppression of ISGs stimulation remains to be elicited.

Role of acetylation in IFNβ gene expression

Deacetylation is important also for initial steps of innate antiviral response. It was reported that susceptibility to virus-induced cytopathic effects was increased in absence of deacetylase activity[117, 155]. Chang et al. demonstrated that HDAC function is required for IRF3-driven gene induction (ISG15 and ISG54) in response to virus infection[117]. Later, Nusinzon and Horvath showed that viral or dsRNA induced activation of IFNβ gene mediated by IRF3, but not by NFκB, needs deacetylase activity, although IRF3 nuclear translocation and DNA binding were not affected by HDAC inhibition[155]. This is consistent with the observations of Chang et al.[117] and Genin et al.[147], who observed unchanged IRF3 binding to DNA in presence of TSA and rather notwithstanding with the observation that IRF3 is acetylated by p300 and this

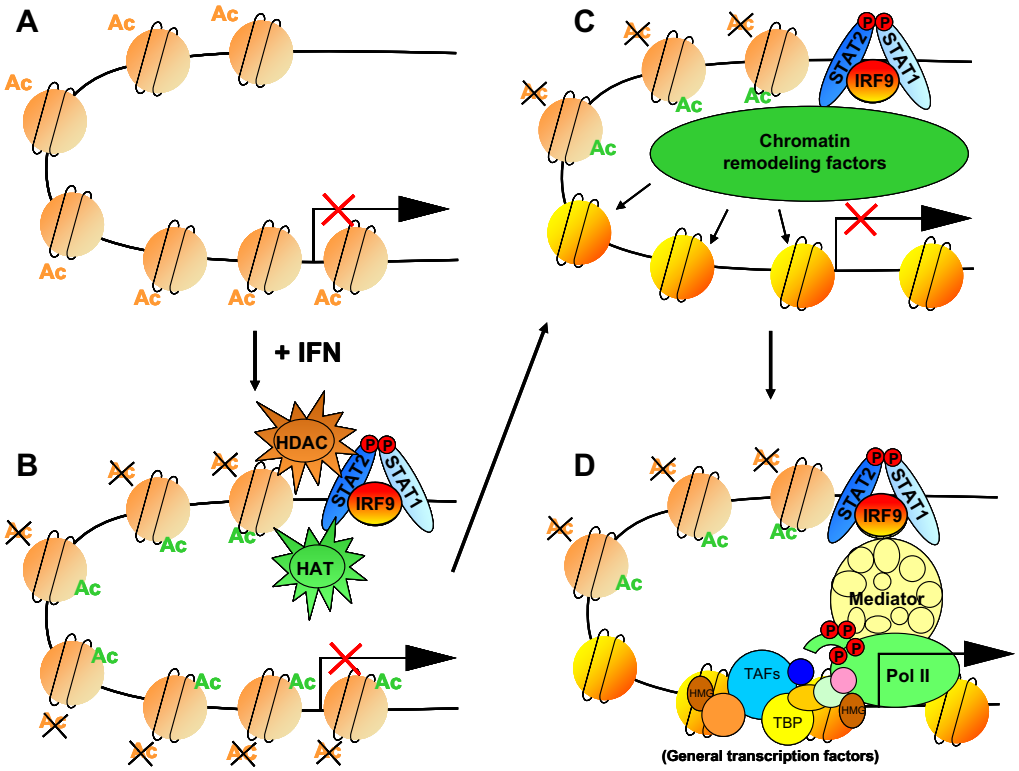

Figure 7: Schematic model for activation of ISG promoters by IFNα/β

(A) In the absence of IFN, histones and other substrates on ISG promoters are basally acetylated that maintain the promoter ready for activation.

(B) Upon IFN stimulation, ISGF3 binds to ISRE recognition element and recruits HATs (p300, CBP, GCN5) and HDACs (HDAC1 and probably others) to modify histones or regulatory proteins and thus changes acetylation profile on the promoter.

(C) Modified chromatin template allows recruitment of additional coactivators to the promoter. For instance, BRG1, the catalytic subunit of SWI/SNF remodeling complex, is recruited and aids to chromatin remodeling and nucleosome repositioning.

(D) The promoter is now available to engage the mediator, general transcription factors, and RNA polymerase II, which results in transcription initiation.
(modified from Nusinzon 2005[154])

acetylation is necessary for DNA-binding[145]. Despite of proclaimed unchanged IRF3 binding to IFNβ promoter, polymerase II did not bind to promoter in presence of TSA[155]. This suggests a role of HDAC enzymes as coactivators required for a regulatory step between IRF3 and RNA polymerase II loading onto IFNβ promoter (similarly to necessity of HDAC action between ISGF3 binding and polymerase II loading in IFN response). siRNA screening of various types of HDACs (HDAC1-10) showed that while HDAC1 and HDAC8 (both class I HDACs) function as repressors of IFNβ gene expression, HDAC6 (class II HDAC) functions as a coactivator of IRF3-dependent transcription of IFNβ gene[155]. HDAC6 is mostly cytoplasmic protein and its

cellular localization appears to be changed under specific signals (e.g. cell cycle arrest results in translocation of fraction of HDAC6 into nucleus[156]). Possibly viral infection could induce specific HDAC6 translocation to nucleus and targeting to promoters of virus-inducible genes. Notably, it was demonstrated previously that HDAC1 function as a critical transcriptional coactivator for ISGF3, a transcription factor downstream of IFNα/β[118]. HDAC1 thus behaves inversely in activation of IFNβ gene and IFNα/β-responsible genes. It supports data from mouse model, where HDAC1 acts as repressor for several genes and simultaneously activates transcription of others[157]. Together, it implies that HDACs have unique and in some cases opposing roles in regulation of individual genes. Similarly to IRF3, IRF7 activity is also dependent on acetylation. IRF7 is acetylated by PCAF and GCN5 in its DNA binding domain. This acetylation negatively modulates DNA-binding activity of IRF7[49], thus further underscoring the importance of deacetylase action in innate antiviral response.

Although the role of deacetylation still remains to be elucidated, some acetylation events in IFNβ gene activation were well documented[158]. During transcriptional activation of IFNβ gene only a small subset of all lysine (K) residues on histones H3 and H4 are acetylated. In response to viral infection, GCN5 first acetylates H3 at K9 and H4 at K8, acetylated H4K8 is recognized by bromodomain of Brg1 (a subunit of SWI/SNF) and chromatin remodeling occurs. Thereafter, but not before phosphorylation of serine 10 at H3, also H3K14 is acetylated and together with acetylated H3K9 they are recognized by double bromodomain of TAFII250 (a part of TFIID); this event timely correlates with TBP (TATA box binding protein) binding, accumulation of first IFNβ mRNA, and subsequent deacetylation of H4K8. Moreover, acetylation regulates also stability of HMG I(Y), a structural component of IFNβ enhanceosome. GCN5 acetylates HMG I(Y) at K71 and thus stabilizes enhanceosome. This acetylation prevents CBP mediated acetylation of K65 that would lead to destabilization of enhanceosome[159].

In summary, these data prove that both acetylation and deacetylation are necessary for innate immune response and that assembly of transcriptional machinery on promoters is highly coordinated process with precise order of histone tails' modifications and recruitment of individual regulatory factors, whose effects are dependent on promoter context.

2.3. CELLULAR SENESCENCE

The cell division is essential for development and survival of multicellular organism. However, the proliferation in renewable tissues necessary for maintenance of the organism's fitness, injury cure and organism longevity brings also a danger of the cancer development. The cells are continuously exposed to environmental insults and reactive products of oxidative metabolism causing errors in the cell genome. Accumulation of errors (mutations) giving a selective advantage to one cell may lead to a malignant phenotype characterized by uncontrolled proliferation (self-sufficiency in growth signals and resistance to growth inhibitory signals) connected with indefinite replicative lifespan (replicative immortality), apoptosis resistance, support of angiogenesis, invasiveness to other tissues and metastasis (reviewed in[160]). The cells evolved several mechanisms controlling genome stability (reviewed in[161]). Depending on level and type of damage they can temporally stop in cell cycle to repair the damage. In case that the damage is unrecoverable and the cell is at a risk of oncogenic transformation, it could be eliminated by a process of programmed cell death – apoptosis, or its growth could be irreversibly arrested by a process known as a cellular senescence. On one hand both apoptosis and senescence protect organism from cancer but on the other hand they have also deleterious effects on the organism. The massive loss of cells by apoptosis or accumulation of senescent cells that are not able to fill their original function lead to overall decline of tissue structure and function and contribute to organism aging (for a review, see[162]). In addition, even though cellular senescence serves as a tumorigenesis barrier by inhibiting the progression of pre-malignant to invasive lesions[163], paradoxically, it seems that senescent cells acquire traits (like secretion of inflammatory cytokines, growth factors and metalloproteinases) that might support tumor promotion, progression and invasiveness (for a review, see[162]).

2.3.1. Regulation of cell cycle, cell cycle checkpoints and DNA damage response

The main goal of cell division cycle is to replicate completely and thoroughly cell genome and equally distribute duplicates into the daughter cells. The molecular machinery controlling cell-cycle progression is based on sequential activation of cyclin-dependent kinases (Cdk) by association with activating proteins named cyclins (cyclin

D/Cdk4,6 – progression from G1 to S phase, cyclin E/Cdk2 and cyclin A/Cdk2 – trigger DNA replication and progression S phase, cyclin B/Cdk1 – enter to mitosis). Considering that uncontrolled proliferation is of high risk of cancerogenesis it is not surprissing that cell cycle progression is tightly regulated (reviewed in[164]).

Action of omnipresent DNA-damaging agens triggers complex genome surveillance machinery called **DNA damage response** that include activation of DNA damage repair mechanisms, delay in cell cycle progression or eventually senescence or apoptosis. DNA repair mechanisms include direct, base and nucleotide excision repair and double-strand break repair by homologous recombination or non-homologous end joining that are carried out by specialized enzymes or enzymes primarily involved in DNA replication (reviewed in[165]). At the same time as DNA damage is recognized and DNA repair takes place, DNA damage activates pathways including several protein kinases leading to inhibition of cyclin dependent kinases and to the delay or arrest of cell cycle progression for the time necessary to fully repair the damage[166]. The mechanisms causing this delay in cell cycle progression are called **DNA damage checkpoints**[167]. Usually, G1/S, intra-S, and G2/M checkpoints are recognized[165]. However, the integrity of the genome is constantly monitored during the cell cycle and the activity of checkpoints is enhanced when the extent of DNA damage rises above certain threshold level. The key players of DNA damage response are sensor proteins that are able to recognize the damage and convey the signal through activation of mediators and transducers to the effector molecules that control the transition to following cell cycle phase, DNA damage repair, apoptosis or senescence (**Figure 8**). ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) kinases belong to the most important sensor molecules. ATM is activated by the damage caused by γ-irradiation, i.e. by resulting dsDNA breaks[168], while ATR is activated by replicative stress or UV light causing mainly base damage[169]. When activated, both kinases phosphorylate signal transducer checkpoint kinases Chk2 (ATM) and Chk1 (ATR) and other proteins including p53, NBS1, BRCA1 (ATM)[165, 170]. Chk1 and Chk2 phosphorylate and thus inactivate Cdc25 phosphatases crucial for the activation of cyclin dependent kinases (Cdk2 and Cdk1) and transition into the next cell cycle stage[171]. Inactivation of Cdc25 represents very fast response to DNA damage and is connected with acute and transient cell cycle delay[167].

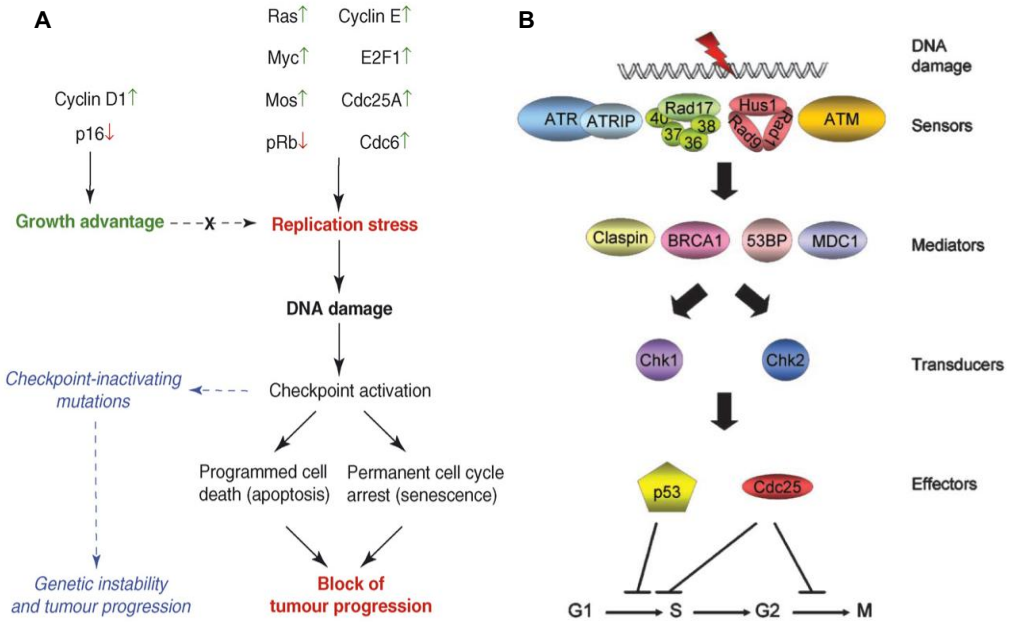

Figure 8: DNA damage checkpoints as a physiological barrier against tumor progression.
(A) Many activated oncogenes (green 'up' arrows) and the loss of some tumor suppressors (red 'down' arrows) deregulate cell cycle progression, causing replication stress and DNA damage that evokes checkpoint response in the early precursor lesions, before gross genetic instability occurs. Such activation of DNA damage response leads to senescence or cell death, thereby blocking tumor progression at its early stages. However, genetic or epigenetic defects that disable checkpoint function may allow escape of the incipient cancer cells from the blockade, and result in proliferation at the expense of increased genetic instability and cancer progression. (from Bartek 2007[172])
(B) Components of DNA damage checkpoints in human cells. (from Sancar 2004[165])

The main target of activated DNA damage checkpoint is p53. p53 is a transcription factor that masters apoptotic, senescent and repair programs in response to cellular stresses (for reviews, see[173, 174]). The amount of p53 protein present in unstressed cells is low, which is determined by high rate of its degradation. The stability of p53 is predominantly mediated by the interaction with MDM2 (in humans HDM2). MDM2 is E3 ubiquitin ligase targeting p53 for proteasome-dependent degradation. Additionally, the transcription of MDM2 is activated by p53 in feedback loop. Upon induction of DNA damage, oncogene activation or other stresses, p53-MDM2 binding weakens (e.g. ARF binds to MDM2 and blocks p53 degradation), p53 is stabilized, and accumulates in the nucleus. Depending on trigger, active p53 is phosphorylated and acetylated on multiple residues and these modifications and their combinations influence the effects of p53 on its target genes[175]. For instance, some posttranslational p53 modifications found in senescent cells overlap and some are distinct from those typically induced by DNA damage [175]. Activation of p53 leads either to p53-dependent apoptosis or to cell-cycle

arrest by induction of p21, which is an inhibitor of cyclin E/Cdk2 and probably also of cyclin D/Cdk4,6. The target of these kinases is pRB protein that is key regulator for S phase entry. Hypophosphorylated pRB binds to E2F and thus blocks E2F-dependent transcription of genes necessary for entry to S phase. Activated Cdks phosphorylate pRB in advanced G1 phase and promote progression through the cell cycle. This process is reverted by action of inhibitors of Cdks (**Figure 9**, reviewed in[176]). Moreover, p53 up-regulates also cell cycle inhibitors GADD45a (growth arrest and DNA-damage-inducible 45 alpha) and 14-3-3 sigma proteins that antagonize activation of cyclinB/Cdk1 and thus block enter to mitosis. The p53 pathway is considered to be responsible for delayed and sustained cell cycle arrest[167].

The outcome of the synchronized activation of both DNA repair and checkpoint mechanisms is the removal of the DNA damage before the cell can enter the next cell cycle phase. When the damage is unreparable or too extensive, effector molecules ensure that the affected cell is removed from the proliferative cellular pool by apoptosis or by entering the senescence.

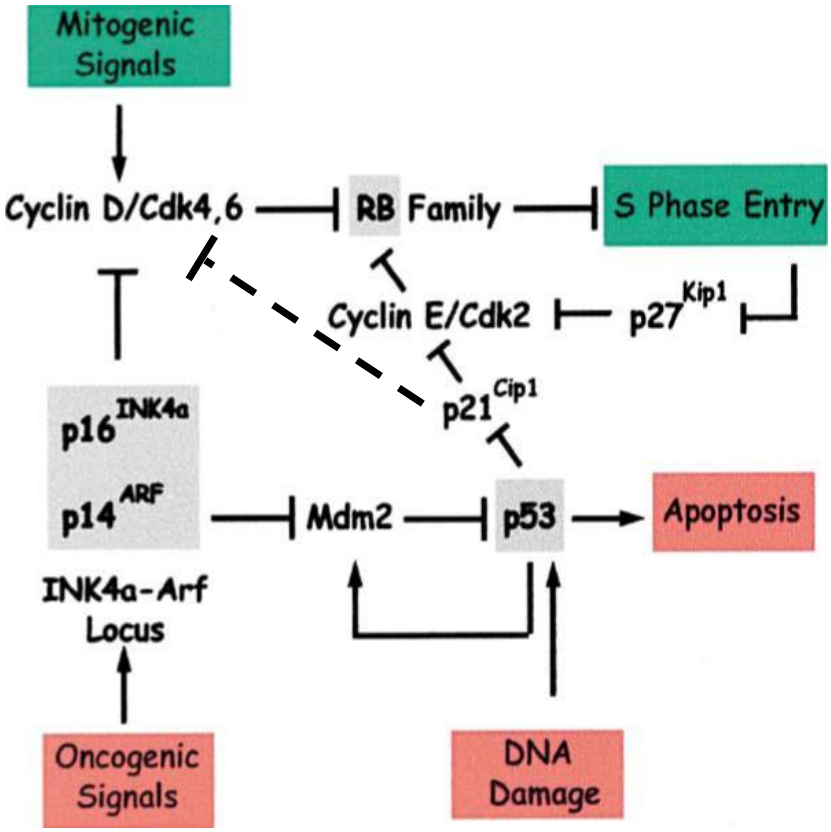

Figure 9: pRB and p53 regulate cell-cycle checkpoint controls
Mitogenic signals activate cyclin D-dependent kinases (Cdk4,Cdk6), which phosphorylate RB family proteins (pRB, p107 and p130) resulting in release of E2F, its binding to target promoters and progression from G1 to S phase. The Cdk2 inhibitor, p27, is expressed in high levels in quiescent cells. In excess of cyclin D/Cdk4,6 complex, p27 is sequestered from cyclin E/Cdk2. Free cyclin E/Cdk2 contributes to pRb phosphorylation and progression to S phase. Moreover, cyclin E/Cdk2 phosphorylates and directs for degradation p27 in late G1. Constitutive oncogenic signals can activate INK4a/ARF locus encoding two structurally unrelated proteins $p16^{INK4a}$ (p16) and ARF (alternative reading frame protein; designed in humans $p14^{ARF}$ and in mouse $p19^{ARF}$). p16 by antagonizing activity of cyclin D/Cdk4,6, activates pRb and blocks transition to S phase. ARF binds to MDM2, thus abrogates MDM2-mediated ubiquitination of p53 and its degradation in proteasome. p53 is also activated in response to DNA damage. Activated p53 induces transcription of variety genes involved mainly in DNA repair, apoptosis or senescence (including p21, an inhibitor of Cdks, resulting in the cell cycle arrest).
(adopted from Sherr 2004[176])

2.3.2. Characteristics of cellular senescence

Senescent cells are permanently arrested. It means that even in presence of supraphysiological mitogenic stimuli they do not progress through cell cycle and do not synthesize DNA but they can remain viable for extended period of time. Cellular senescence is characterized by morphological and functional changes in cellular phenotype: enlarged and flattened morphology, changed nuclear structure (e.g. heterochromatinization), the expansion of lysosomal compartment revealed by senescence associated ß-galactosidase (SA-β-gal) activity, altered protein processing and degradation, enhanced oxidative stress, accumulation of lipofuscine, resistance to apoptosis, and specific gene expression pattern[177] (**Figure 10**; for reviews, see[162, 178]). Levels of cell cycle inhibitors such as p53 and cyclin dependent kinase inhibitors p21

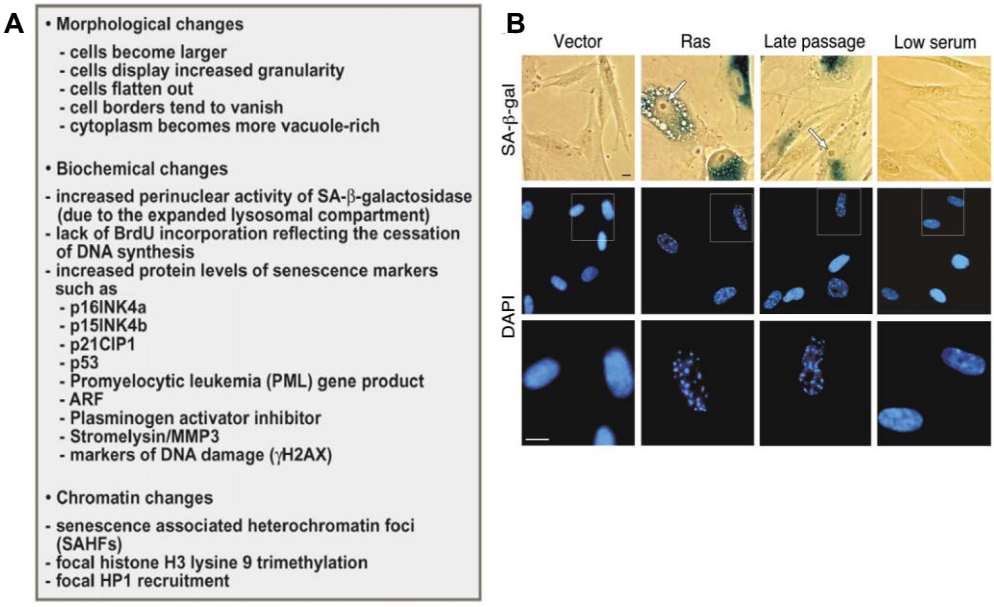

Figure 10: Features of cellular senescence
(A) Table of changes typical for senescence onset (from Schmitt 2007[162]).
(B) Human diploid fibroblasts (IMR90) undergoing oncogene-induced senescence or replicative senescence induced by expression oncogenic Ras or by extensive passaging, respectively, exhibit typical senescence features. They are positive for the SA-β-gal staining, form senescence associated heterochromatin foci (SAHF; DNA is visualized by DAPI staining), are enlarged and display one prominent nucleolus (indicated by arrow). In comparison, exponentially growing cells (transfected with an empty vector) and quiescent cells (induced by serum withdrawal) lack these features. Scale bars are equal to 10μm. (adopted from Narita 2003[227])

and p16 gradually increase during the progress toward senescence, whereas the levels of growth promoting factors such as c-Fos decline (reviewed in[179]). Although the list of senescence markers rapidly increases, none of them is so exclusive to specifically distinguish between truly senescent and long-term arrested cell. Although SA-β-gal is dispensable for cells to enter senescence[180], SA-β-gal assay, visualizing SA-β-gal activity as blue perinuclear staining (**Figure 10**), is the most commonly used method to determine senescent cells[181].

2.3.3. Forms of cellular senescence

Cellular senescence can be induced by several types of external and internal stimuli. Historically for the first time was cellular senescence described by Leonard Hayflick and Paul Moorhaed[182]. They observed that normal human diploid cells can undergo only limited number of cell divisions in culture and after exhausting this genetically determined dividing capacity, their growth is permanently arrested. This so called **replicative senescence** is a common hallmark feature of primary mammalian cells. As a critical factor responsible for triggering replicative senescence is considered shortening of telomeres to critical length[183] (**Box 3**), which evokes DNA damage response[184]. The attrition of telomeric DNA could be accelerated by oxidative stress[185]. Lately, it was found that senescence could be induced by various other stimuli independently on replication history of the cell. Therefore, it is termed **premature senescence**. Exposure of cells to sublethal stress (γ-irradiation, UV, H_2O_2, ethanol, hyperoxia, DNA topoizomerase inhibitors, analogs of nucleotides and others) can result in stress-induced premature senescence (for reviews, see[186, 187]). Also aberrant oncogenic and mitogenic signals such a mutation causing permanent activation of Ras[188] or overexpression of cyclin E, mos or cdc6 leads to arrest of cellular growth and to **oncogene-induced premature senescence**[163]. Initially, the oncogenic activation results in a burst of cellular hyperproliferation accompanied by elevated intracellular levels of reactive species, augmented numbers of active replicons, alterations in DNA replication fork progression, and the appearance of DNA single- and double-strand breaks that initiate DNA damage response followed by senescence establishment[163, 189-192]. Notably, senescence response is not induced by every oncogene. For instance, activated Myc provokes rather apoptosis and seems to block pathway leading to senescence[179]. In addition, also ceramides[186, 193] and inhibitors of histone deacetylases[194-196] are able to induce premature senescence. The phenotype and expression pattern of premature

senescence is very similar to replicative senescence, although differences in expression of several genes were found[197]. This resemblance implies that cellular senescence

Box 3: Telomeres
Telomeres are highly specialized chromatin structures at the ends of linear chromosomes (reviewed in[198]). They are made up of many double stranded tandem repeats (in vertebrates 5'-TTAGGG-3'), 3'-single strand overhang and a number of telomere associated proteins. The very end of a telomere is concealed by forming loop (cap) structures. This loop prevents recognition of telomere end as a DNA break and thus protects chromosomes from end-to-end fusions, misrepair and degradation. As implies from DNA replication biochemistry, telomeres get shorten (50-200bp) by each cell division. If telomeres become critically short they loose their capping function, become sticky, and are prone to illegitimate chromosome end-to-end fusions and subsequent chromosome rearrangements. The telomeres attrition, which could be accelerated by oxidative and other stresses, is thought to be responsible for the onset of genomic instability, activation of DNA damage response and induction of replicative senescence, which was believed to serve as an intrinsic mechanism to prevent normal somatic cells from replicating indefinitely[184, 199]. Telomere length can be maintained by RNA-dependent enzyme called telomerase[200]. Although the telomerase expression is very weak or undetectable in most normal human somatic cells, it is activated in many cancer cells. Cancer cells can also maintain telomeres by telomerase-independent mechanism termed alternative lengthening of telomeres (ALT), which is based on recombination. Despite of intensive research the role of telomere shortening in senescence onset remains unclear[201].

represents a common growth arrest program that can be activated by diverse stimuli. Commonly in tumor cells, some members of key pathways directing cells to senescence or apoptosis are abrogated and these cells are broadly resistant to various types of stress. Searching for and use of treatments that selectively induce cellular senescence in tumor cells appears to be a promising aproach to inhibit cancer progression. This idea is supported by recent reports demonstrating that DNA-damaging treatment *in vivo* is accompanied by the induction of cellular senescence in tumor cells correlating with extended overall survival of the host[178].

BrdU-induced premature senescence

Between compounds that were reported to effectively induce senescence not only in normal mammalian cells but also in most types of tumor cells belong halogenated analogs of thymidine: 5-bromo-2'-deoxyuridine (BrdU)[202], 5-chloro-2'-deoxyuridine[203],

and 5-iodo-2'-deoxyuridine[203]. BrdU is incorporated into DNA as 5-bromouracil instead of thymine. BrdU-induced premature senescence resembles replicative senescence, cells become enlarged, flatten, positive for SA-β-gal staining, and there is a great overlap in genes upregulated in BrdU-induced and replicative senescence[204, 205].

The mechanism how BrdU induces premature senescence is not completely understood. Telomere shortening does not seem to be accelerated by BrdU, however, the presence of BrdU in DNA changes its interactions with regulatory proteins that may lead to altered expression of some genes[202]. Suzuki et al.[206] observed that BrdU promotes decondensation of constitutive chromatin and AT-rich Giemsa-dark bands in mitotic chromosomes. The authors suggested that BrdU-induced senescence-like phenotype in immortal cell lines is caused by activation of limited number of genes located on or near AT-rich normally inactive chromatin. They hypothesized that AT-rich scaffold/matrix attachment regions (S/MAR, noncoding DNA regions contributing to chromatin organization by attaching chromatin loops to nuclear matrix or scaffold strongly affect gene expression[207]) are involved in this phenomenon. Moreover, they showed that distamycin A (DMA), netropsin or Hoechst 33258, that bind to the minor grooves of AT-rich regions, enhance decondenzation of herterochromatin and compete with DNA-binding proteins, strongly synergize senescence inducing effect of BrdU[206]. Although originally Suzuki et al. did not attributed a role in BrdU-induced senescence to DNA damage[206], indeed, very recently it has been shown that BrdU evokes the DNA damage response and activates Chk1, Chk2 and p53 that are involved in DNA damage checkpoints[208], which is in agreement with unpublished results from our laboratory. Importantly, it is known that 5-bromouracil incorporated into DNA is converted to uracil after exposure to light, uracil is removed by uracil glycosylase and this may cause a nick or gap in the DNA due to incomplete process of excision repair[209]. Moreover, BrdU has a mutagenic potential, since bromouracil is not recognized by cellular repair enzymes and may pair with cytosine instead of adenine. This causes highly specific transition from AT to GC pairs during subsequent replication and increases a frequency of point mutation[210, 211]. Additionally, BrdU and DMA are known inducers of fragile sites (for a review, see[212]). The fragile sites are AT-rich regions of DNA that can form secondary structures complicating progression of a replication fork and delaying replication. The fragile sites are normally stable in somatic cells, but under conditions of replication stress or after treatment with inhibitors of replication (e.g. aphidicolin, 5-azacytidin, BrdU, and DMA) they display site-specific deletions, breaks, chromosome

rearrangements, increased rates of sister chromatid exchanges (suggesting attempt to repair damaged DNA by homologous recombination), plasmid or viral integration and intrachromosomal gene amplification (reviewed in[213]). Loss of heterozygosity at fragile sites and activation of DNA damage checkpoints were reported as one of the first events in early stages of cancer development[214, 215]. Indeed, BrdU was reported to substantially elevate the number of chromosomal aberrations and sister-chromatid exchanges[216]. Importance of fragile sites is further supported by reports that also other inducers of fragile sites like aphidicolin[217], adriamycin[218], or 5-azacytidin[219, 220] are able to establish premature senescence phenotype tumor cell lines. In summary these data indicate that DNA damage and activation of DNA damage response are likely to be critical for BrdU-induction of senescence.

The model of BrdU/DMA-induced senescence has two main advantages: BrdU is capable to induce senescence in immortalized cell lines and the onset of senescence is fast (within a few days). Therefore, we employed this model in our studies of PML expression (Research Paper II and III).

2.3.4. Mechanism of cellular senescence

Although exact molecular mechanisms connecting initiatory stimuli and main pathways leading to senescence remain to be revealed, senescence-associated growth arrest is executed independently on the nature of trigger by the several tumor-suppressor genes. The most important role is ascribed to p53, pRB and inhibitors of cyclin dependent kinases p16 and p21 (reviewed in[179]; for mechanism of p16-pRB and p53-p21 pathways, see **Figure 9**). Expression of p16, p21 and p53 genes is progressively upregulated by both accumulation of population doublings and oncogenic activation. pRB is in senescence held predominantly in the active (hypophosphorylated) state[179, 188]. Ectopic expression of p16 and p21 in normal human diploid fibroblast is sufficient for induction of senescence[221]. However, various studies (including our observation on p53-defective cell lines) indicate that neither p53 nor p21 seem to be absolutely required for induction of senescence, as their inactivation only makes cells less sensitive to senescence triggers or delays senescence onset, but does not completely abrogate the program[217, 222, 223]. It was reported that engagement of tumor suppressor pathways differs in humans and mice (for a review, see[224, 225]). In human cells, telomeric signals engage p53-p21-pRB pathway, while non-telomeric signals trigger both p53-p21-pRB

and p16-pRB. In mouse cells, ARF is suggested to play important role and dominant pathway of senescence induction is ARF-p53-p21-pRB pathway[226].

Activation of the above mentioned tumor suppressors explains initial phase of senescence – the block of progression through the cell cycle. But only the withdrawal from the cell cycle not necessarily means that cell could not later (e.g. after DNA damage repair) continue in proliferation. Thus it is assumed that the stabilization of cell cycle arrests to irreversible senescence involves other yet unknown critical events. Recently, it was reported that cellular senescence in human cells leads to a global alternation in chromatin structure connected with widespread epigenetic changes and silencing of genes involved in cell proliferation[227, 228]. The appearance of DNA-dense structures in cell nucleus termed senescence-associated heterochromatin foci (SAHF) reflects these changes[227] (reviewed in[229, 230]). Markers of heterochromatin like di- and tri-methylated H3K9 and heterochromatic protein (HP1) are associated with SAHF, whereas RNA polymerase II is excluded. Tumor suppressor pRB contributes to SAHF formation by binding SUV39H1, a histone methyltransferase responsible for H3K9 methylation, and its recruitment to E2F-target promoters. Indeed, chromatin immunoprecipitation experiments showed that some E2F-dependent genes (e.g. PCNA and cyclin A) exhibit heterochromatin features in senescence state[227]. However, as formation of SAHFs was not observed in all cases of senescence types, it is not clear whether it could be a primary cause of senescence onset or just an accompaniment feature. The formation of SAHF is stepwise process (**Figure 11**) and a key role plays complex of histone chaperones, histone repressor A (HIRA) and antisilencing function 1a (ASF1a)[231]. HIRA/ASF1a bind to histone H3 and cause chromatin condensation upstream from SAHF formation. Moreover, a linker histone H1 is substituted with high mobility group A (HMGA) protein[232, 233]. In later step, histone H3 is methylated by SUV39H1 generating a docking site for HP1. Finally, a histone variant macro-H2A resistant to chromatin remodeling and acting as transcriptional silencer is incorporated into chtomatin to stably repress transcription[228]. Both HIRA and HP1 proteins pass transiently through PML NBs that is necessary for their activation. The disruption of PML NBs prevents SAHF formation and senescence establishment[234]. Participation of PML NBs in SAHF formation could at least partially explain the onset of senescence in response to PML overexpression (see section 2.4.7).

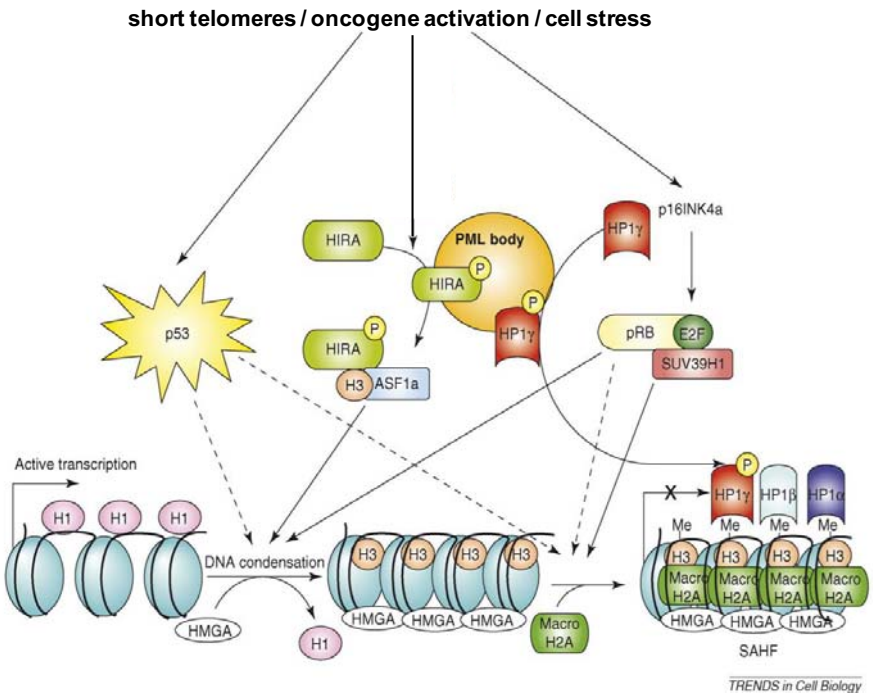

Figure 11: A model for formation of SAHF in senescent human cells.
Senescence is triggered by short telomeres, activated oncogenes and other cell stresses. The HIRA/ASF1a pathway cooperates with the p16INK4a/pRB pathway to drive chromosome condensation. HIRA translocates into PML NBs prior SAHF formation and cell-cycle exit. It is probable that PML NBs are place of HIRA-containing complexes assembly and/or modification. pRB contributes to chromatin condensation by recruitment of histone methyltransferase and histone deacetylases to E2F-target genes. After chromosome condensation, HP1 proteins and histone variant macroH2A are incorporated into SAHF resulting in constitutive heterochromatinization. Recruitment of HP1γ to SAHF depends on HP1γ phosphorylation that is hypothesized to occur in PML NBs. Dashed lines indicate steps that are poorly defined at present. (adopted from Di Micco 2007[229])

2.3.5. Cytokines in cellular senescence

There is a growing line of evidence that senescent cells exhibit specific secretory phenotype. Recently, several research groups have showed that senescent cells produce chemokines and cytokines of pro-inflammatory character (e.g. IL-1α [235, 236], IL-1β[236], IL-6[236, 237], IL-8[236], IL-11[238], monocyte chemoattractant protein MCP1[236]). Moreover, we described increased expression of IFNβ and its secretion in response to BrdU/DMA-induced senescence in HeLa cells and suggested that sustained IFNβ signalization may be responsible for senescence promotion (Research paper III). Our results are in concordance with an observation of Shuttleworth et al. reporting that BrdU increases expression of interferon in Namalwa cells[239] and with several other reports describing

antiproliferative effects of IFNβ. Treatment of certain tumor cells with IFNβ results in accumulation in G0/G1[240] or S phase[241] of the cell cycle and in some cases is followed by induction of senescence-like phenotype[242]. Recently, Moiseeva et al. have shown that while a transient IFNβ-treatment induces a reversible cell cycle arrest, a long-term treatment triggers oxidative stress and DNA damage response and premature senescence of normal human fibroblasts p53-dependent manner[243]. Taken together, IFNβ can play an important role in induction of senescence phenotype.

Intriguingly, the production of pro-inflammatory cytokines by senescent cells is supposed to promote cancer development[162]. Although senescent cells seem to be partially removed from tissues[178], their accumulation during organism aging could evoke immune reaction. The chronic inflammation caused by pro-inflammatory cytokines could initiate cancer development due to the tissue damage and increased production of reactive oxygen species and lipid peroxidation that induce the DNA damage[244, 245]. The microenvironment of many cancers was found to be rich in cytokines, chemokines and inflammatory enzymes (reviewed in[246]). In addition, direct link between several cytokines and promotion of cancer has been described. TNF-α as a major mediator of inflammation plays a role in both tissue destruction and recovery from the damage. It induces other inflammatory mediators and it was described to be produced by cancers[247]. IL-1 was found to increase tumor invasiveness and metastasis[248]. IL-6 is a pleiotropic cytokine modulating transcription of several genes during inflammation. Elevated blood levels of IL-6 are associated with several types of tumors[249]. Furthermore, chemokines induced by inflammatory cytokines cause infiltration of leukocytes into the tumor and regulate movement of cells into and out of the tumor[246]. In addition to inflammatory cytokines, extracellular matrix remodeling enzymes like metalloproteinases[177, 250-252] and growth factors[253] are produced by senescent cells and can stimulate tumor growth[162]. Indeed, several groups reported that senescent cells stimulate preneoplastic cell growth both in vitro and in vivo[252, 254-256]. Consistently, the cells exhibiting senescence phenotype were found at sites of hyperplasic or premalignant lesions[214, 257-259]. These findings point out that senescence as safeguard mechanism against uncontrolled proliferation can have undesirable side effects, which paradoxically support tumor growth.

2.4. PROMYELOCYTIC LEUKEMIA PROTEIN

The nucleus of the eukaryotic cell is a complex membrane-bound organelle compartmentalized into subdomains that represent morphologically, structurally and functionally distinct structures, although they are not separated by membrane (reviewed in[260-262]). One type of these nuclear subdomains is represented by promyeolocytic leukemia nuclear bodies (PML NBs), matrix-associated multi-protein complexes. The principal component of PML NBs, which is essential for their proper formation and integrity, is promyelocytic leukemia protein (PML). For the first time was gene coding PML protein described in early 1990s in a connection with acute promyelocytic leukemia and the role of PML as a tumor suppressor was predicted[263-265]. Since then wide range of proteins with heterogenous biological functions was found to associate with PML, accumulate and/or get modified in PML NBs. These findings implicated PML and PML NBs in tumor suppressive mechanisms at several levels, including regulation of cell cycle progression, DNA repair, senescence, and apoptosis (reviewed in[266]). Moreover, their role in antiviral defense was suggested (reviewed in[267]). Despite of the plethora of biological functions in which PML contributes, it remains to be elucidated whether PML has some main and exclusive function and which events drive its expression. Also it is not clear what is an evolutionary advantage of existence of PML NBs compiling proteins of very heterogeneous functions and how could single organelle efficiently utilize these function.

2.4.1. PML gene

PML gene and protein can be found under alternative names MYL, PP8675, RNF71, or TRIM19. Human PML gene is located on chromosome 15 (according to Ensembl or Entrez Gene genomic location is 15q24.1 or 15q22, respectively). The PML genomic locus spans approximately over 53 kb and is subdivided into nine exons[268]. The primary PML transcripts may undergo extensive alternative splicing[269, 270]. To date, at least 11 different mRNAs encoding various PML protein isoforms were found in humans[270, 271].

PML gene is not evolutionary conserved among eukaryotes as no its homologs were found in *Drosophila melanogaster*, *Saccharomyces cerevisiae* or *Arabidopsis thaliana*[272]. PML expression is restricted to higher eukaryotes, which correlate with its

proposed function in tumor suppressive pathways that evolved in multicellular organisms with renewable tissues to protect organism against uncontrolled cell proliferation.

The tumor suppressor properties of PML are inferred from facts that disruption of PML gene leads to leukemia or at least increased proliferation and susceptibility to tumor development. Chromosomal translocation of PML gene with gene for retinoic acid is a cause of majority cases of acute promyelocytic leukemia (for details see section 2.4.8, reviewed in [273]). A valuable research tool for understanding the role of PML in context of the whole organism brought generation of PML deficient mice (PML-/-). PML gene is not necessary for survival, because PML-/- mice develop normally, are viable, fertile, and at the gross phenotypic level are indistinguishable from PML+/+ littermates. However, they are highly sensitive to spontaneous botryomycotic infections and they succumb due to infection within the first year of their life[274]. PML-/- mice and derived cells were resistant to apoptosis triggered by a number of stimuli such as ionizing radiation, interferon, ceramide, Fas ligand and TNFα[275]. Despite of this inability to remove damaged cells, the incidence of spontaneous tumors in PML-/- mice was not increased during the first year of life[274]. As the long-term assessment of tumor incidence was compromised by early infection-caused death of mutant mice, the experimental models accelerating the tumor formation were used. Indeed, it was revealed that PML-/- mice develop more tumors after treatment with tumor-promoting agents than their wild type littermates[274]. Furthermore, in vitro studies of PML-/- cells demonstrated a role of PML in regulation of cell proliferation. PML deficient mouse embryonic fibroblasts grow substantially faster, more easily form colonies and grow to higher densities than their wild type counterparts. However, they are still unable to grow in a semi-solid medium as fully transformed cells[274]. Unlike in PML+/+ cells, retinoic acid is not able to inhibit the growth of cells with inactivated PML gene[274]. Additionally, retinoic acid-induced terminal differentiation of progenitors to myeloid cells was abrogated in PML-/- cells. This is in concordance with the observation that PML-/- mice exhibit a marked reduction of circulating myeloid cells[274].

2.4.2. Regulation of PML gene transcription

Although recognition elements for several transcription factors were found in PML promoter and its first exon and intron[276, 277], currently almost no data are available about

regulation of basal expression of PML. PML promoter lacks a classical TATA box[276]. The region of 1.44 kb upstream from the first ATG contents 56% of GC (enhanced GC content was found in promoters of genes involved in regulation of growth control) and consensus sequences for AP-1, AP-2, AP-4, est-1, GATA-1, GATA-2, SP-1, Oct-1, Oct-2, CTF, NF-IL6, PEA-3, SRE (CArG-box), and steroid response element were found within this region in silico[276]. However, significance of these potential regulation sites remains to be determined. Interestingly, PML is readily induced in response to various types of stresses (viral infection, DNA damage, aberrant oncogene activation or overexpression) and some mechanisms of induced PML transcription were revealed.

First described and foremost inducers of PML transcription are interferons[99, 278, 279]. IFN-enhanced expression of PML consequently causes increase in both size and number of PML NBs[278]. Type I IFNs, IFNα and IFNβ, strongly elevate PML mRNA levels through ISRE element located in PML gene regulatory region, whereas less potent inducer IFNγ acts through relatively weak GAS element (PML GAS element was not able to compete for STAT1 homodimer with strong GAS from IRF1, however a competition was reported for weak elements like from IFP-53)[276]. Both PML ISRE and GAS elements are located in the first untranslated exon[276]. IFNγ was able to induce reporter gene containing in its promoter only PML ISRE element, suggesting that IFNγ can stimulate PML expression indirectly though some IFNγ-inducible transcription factor(s)[276]. Indeed, it was found that IRF1 binding sequence overlaps with PML ISRE site[103] and we confirmed IRF1 binding to this element by chromatin immunoprecipitation (our unpublished results). Recently, it was reported that IRF1 and two haematopoietic specific transcription factors - IRF8 and PU.1 (est-related factor) - play an important role in IFNγ-induced expression of PML in activated macrophages[280]. IRF8 was necessary for basal PML expression in haematopoietic organs in vivo[280]. In consistence with our data (Research Paper I and II), the kinetic studies show that PML mRNA levels peaks between 4 and 8 hours in response to IFNα and IFNγ and than decline steadily for following 12 hours[276]. The decline in PML mRNA level implies existence of some negative loop. Notably, truncation of 5' end of promoter region with retained ISRE and GAS elements (about 280bp upstream from transcription start was retained) led to enhanced baseline expression of reporter gene implicating presence of a silencing domain[276]. In addition, we have found that induction of PML by IFNs is dependent on the activity of histone deacetylases (Research Paper I). Recently, PML

has been described as a negative regulator of IFNγ-signaling pathway, which indicates existence of negative feed-back loop[281]. As interferons are cytokines produced in response to viral infection, a role of PML was suggested in antiviral defense.

Additionally, PML levels were found to be elevated in response to DNA damage[277, 282] and during the onset of oncogene-induced senescence[225, 283]. Plausible explanation for the mechanism of this increase is the recently reported fact that PML is a direct target gene of p53[277], which is important tumor suppressor integrating various stress signals and converting them into one of antiproliferative responses. Several p53 response elements were found in both human and mouse PML promoter and first intron[277] (**Figure 12**). However, only the elements in first intron (3 in mouse and 1 in humans) seem to be relevant for induction of PML transcription. The binding of p53 to these elements after stimulation by oncogenic ras was confirmed both in vitro and in vivo. Intriguingly, only activated p53 binds to these response elements[277]. As PML was previously described as upstream regulator of p53 and activation of p53 depends on the acetylation occurring in PML NBs[225], it is likely that PML and p53 influence each other by positive feedback loop. Of note, IFNα/β signaling induce transcription of p53 gene and increase of p53 protein levels, but on its own does not activate p53[98]. Therefore it is improbable that this induction of p53 would substantially contribute in increased transcription of PML gene in response to IFNα/β.

PML expression seems to be also stimulated by estrogenes. The highest expression of PML in the endometrium is during the proliferative (estrogenic) phase, while minimal is during the luteal (secretory) phase, when only a few cells exhibit positive staining for PML[284, 285].

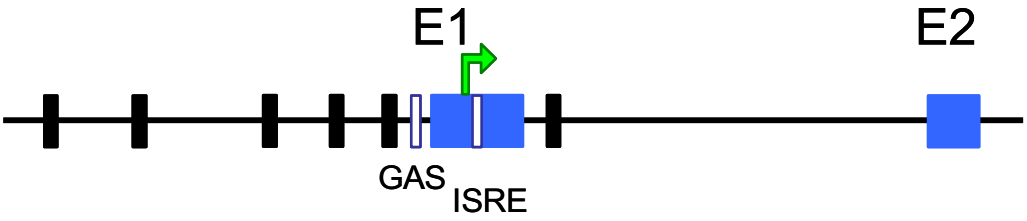

Figure 12: Scheme of promoter and proximal part of human PML gene.
Exons are represented by blue boxes, putative p53 response elements are indicated by black and GAS/ISRE elements by white boxes. Transcription start is indicated by the arrow.
(adopted from de Stanchina 2004[277])

2.4.3. Expression of PML

Although PML is expressed ubiquitously in cells of different origin *in vitro*, its expression *in vivo* is more restricted. PML expression is generally suppressed in tissues with high proliferative index (including tumor cells) and certain terminally differentiated cells[286, 287]. Specifically, PML NBs are absent in rapidly growing epithelia (normal breast, colon, stomach, parathyroid, lung), large neurons and other cells of neuronal lineage such as neuroblastoma cells[286, 288]. PML expression is however reestablished in neurons under certain pathological conditions which might suggest involvement of PML bodies in the repair processes after axonal injury[288, 289]. Importantly, PML seems to be prevalently down-regulated in tumor cells, although certain tumor types exhibit normal or high PML expression[285, 290]. From normal cells, the highest PML levels were found in macrophages, especially in activated (by IFNγ), which corresponds with the PML inducibility by interferons[285, 289, 291]. In concordance, PML was found to be highly expressed in inflammatory diseases as psoriasis and hepatitis, inflammatory cells surrounding epithelial cancers and Hodgkin's disease, in inflammatory lesions of graft-versus-host disease[284-286, 292].

2.4.4. PML protein structure and PML isoforms

PML belongs to the TRIM (tripartite motif) protein superfamily according to the main motif known also as RBCC (RING B-box coiled-coil) family that members are frequently involved in regulation of transcription (reviewed in[293]). RBCC motif is characterized by the presence RING finger (a zinc binding domain with the C_3HC_4 configuration of cysteine and histidine residues), one or two B boxes (alternate cysteine-histidine rich zinc binding domains; PML has two: B1 and B2 box), and α-helical coiled-coil dimerization domain. RING finger mediates protein-protein interactions and coiled-coil domain is responsible for PML multimerization[270]. RBCC motif is essential for PML NBs formation as well as for PML antiviral, tumor-suppressive, and apoptotic activities[270, 293-297]. RBCC motif is localized in N-terminal part of PML protein and is retained in all PML isoforms (**Figure 13**). The C-terminal part contains nuclear localization signal (NLS) responsible for prevalent localization of PML in nucleus. NLS might by skipped due to alternative splicing and cytoplasmic PML isoforms are produced[270]. Moreover, nuclear export signal (NES) was found in C-terminal part of

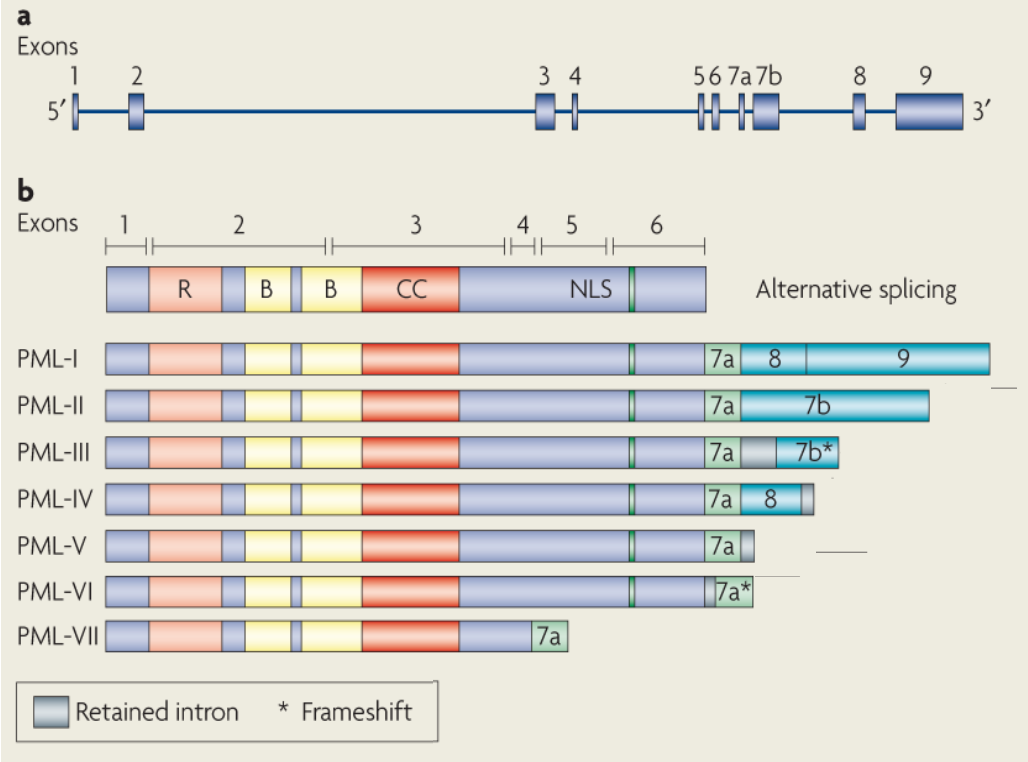

Figure 13: The PML gene and PML isoforms
(a) Schematic view of PML gene spanning over about 53 kb and consisting of nine exons (represented by blue boxes).
(b) Alternative splicing of C-terminal region leads to the generation of several PML isoforms, main seven isoforms are shown, other isoforms vary in splicing of exons 4, 5, and 6. As indicated all of them have retained RBCC motif constituting of RING domain (R), two B-boxes (B) and coiled-coil domain (CC).
(adopted from Bernardi 2007[266])

PML of some isoforms suggesting that their function may be dependent on ability to shuttle between nucleus and cytoplasm[298]. Alternatively spliced mRNAs generate proteins with predicted molecular weight from 48-97 kDa, which however do not correspond to observed migration speeds in electric field that are higher (up to 220 kDa). This shift is the result of various posttranslational modifications[270, 271], most probably multi- and polysumoylation. According to Jensen et al., PML isoforms were classified into seven classes marked with Greek numbers PML I to PML VII[270] (**Figure 13**). A further sub-classification a, b, or c reflects alternative splicing within exon 5, exons 5 and 6, or exons 4, 5, and 6, respectively. Since the b and c variants lack NLS, they are likely to be cytoplasmatic[267, 299]. Although prevailingly is PML studied as one molecule, regardless of its many isoforms, it is becoming increasingly clear that the different PML isoforms have different functions, localization pattern and are differently

expressed[271, 300]. The longest PML isoforms PML I and PML II are expressed at the highest levels, while PML III, PML IV and PML V are quantitatively minor isoforms[271]. PML I is a prevalent isoform in nontransformed cells (up to 80% of total PML mRNA), however its abundance is significantly lowered in transformed cell lines suggesting link between PML I and tumor transformation[271]. Other isoform with ascribed specific function is PML IV that is the only isoform able to induce senescence when overexpressed[301]. Although major interest was paid to nuclear PML, recent studies have shown that cytoplasmic PML isoform is essential for the TGFβ signaling[302].

2.4.5. Posttranslational modifications of PML protein

Probably the most important modification for formation and integrity of PML is sumoylation. PML might be modified by all three members of small ubiquitin-like modifier family (SUMO1, SUMO2 and SUMO3) and directly interact with UBC9, an enzyme catalyzing the sumoylation of PML on three lysine residues[303]. The sumoylation status of PML can be finely tuned by UBC9 counterparts SUMO-specific proteases (SENP1, SENP2, and SENP 5) that are responsible for removal of SUMO from PML[304]. Unmodified PML is associated with the soluble nucleoplasmic fraction, whereas sumoylated PML fraction is tightly associated with the nuclear body[305]. Mutant PML that is not able to bind SUMO forms aberrant aggregates in nucleoplasm and is unable to recruit PML NBs associated proteins indicating that PML sumoylation is necessary for PML NBs formation[306]. However, this mutant retains tumor suppressive properties when overexpressed, thus it is unclear whether sumoylation affects antitumor functions of PML[301]. Interestingly, RING domain of PML is required for efficient PML sumoylation[307] and PML was suggested to function itself as SUMO E3 ligase (it means that provide platform for type E2 enzymes like UBC9 and specific substrates)[307, 308]. Furthermore, *in situ* sumoylation assay revealed PML NBs as site of active sumoylation [308]. In fact many proteins colocalizing with PML NBs (e.g. Sp100, Daxx, CBP) are sumoylated[309]. Recently, it has been revealed that PML contains SUMO–binding domain, which is necessary for PML NBs formation[307](**Figure 14**). Based on above-mentioned observations, Shen et al. suggested model explaining mechanism of PML NBs formation[307]. In mitosis, PML protein is desumoylated, its molecules homo-multimerize through RBCC motif and form PML aggregates, PML NBs-associated

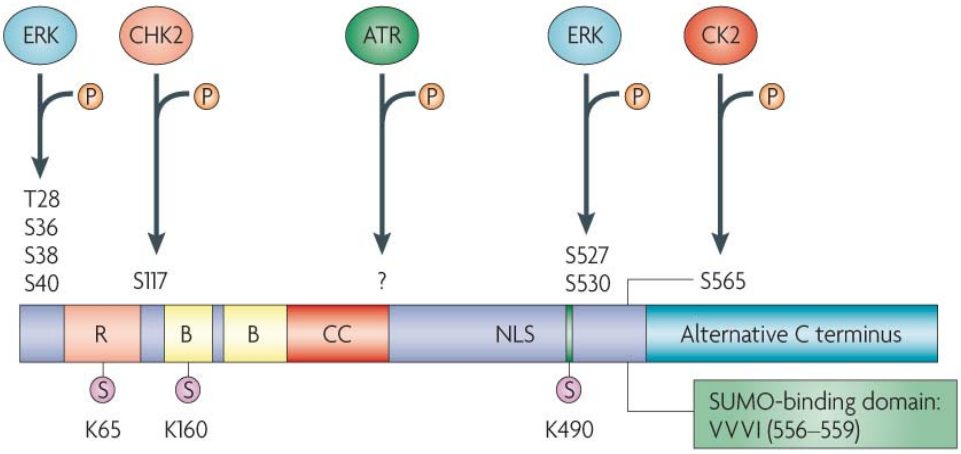

Figure 14: Post-translational modifications of PML protein
A schematic representation of the PML protein with its main functional domains is depicted. Several kinases that are known to phosphorylate PML are shown, including extracellularregulated kinase (ERK), checkpoint kinase‑2 (Chk2), ataxia telangiectasia mutated (ATM)- and Rad3-related (ATR), and casein kinase-2 (CK2), along with the amino-acid residues on PML that they phosphorylate. The three sumoylation sites (S) of PML are also indicated together with the SUMO-binding domain that comprises amino acids VVVI at residues 556–559.
(adopted from Bernardi 2007[266])

proteins are dispersed. In interphase, sumolyation of PML molecules triggers nucleation event: sumoylated PML molecules noncovalently interact each to other through SUMO-binding domains. Subsequently also other sumoylated proteins are recognized by PML (and vice versa some of them such as Daxx[310] having also SUMO-binding domain can recognize sumoylated PML)[307].

Phosphorylation is dynamic modification regulating functions, stability and localization of various proteins. Also PML protein is phosphorylated on serine and tyrosine residues[311] and is a substrate of several kinases (**Figure 14**). PML is specifically phosphorylated during mitosis (this state is connected with desumoylation) and reversal of mitosis-specific modifications in G1 phase leads to reassembly of PML NBs implicating that they are important factors of PML localization during the cell cycle[312]. Futhermore, phosphorylation of PML was found to be important for its relocalization into nucleolus and for redistribution of PML bodies in response to DNA damage[313, 314]. Following DNA damage, ATR[313] and Chk2 kinases phosphorylate PML[315]. Finally, crucial modification for PML protein stability is phosphorylation of Ser517 by casein kinase-2 (CK2) that promotes ubiquitin-mediated degradation of PML protein[316]. In cancer cells, PML protein levels are mostly undetectable, despite of the

presence of PML mRNA[284, 290, 317]. Therefore it seems that this down-regulation of PML expression is rather due to increased PML degradation than lack of PML transcription. In line with this hypothesis, CK2 is frequently activated in cancer cells[316].

2.4.6. PML nuclear bodies

The distribution of PML protein shows a unique pattern in nucleus. Beside weak nucleoplasmic staining typically excluding nucleoli the majority of protein concentrates in clearly distinguishable dots called nuclear bodies (NBs)[318]. As these bodies are simply defined by presence of PML they are most commonly referred as PML NBs. But in literature they are also known as PML oncogenic domains (PODs), nuclear domain 10 (ND10), or Kremer bodies[319, 320]. Typical PML NBs are small spheres of 0.2-1 μm diameter. They are present in most mammalian cell, there are usually 1-30 PML NBs per nucleus depending on phase of the cell cycle, cell type and differentiation stage (reviewed in[273, 321]). Although data concerning the cell cycle phase with highest number of PML NBs are controversial[285, 312, 322, 323], the latest observation of Dellaire et al. [323] showed that number of PML NBs increases about twice during S phase when compared to G1 phase. This duplication occurs through fission mechanism as a result of tight binding of bodies to chromatin[323]. A physiological dispersion of PML NBs and associated proteins occurs during mitosis[312, 318], when PML protein accumulates into a few large aggregates distinct from normal PML NBs (they do not contain PML NBs-associated proteins like Sp100, SUMO-1 or Daxx)[324]. In electron microscope, PML NBs appear to be electron dense structures[325]. The periphery of PML NB is in extensive contact with chromatin fibers through protein threads running out from nucleic acid free center[326]. These contacts are important for integrity and positional stability of PML NBs. Position of PML NBs in the nucleus is relatively stable, only a small subset of bodies (12 %) shows rapid ATP-dependent motion. Fusion or fission of PML NBs is often observed[327]. PML NBs remain tightly bound to nuclear matrix after removing of chromatine and RNA (high salt extraction and extensive DNase and RNase treatment)[325]. However, depending on internal and external stimuli, PML NBs can become highly dynamic structures changing its number, size, shape, and protein content. Striking changes are observed in response to stress (heat shock[328], heavy metal exposure[328], DNA damage[314], viral infection[329-332]).

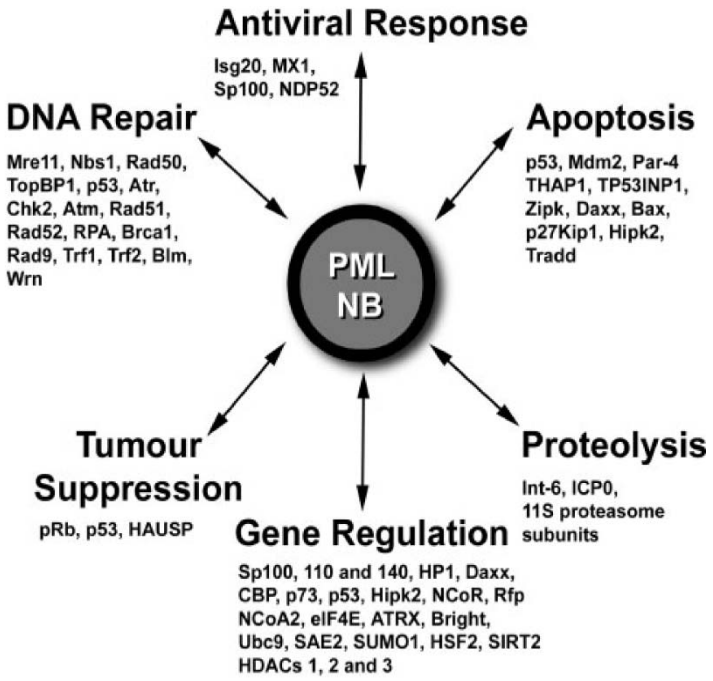

Figure 15: PML associated proteins and PML nuclear body functions
The figure summarizes the many diverse cellular functions attributed to PML bodies and lists the proteins implicated in those processes that localize at PML NBs or associate with PML directly. Note: the listed proteins may either localize to only a subset of PML NBs, may localize only under stress conditions (e.g. viral infection or DNA damage), or may be distributed both throughout the nucleoplasm and within PML NBs.
(from Dellaire 2004[314])

List of proteins associated with PML NBs is rapidly expanding, up to date 77 proteins have been identified as transient or permanent components of PML NBs (reviewed in[314, 266], **Figure 15**). The proteins associated within PML NBs are functionally very heterogenous, they belong to transcription factors (Sp 100[328], Daxx[319, 335], pRB [283, 336], p53[225, 283, 337, 338]), proteins involved in DNA damage repair (BLM[319, 339], WRN[340], Mre11[282, 341, 342], Rad50[282], NBS1[282, 341, 342], telomere-binding proteins (TRF1[343], TRF2[343]), chromatin modifying/remodeling proteins (HDACs[344], CBP[345-348], HIRA[228], HP1[349, 350], NDHII[351]) and many others. PML plays a key role in the integrity of PML NBs, because in absence of PML the majority of PML NBs-associated proteins is not able to form bodies and is dispersed throughout nucleoplasm[306, 319, 352]. Various functions of PML and PML NBs were deduced from function of associated proteins. The clue for puzzling involvement of PML NBs in so many heterogenous functions lies probably in fact that not all PML NBs are structurally equal. Different PML isoforms give rise to different PML NBs and not all PML NBs within one nucleus have the same

shape or protein composition[271, 301]. For example, normal PML NBs do not colocalize with telomeres in either primary cells or cells immortalized upon reactivation of telomerase. However, in low but constant percentage of cells using alternative telomere lengthening (ATL) mechanism specialized type of PML NBs was reported[201, 353]. These PML structures differ from others in bigger size, annular shape and colocalization with telomere repeat DNA. Moreover, telomere binding proteins TRF1 and TRF2, and DNA damage response proteins Rad51, Rad52, RPA, Mre11, NBS1and BRCA1 (proteins not typically found in normal PML NBs) are constitutively associated within these ATL-related bodies[342, 353]. Other "giant" multiprotein structures containing PML and repair proteins were described by Lucciani et al. 2006[350]. They are generated during G2 phase in lymphocytes of patients with immunodeficiency, centromeric instability and facial dystrophy (ICF) syndrome[350]. Furthermore, different types of stress can induce variety of PML structures. In response to heat shock or Cd^{2+} treatment, PML NBs disperse into hundreds of small spots throughout nucleoplasm[328]. These spots are called "microstructures" and contain PML, but lack both Sp100 and SUMO[354, 355]. Similarly, treatments causing DNA damage lead to dispersion of PML NBs into smaller bodies or even into diffuse nucleoplasmic pattern (UV-irradiation, cisplatin, alkylating agents)[356-358] or can result in accumulation of PML around nucleoli accompanied by preservation of majority of the typical PML NBs (doxorubicin, mitomycin C)[313]. In addition, we have reported for the first time the association of PML donut–like structures with nucleoli in hMSC under normal growth-permitting conditions. Moreover, we have shown that inhibition of rRNA synthesis by low concentration of actinomycin D causes accumulation of PML protein around the segregated nucleolus in structures termed PML nucleolar coats that are structurally and morphologically distinct from above described PML donut-like structures (Research Paper II).

2.4.7. PML functions

Huge effort of many research groups has been spent to elicite what is function of PML and PML NBs. Mainly based on colocalization and coimmunoprecipitation assays, many proteins of various functions were found to partially or temporally associate with PML and the function of PML is deducedon the basis of these interactions. PML, and by inference PML NBs, were implicated to play the role in virtually all biological functions, including regulation of gene transcription, antiviral

response, induction of apoptosis and cellular senescence, inhibition of proliferation, and maintenance of genome stability (reviewed in[314, 333, 334], **Figure 15**). Despite of described partial contribution of PML in mentioned processes and unexceptionable role in tumor suppression, the specific function of PML remains unclear. Three general hypothesis how PML NBs could exert its biological functions were suggested. According to first model, PML NBs serve as storage places where proteins are accumulated in both pathological conditions (foreign or misfolded proteins sequestration) and normal conditions and released when necessary[333, 354, 359]. In another model, PML NBs can form a catalytic surface and represent places where multi-subunit complexes are formed and where regulatory proteins, such as p53, are posttranslationally modified. And finally, PMN NBs are supposed to be active sites of regulating transcription and chromatin organization[321]. These models are not mutually exclusive and it appears increasingly unlikely that PML NBs are random aggregates of nuclear proteins.

PML and transcriptional regulation

Role of PML in transcription is still unclear and is a matter of a debate. PML NBs were found near genomic regions that are particularly gene rich and are transcriptionally active [360]. Association with major histocompatibility complex I (MHC I) gene cluster region[361, 362], histone-encoding gene cluster[360] and p53gene locus[363] was reported. However, PML NBs do not overlap with sites of RNA transcription[360, 364] and they do not contain transcription factors (TFIIH, E2F, or glucocorticoid receptors)[364]. Conversely, one group was able to detect nascent RNA polymerase II transcripts in the center of the NB structure[345]. Moreover, transcriptional coactivator CBP was found in PML NBs. Later, Bazett-Jones' group using electron spectroscopic imaging (ESI), which is very sensitive method enabling precise localization of proteins and nucleic acids, demonstrated that the core of the PML nuclear body is protein-based structure and does not contain detectable nucleic acid[365]. However, they found the newly synthesized RNA associated with the periphery of the PML nuclear body[365]. Their results dismiss the hypothesis that the PML nuclear body is a site of transcription, but support the model in which the PML nuclear body may contribute to the formation of a favorable nuclear environment for the expression of specific genes. Moreover, PML NBs are frequently found in juxtaposition with Cajal bodies, cleavage bodies, and

splicing speckles suggesting that PML may play some role in RNA-processing events[364].

PML in viral defense

Interferons that play crucial role in establishment of intracellular antiviral state increase expression of PML and other structural compartments of PML NBs (Sp100, Sp110, Sp140, ISG20, PA28) resulting in multiplication of these structures (reviewed in [366]). While in majority of normal tissues PML amounts are low, its levels dramatically increase during inflammation[285]. This suggests that PML NBs can play a role in IFN response. Other way around, the members of PIAS (protein inhibitor of activated STATs) family that are SUMO E3 ligases and negative regulators of JAK-STAT signaling pathway were found localized within PML NBs[367, 368]. These findings rise the possibility that PML NBs might also contribute to suppression of IFN signaling.

Importantly, PML-/- mice and derived cells exhibit increased sensitivity to viral infection[274, 369]. On the contrary, overexpression of PML dramatically decrease virus gene expression and replication of virus (e.g. human foamy virus, vesicular stomatitis virus, influenza virus)[370-372]. Further link between PML, PML NBs and viral infection is manifested by fact that some viral parental genomes associate with PML NBs and early transcription and genome replication of several viruses occur in the vicinity of PML NBs[331, 373-375]. Moreover, proteins expressed by a wide range of viruses colocalize to PML NBs and cause their disruption by a variety of mechanisms (for a review, see[267]). Well described is the case of herpes simplex virus 1 (HSV-1) early protein ICP0, which contains RING finger domain exhibiting ubiquitin E3 ligase activity[376]. ICP0 induce the degradation of PML and the SUMO-modified isoforms of SP100, resulting in destruction of PML NBs[377]. As ICP0 is a key derepressing agent of HSV-1 viral genome, it seems that disruption of PML NBs can be a critical event for the expression of viral genes[378]. Also other herpesvirus sub-families, adenoviruses and papovaviruses encode proteins that target PML NBs (reviewed in[267, 375]).

Finally, PML was shown to be important for p53 activation (discussed in following paragraph). p53 is known to be induced by IFN and is thought to be important for antiviral defense and IFN-induced apoptosis[98, 379]. An example of cooperation of PML and p53 is the case of poliovirus infection. After infection, PML is phosphorylated by ERK and is modified by SUMO, this events lead to recruitment of p53 to PML NBs, p53 phospohorylation, activation of p53 target genes, apoptosis and inhibition of virus

replication[380]. However, this is only transient state, because later poliovirus counteracts p53 activity by recruitment of MDM2 and 20S (a proteasome component) into PML NBs followed by degradation of p53 in MDM2- and proteasome-dependent manner[380].

PML as a tumor suppressor

Although PML knockout mice do not display spontaneous tumor formation, such as observed in p53 or p19 deficient mice, deletion of PML led to greater susceptibility to tumor promoting agents[274, 320]. Similarly to many other tumor suppressors, PML protein is completely or partially lost in a large fraction of human cancers and this loss correlates with tumor progression[285, 290]. As mRNA transcripts of PML gene were consistently detected and sequence analysis did not revealed inactivating mutations, it is suggested that PML is aberrantly degraded in human cancer [284, 290]. In agreement with tumor-suppressive potential of PML, its overexpression results in strong growth suppression that is connected with the establishment of cellular senescence or with the induction of apoptosis[283 222, 302, 381, 382]. In vivo, this is manifested by lower potential of PML overexpressing cells (breast and prostate cancer cells) to initiate tumor formation when injected into nude mice[383, 384]. In vitro, the cells (HeLa, breast cancer cell line) overexpressing PML accumulate in G1 phase and their entry into S phase is delayed due to the decreased expression of cyclin D/E and Cdk2[385]. Intriguingly, the decreased expression of cyclin D1 may be at least partly directly regulated by PML through its interaction with eIF4E (eukaryotic initiation factor 4E) that is responsible for the transport of cyclin D1 mRNA from the nucleus to the cytoplasm[386-389]. PML was shown to be an essential regulator of this eIF4E function as the interaction of eIF4E with PML reduces the affinity of eIF4E to m7G-cap of cyclin D1 mRNA that subsequently leads to nuclear retention of cyclin D1 mRNA and the abrogation of its translation[272]. Nevertheless, more general effects of PML on the cell cycle progression are now attributed to the ability of PML to interact with or influence the proteins involved in p53 and pRB tumor suppressor pathways[336].

In dependence on variety of stimuli, p53 is heavily posttranslationally modified and these modifications and their combinations determine p53 activity and stability (for reviews, see [38, 390]). Strikingly, vast majority of key proteins regulating posttranslational modifications of p53 (MDM2, CBP, HAUSP, Sir2-related deacetylase, PIAS, ARF) have been found in PML NBs, at least under certain conditions, and direct interaction and functional links between PML and some of these proteins have been evidenced

(**Figure 16**, reviewed in[382]). Furthemore, p53 is actively recruited to PML NBs in response to oncogene overexpression, arsenic trioxide, UV- and γ-irradiation[225, 282, 314, 391]. Importantly, only certain PML isoforms can recruit p53 into bodies since p53 interaction with PML is mediated by C-terminal domain that is not shared by all PML isoforms[338].

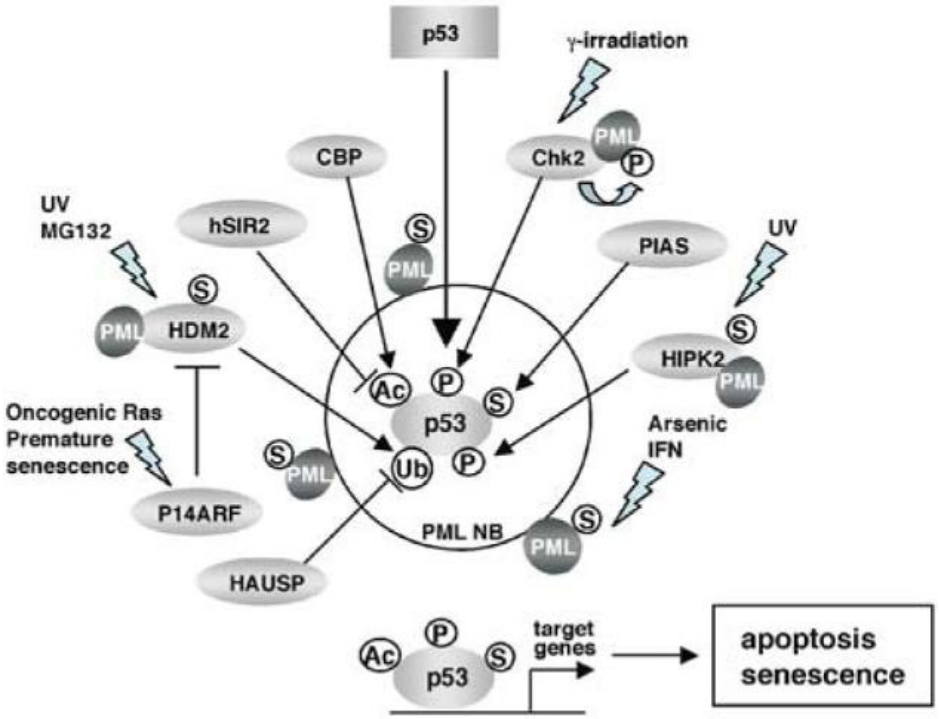

Figure 16: p53 and its posttranslational modifications in PML NBs
S, SUMOylation; P, phoshorylation; Ac, acetylation; Ub, ubiquitination .
(adopted from Takahashi et al. 2004[382])

PML potentiates the function of p53 by regulating its CBP-dependent acetylation[225, 337] and Chk2-dependent phosphorylation in the PML NBs[392]. PML affects not only the activation but also the stability of p53 through the interaction with MDM2 and HAUSP (herpesvirus-associated ubiquitin specific protease). It was demonstrated that PML directly interacts with MDM2, the key p53 negative regulator[391, 393]. This interaction occurs predominantly in the absence of PML sumoylation of Lys160, so it can be expected that it is the free nucleoplasmic PML form that binds MDM2[393]. Additionally, in response to specific DNA-damaging agents (e.g. doxorubicin, but not γ-irradiation) PML was found to sequester MDM2 to nucleolus in ARF-independent manner and thus to enhance p53 stability[313]. On the other hand, the activity of HAUSP

is linked to PML nuclear bodies; HAUSP is able to remove ubiquitin residue from p53 molecule thus protecting it from proteasome-dependent degradation[332, 394].

Notably, the relationship between PML and p53 is reciprocal since p53 transcriptionally activates PML expression (see section 2.4.2.) and may also inhibit CK2-dependent PML destabilization[316]. Together, these two tumor suppressor proteins potentiate activity of each other in a positive feedback loop that reinforces their downstream effects leading to the establishment of the growth arrest including senescence or the induction of apoptosis. Despite of the above described functional interaction between PLM and p53, a few studies have suggested that in fact pRB, rather than p53, may be the essential protein for PML-triggered apoptosis or senescence in human cells. Firstly, forced expression of PML was able to induce cell cycle arrest in liver tumor cell regardless of p53 status[395]. Secondly, the inactivation of pRB in human fibroblasts prevented PML-induced senescence, while the inactivation of p53 was not sufficient to block it and maximally delayed the process[222]. However one study showed that although PML was shown to physically interact with pRB, functionally, PML and pRB did not appeared to be mutually necessary to exert their growth suppressor activities[336]. Therefore it is likely that PML can simultaneously affect multiple pathways involved in cell-cycle regulation and further research is necessary to reveal PLM, p53 and pRB crosstalk.

PML in DNA-damage response

As was discussed in previous section 2.3., maintaining of genome integrity is essential for proper cellular functions and organism homeostasis. Although precise function of PML and PML NBs in DNA repair and DNA damage checkpoints remain to be elucidated, it is clear that PML is at least involved in modulation of the cellular response to DNA damage and linked processes: senescence and apoptosis.

One of the strong evidence for the role of PML in DNA damage response is the fact that PML is the direct target of key regulators of this process. PML is phosphorylated by Chk2 (S117)[315] and by ATR[313] in response to genotoxic stress. Interestingly, both kinases are targeted to PML NBs following DNA damage[314]. Additionally, recent study has demonstrated that PML interacts with Chk2 and mediates its autophosphorylation, which is an essential step for Chk2 activity that occurs after phosphorylation by the upstream kinase ATM[396]. Secondly, number of PML NBs increases in response to genotoxic stress in ATM- and ATR-dependentent manner[314, 397].

And a subset of PML NBs colocalizes with sites of new DNA synthesis, which is considered as marker for active DNA repair[356], as well as with sites of single[398, 399] and double stranded breaks and γH2AX (phosphorylated histone H2AX, a marker of ss and ds breaks, a sensor of DNA damage) foci[282, 397]. Thirdly, several proteins involved in DNA repair and DNA damage response partially colocalize or pass through PML NBs in a temporary regulated manner and their activity can be altered upon transition through them[282, 319, 396, 400]. A subset of PML NBs colocalize with BLM, a member of the RecQ DNA helicase family, at sites of nucleotide excision repair (NER) induced by UV-C radiation [356, 400]. Another RecQ DNA helicase, WRN, whose loss results in premature aging (Werner syndrome), alters its regular location in the nucleolus and moves into nucleoplasmic foci containing Rad51 and RPA that partially overlap with PML NBs upon DNA damage caused by irradiation[314, 340]. Also the DNA damage response protein TopBP1 that under normal conditions does not associate with PML NBs translocates into them in response to ionizing irradiation[399].

Double stranded breaks are repaired in eukaryotes by the concerted action of mechanisms based on homologous recombination (HR) or non-homologous end joining (NHEJ) [401, 402]. The highly conserved MRN complex, whose core contains the proteins Mre11, Rad50 and NBS1, plays a role in both modes of DSB repair, particularly in the HR pathway[403, 404]. The members of MRN complex were found to colocalize with a subset of PML NBs in unstressed cells[282, 341, 342]. Intriguingly, Mre11 and NBS1 rapidly dissociate for PML NBs after γ-irradiation and reassociate later during recovery or in cells arrested in G2 phase[314]. Similarly, many other DNA repair factors colocalize with PML NBs in late time points of DNA damage suggesting that PML could rather play a role in late phases of DNA repair[282, 397]. Or taken together with the fact that colocalization of PML NBs and ssDNA is particularly efficient in cells with compromised DNA repair mechanisms[398], it is assumed that PML NBs might mark sites with irreparable DNA damage and promote signaling to checkpoint pathways. Nevertheless, the role of PML NBs in HR is supported by existence of specialized PML structures in ATL cells (described in 2.4.6.), that are supposed to use HR for maintenance of their telomere length[353]. Approvingly, in comparison to wild type cells, PML deficient cells exhibit a high frequency of sister chromatid exchange, which is disorder found in cell with defective homologue recombination[339].

PML and regulation of apoptosis

Many studies have established that PML protein is an important factor in the regulation of both p53-dependent and p53-independent apoptotic pathways[275, 337, 382, 405, 406]. The direct demonstration for a physiological role of PML in apoptosis control came from the phenotypic analysis of PML–/– mice[274, 275]. Cells derived from PML–/– mice presented defects in apoptosis induced by Fas, TNF, interferons and ceramides[275]. Apoptosis induction was reduced, but not abrogated, implying a role for PML as a modulator, rather than as an essential trigger. Although the role of PML in apoptosis is still under investigation, one of the possible mechanisms may involve Daxx. Daxx is protein exhibiting both pro- and anti-apoptotic activities (for a review, see[407]). The majority of Daxx appear to colocalize with PML NBs[319, 335]and the C-terminal region of Daxx directly interacts with PML[335]. Daxx localization to PML NBs correlates with its ability to sensitize cells to Fas- and splenocyte activation-induced apoptosis[335, 408]. Therefore it was suggested that pro-apoptotic function of Daxx may require PML NBs locatication[335, 408], whereas its anti-apoptotic functions are exerted outside of PML NBs[409]. However, one recent study has proposed that also apoptosis opposing action of Daxx can be connected with PML NBs in cell type specific manner[410].

PML and regulation of cellular senescence

Originally, PML was implicated in induction of cellular senescence because it was found to be one of the genes up-regulated upon oncogenic ras-induced arrest in human diploid fibroblasts IMR90[283] and mouse embryonic fibroblasts[225]. Together with elevated levels of PML mRNA and protein, also number and size of PML NBs increased[225, 283]. Furthermore, replicative senescence induced by extensive passaging of IMR90 cells led to upregulation of PML and PML NBs[283]. Additionally, we have described that replicative senescence in hMSC or premature BrdU/DMA-induced senescence in several human cell lines led to enhanced expression of PML and subsequent increase of PML NBs number (Research paper II and III). In contrast to wild type cells, oncogenic ras did not induced growth arrest and senescence phenotype in PML-/- cells, suggesting that PML is required for ras-induced senescence[225]. On the other hand, overexpression of PML induces either growth arrest associated with premature senescence onset[225, 283] or apoptosis depending on the cell type and/or level of expression (reviewed in[405]). The subsequent detailed study showed that overexpression of one specific isoform of PML (PML IV), but not other isoforms, is

capable to induce senescence in human and mouse fibroblasts[301]. PML IV-induced senescence cannot be bypassed by ectopic expression of catalytic subunit of telomerase, indicating telomerase independence of this process[301]. Interestingly, PML-/- cells were resistant to PML IV–induced senescence, implying that PML IV alone is necessary but not sufficient for this process to occur and cooperation of other isoforms (or some factor not expressed in PML-/- cells) is required[301].

PML has been shown to influence activation of p53, a central regulatory switch in a network controlling cell proliferation and apoptosis. In ras- and PML IV-induced senescence, PML contributes to stabilization and transcriptional activation of p53 by mediating its acetylation and phosphorylation[225, 301]. Phosphorylation of p53 at S46 is mediated by HIPK2 (homodomain-interacting protein kinase 2), which is recruited to PML NBs exclusively by PML IV isoform[411]. This phosphorylation facilitates CBP-mediated acetylation of p53 at K382 that is required for optimal activation of p53 and expression of p53-dependent genes[225]. In both types of senescence, a ternary complex of PML/p53/CBP is formed and presence of PML elevates acetylation of p53 by CBP[225, 283]. Although recruitment of p53 and CBP to PML NBs is enhanced in response to senescence[225, 283], they can form the functional pro-senescent complex with PML also independently of PML NBs [301]. Intriguingly, PML- and ras-induced senescence can be antagonized by action of deacetylase SIRT1 (Sir2-like protein 1) that reverts acetylation of p53 and is recruited to PML NBs[412].

In addition, PML was reported to colocalize within nuclear bodies with the nonphosphorylated fraction of the pRB, other key player in senescence[336]. In ras-induced senescence, the fraction of pRB colocalizing with PML NBs was remarkably increased[283]. However, the necessity of PML and pRB interaction for senescence onset is in question (discussed above).

Finally, the recent studies proposed that PML NBs are required for activation of HIRA and HP1γ, i.e. the proteins that are involved in senescence-induced heterochromatinization and formation of heterochromatin foci, SAHF (for detail see section 2.3.4, reviewed in[228]).

2.4.8. Acute promyelocytic leukemia

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) comprising of about 10% of AML[413]. Molecular basis of APL is chromosomal

translocation invariably involving retinoid acid receptor alpha (RARα) gene on chromosome 17. Approximately in 98% of all cases of APL a translocation partner for RARα is promyelocytic leukemia protein (PML) gene located on chromosome 15. This t(15;17)(q22;q21) translocation leads to production of reciprocal fusion proteins PML-RARα and RARα-PML[263-265]. PML fusions proteins are not able to form PML NBs, thus in APL cells PML NBs are dispersed and form a microparticulate pattern in the nucleus and cytoplasm[414-416]. However, the retinoic acid treatment (discussed below) restores formation of PML NBs[414-416], which correlates with disease remission. Other APL associated gene rearrangements were reported with promyelocytic leukemia zinc finger protein, PLZF (11q13)[417], nucleophosmin, NPM (B23, 5q35)[418], nuclear mitotic apparatus protein, NuMA (11q23)[419], signal transducer and activator of transcription, STAT5b (17q21)[420], and newly also the regulatory subunit of protein kinase A, PRKAR1A (17q24)[421]. These fusion proteins are considered to be responsible for differentiation block of myeloid line and for following accumulation of immature cells (at promyelocytic stage) in bone marrow (for a review about APL, see[273]).

Complete remission of the disease is usually achieved by treatment with all-*trans* -retinoic acid (RA) and chemotherapy. However, the therapy is not successful in all cases. For example, APL associated with production of PLZF-RARα or STAT5b-RARα fusion proteins is resistant to this treatment[420, 422]. Moreover, even after achieving remission in RA-responsible types of APL, the disease inevitably relapses and becomes soon resistant to RA treatment. This acquired resistance correlates with mutations in ligand-binding domain of fusion protein in about 25% of RA-refractory patients[423, 424], but in remaining cases the mechanism is unknown. To cover RA-resistant cases and to make the cure more effective, other drugs such as As_2O_3 (triggering the proteasome-dependent degradation of PML/RARα, leading to complete remission of majority RA-resistant patients[425]) and inhibitors of HDACs and histone methyltaransferases are tested in combination with RA.

Molecular mechanism of APL

The molecular mechanism underlying APL and proposing new possibilities in APL treatment was described previously[426-428]. It was shown that RARα in normal cells binds to retinoic acid response elements (RARE) located in the promoters of retinoic acid (RA) target genes and modulates transcription through an interaction with various specific cofactors. In the absence of ligand (RA), RARα associates with corepressor

molecules such as N-CoR and SMRT recruiting histone deacetylases to the target genes. Resulting histone deacetylation leads to chromatin reorganization and repression of transcription. On the other hand, in the presence of RA, corepressor complex dissociates, and a number of transcriptional coactivator proteins (including HATs) binds to RARα leading to the activation of RA-inducible gene transcription. In APL cells, RARα portion of fusion proteins retains functional DNA- and RA-binding domains. Thus, fusion proteins can still bind to RARE in gene promoters and recruit corepressor complex comprising histone deacetylases. In case of PML-RARα, the repressor complex is aberrantly not released after the treatment with physiological levels of RA (10^{-9}-10^{-8} M) due to the attached fusion partner that causes a stronger interaction of fusion protein with corepressor complex. Thus, higher pharmacological doses of RA (10^{-7}-10^{-6} M) are required to induce dissociation of corepressor complex and to activate transcription[429]. However, in APL associated with PLZF-RARα even pharmacological doses of RA fail to induce transcription of RA-activated genes. This resistance is explained by the presence of two N-CoR binding sites in PLZF-RARα. One is in RAR part of fusion protein and pharmacological doses are able to release repressor complex from it. The second N-CoR binding site is in PLZF part and corepressor complex with histone decatylases bound to this site is resistant to RA. Moreover, RAR fusion partners are able to oligomerize causing stochiometric increase of HDAC-containing repressor complexes on RA-responsible promoters[430, 431]. Based on this model, it has been suggested that in addition to RA, HDAC inhibitors could be used for APL treatment in order to eliminate the repressing effect of HDACs. The observation that TSA, an HDAC inhibitor, caused reactivation of RA-inducible genes in APL cells[427] strongly supports this hypothesis.

Later, it was shown that not only HDACs are recruited to RAR fusion proteins to block transcription, but whole chromatin remodeling machinery is involved to establishing and maintenance of silenced chromatin state on RA target genes. Recruitment of histone methyltransferase SUV39H1[432], DNA methyltransferases[433], methylated DNA binding protein MBD1 (through HDAC3)[434], and polycomb repressive complex 2[435] was reported.

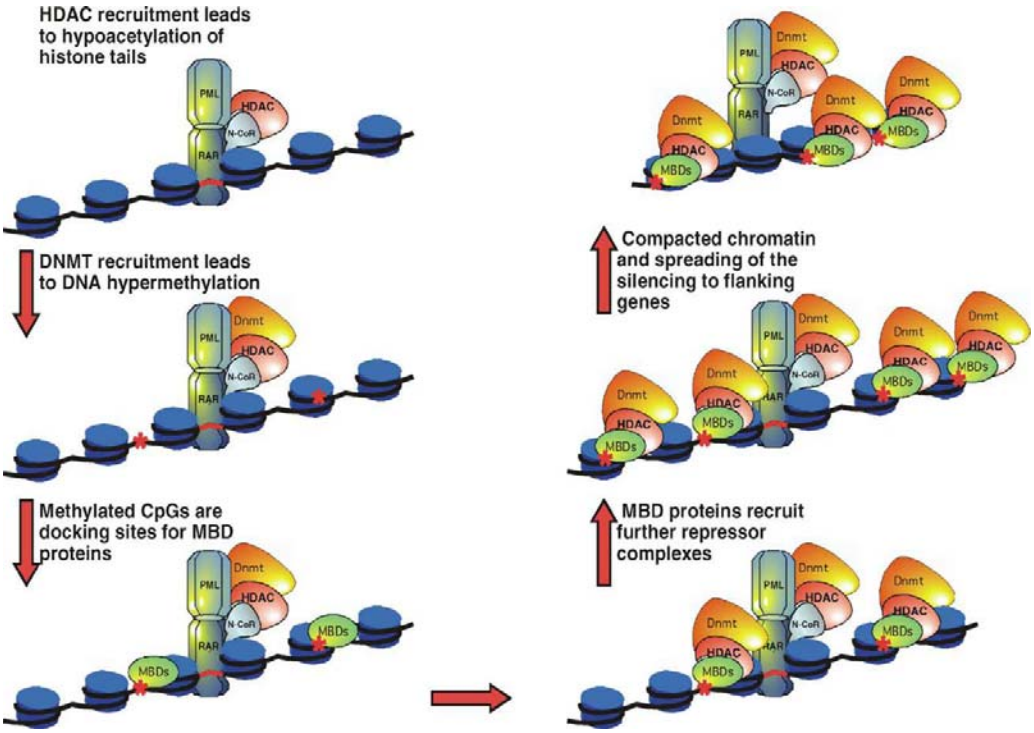

Figure 15: PML-RARα mediated gene repression.
Schematic representation of the step-wise silencing of PML-RARα target genes. The oncoprotein recognizes a well-defined DNA sequence (depicted in red) and recruits repressor enzymes, such as HDACs and DNA-methyltransferases (DNMTs), the activity of which leads to hypoacetylation of histone tails, DNA methylation, and transcriptional silencing. Methylated CpGs (red asterisk) are docking sites for methylated DNA binding protein (MBD) proteins, which can in turn recruit further repressor enzymes. The progression wave of the proposed mechanism might ''close'' the chromatin structure and could even influence neighboring genes.
(from Villa et at. 2004[436])

3. AIMS OF THE STUDY

The presented PhD thesis was elaborated as a part of long-term project carried out by the group of Zdeněk Hodný in the Laboratory of Biology of the Cell Nucleus studying the role of PML and PML nuclear bodies in response to genotoxic and non-genotoxic stresses. The objective of this PhD study was to contribute to clarification of mechanisms regulating PML expression during interferon response and in senescent cells and to evaluate hMSC cells as a model for PML studies.

The specific aims of this PhD study were the following:

1. To examine the role of acetylation in IFNα-induced expression of PML gene.

2. To describe PML expression and nuclear compartmentalization in growing, differentiated and senescent hMSC.

3. To follow the effect of drug-induced premature senescence on PML expression and to reveal a molecular mechanism of its regulation.

4. COMMENTS ON PRESENTED PUBLICATIONS

Research Paper I

Vlasáková J, Nováková Z, Rossmeislová L, Kahle M, Hozák P, Hodný Z: Histone deacetylase inhibitors suppress IFNalpha-induced up-regulation of promyelocytic leukemia protein. Blood 2007;109(4):1373-80

One of the proposed functions of PML NBs is that they play a role in antiviral defence[366]. Interferons of both types (IFNα/β and IFNγ) dramatically increase expression of PML gene and other interferon-stimulated components of PML NBs (e.g. Sp100 and ISG20) resulting in increase of size and number of PML NBs. PML gene possessing two IFNs-responsible elements (ISRE and GAS) in its promoter was thus assigned to a group of ISGs[276]. The transcription of several ISGs was reported to be impaired by inhibitors of histone deacetylases suggesting that deacetylation could be required for their expression (reviewed in[154]).

In this paper we concentrated on deciphering a role of acetylation in regulation of PML expression. We explored whether and how HDACIs, the agents causing overall protein hyperacetylation, influence induction of PML by IFNα. By indirect immunofluorescence using antibodies against two main components of PMN NBs, PML and Sp100, we showed that IFNα-induced increase of number of these structures is blocked by presence of TSA, a HDAC inhibitor. Pretreatment with TSA and subsequent treatment with IFNα did not block the increase of PML NBs, suggesting that the TSA effect is reversible. In inverse experimental setting, number of PML NBs elevated by pretreatment with IFNα remained almost unchanged by subsequent exposition to TSA. This observation led us to the hypothesis that TSA does not cause dissociation of once assembled PML NBs, but rather causes a lack of their structural components and thus blocks their multiplication. We have confirmed this assumption by performing quantitative RT-PCR and western blot analysis. Although IFNα dramatically elevated both PML mRNA and PML protein levels, the simultaneous treatment with TSA abolished this increase. We reported the suppressing effect of TSA on IFN-induction of PML levels in human diploid fibroblasts, several human lines (HeLa, SaOS-2, HEK293T, K562, and Jurkat), and mouse NIH-3T3 cells indicating operation of the

same mechanism independently on the tissue origin and in different species. Interestingly, in contrast to IFNα-induced expression of PML, the basal expression of PML and number of PML NBs were influenced only moderately by presence of HDACIs. We therefore hypothesize that basal levels of PML are maintained independently of Jak-STAT signaling pathway. This is further supported by the observation that mouse cells deficient of STAT1 (the main mediator of IFN signaling), readily express PML and form PML NBs (our unpublished data).

In next step, we tried to reveal mechanism of TSA mediated suppression of IFNα-induced PML expression. Previously published data concerning the effect of HDACIs on Jak-STAT pathway transiting IFN signal to its target genes were highly controversial, both impairment and preservation of this pathway were reported. To check intactness of Jak-STAT pathway, we used antibody recognizing STAT2, a component of ISGF3 complex, which is a transcription factor localizing into nucleus and binding to ISRE elements of target genes after IFNα/β stimulation. Cellular fractionation and chromatin immunoprecipitation revealed that TSA does not block IFNα-induced translocation of STAT2 into nucleus and its binding to ISRE element of PML promoter. Therefore we assume that the deacetylation event inhibited by HDACIs and necessary for initiation of transcription of PML lies downstream of Jak-STAT signaling pathway, which supports and is consistent with the results of Nusinzon et al.[118] and Chang et al.[117]. The identification of executive HDAC(s) would also contribute to clarifying the mechanism of HDACIs effect on IFN pathway. Unfortunately, majority of HDACIs are poorly selective or nonselective inhibitors blocking activity of most or all HDACs of class I and class II[76]. In our study all tested HDACIs, TSA, sodium butyrate, SAHA, valproic acid, and MS-275 (at low concentration selective for HDAC1), were able to block IFNα-induced expression of PML at mRNA and protein level, although in different extent. MS-275 exhibited weaker inhibiting effect than TSA or butyrate suggesting that HDAC1 could be one, but not the only one, of HDAC enzymes required for full transcriptional activation of ISGs.

Finally, we showed that also IFNα-activation of two other ISGs, Sp100 and IRF1, is negatively affected by the presence of TSA. Sp100 is a structural component of PML NBs and its lack most likely contributes to the observed impairment in multiplication of PML NBs in IFNα response. IRF1 plays a role in delayed IFN response and can bind to PML promoter and stimulates its transcription. Although TSA-

suppression of IFR1 induction by IFNα was modest, we can hypothesize, that it at least partially has impact on PML expression.

In summary, we have shown for the first time that IFNα-induction of PML, Sp100 and IRF1 is suppressed by HDACIs. Our findings further support and extend studies that deacetylation event is necessary for full transcriptional activation of many if not all ISGs and that HDACIs are able to block it. Moreover, our study may have important clinical impact. HDACIs are currently intensively tested for clinical praxis as potent anticancer drugs, some of them entered advanced phases of clinical trials and SAHA has been recently approved for treatment of one type of lymphoma. However, the mechanisms of their effects are often unclear and potential interference with fundamental cell signaling pathways is only foreshadowed. Understanding the biological activity of HDACIs will help to predict the long-term effects of these drugs on normal cells and to avoid potential side effects. Specifically, the effect of HDACIs on IFN pathway seems to be important since it can interfere with physiological functions of IFNs that play a critical role in cellular antiviral defense and are also widely used for treatment of various cancer types. Although, the specific histone deacetylase(s) involved in activation of ISGs remain(s) to be identified, this finding can lead to design or to selection of anti-tumor HDAC inhibitors that would not perturbe the interferon pathway.

In addition, as PML expression and IFN response pathway are activated in senescent cells (Research Paper III), the understanding of regulation of interferon stimulated genes expression could help to reveal molecular mechanisms lying behind this process.

Research Paper II

Janderová-Rossmeislová L, Nováková Z, **Vlasáková J**, Philimonenko V, Hozák P, Hodný Z: PML protein association with specific nucleolar structures differs in normal, tumor and senescent human cells. J Struct Biol. 2007;159(1):56-70

A role of PML as a tumor suppressor is underscored by the fact that PML protein level is diminished in majority of tumors. PML gene expression seems to be differently regulated in the tumor cells[290]. Nevertheless, the most of in vitro studies of

PML and PML NBs have been carried out on immortalized aneuploid cell lines with compromised ability to regulate their growth. This encouraged us to study PML expression and PML NBs in normal diploid cells.

In the presented paper we employed the model of normal diploid human mesenchymal stem cells (hMSC) that are pluripotent precursor cells readily proliferating and retaining the ability to differentiate[437]. By indirect immunofluorescence we followed expression of PML and localization of PML NBs in hMSC. Exponentially growing, confluent, and terminally differentiated (into adipocytes) hMSC express PML protein that localize in PML NBs and also is found free in nucleoplasm. Similarly as in other cells, PML mRNA and PML protein levels in hMSC are inducible by treatment with IFNα. Interestingly, number of PML NBs strongly increases with proliferative age of hMSC. The number of PML NBs in replicatively senescent cells is almost three-time higher than in early passages.

Furthermore, we reported various forms of novel association of PML with nucleoli upon standard growth conditions as well as after inhibition of rRNA synthesis in hMSC and normal diploid human fibroblasts. Apart from observed PML protein positive spots on the surface of nucleus, we often reported donut-like structures, positive for several PML NBs associated proteins (PML, Sp100, SUMO-1, Daxx). The PML donut structures were attached to or in close proximity of nucleoli and appearance of typical nucleolar proteins within these donut structures confirmed the relationship of PML NBs and nucleolus. Inhibition of polymerase I by actinomycin D resulting in nucleolar inactivation and segregation led to formation of PML coats, structurally different from PML donut structures, on the surface of segregated nucleoli (often engulfing majority of or whole segregated nucleolus). Interestingly, in contrast to hMSC and normal human fibroblasts we have found that several immortalized human cell lines with defects in the p53 and pRB pathways (HeLa, U-2 OS, A549, SaOS-2, H1299) do not form PML structures associated with neither active nor inactive nucleoli even though they express PML protein. The absence of nucleolar PML compartment in rapidly growing tumor-derived cells suggests that PML association with the nucleolus might be important for cell-cycle regulation. In line with this, the formation of PML structures associated with nucleoli was reestablished in cell lines prematurely senescent by drugs causing genotoxic stress (5-bromo-2'-deoxyuridine and distamycin A), which correlated with reactivation of p53 and pRB pathway and subsequent withdrawal from the cell cycle. Importantly, the binding to the nucleolus was enhanced in replicatively

senescent hMSC. These findings indicate that PML may be involved in nucleolar functions of senescent cell.

Till today, PML relocalization to nucleolus was reported only under stress conditions like inhibition of proteasomal degradation[438] or extensive DNA damage[313]. Our paper brought the first evidence that PML associates with nucleoli also under normal growth-permitting conditions and that this association is increased in senescent cells. Our results were confirmed by later published paper describing the presence of nucleoli-associated PML donut structures in unstressed WI-38 human primary fibroblasts and their increasing abundance during the progression toward senescence[439]. Furthermore, they reported translocation of PML I, PML IV, and PML V isoforms to the nucleoli in response to various types of cellular stress (UV-C- and γ- irradiation; doxorubicin, a topoisomerase II inhibitor; actinomycin D). Doxorubicin treatment and γ-irradiation lead to appearance of PML donut structures containing nucleolar proteins[439].

Taken these findings together, we propose that in normal and senescent tumor cells PML responds to changes in the transcription activity and structure of the nucleoli by translocation to the nucleolar surface and that this association could be important for the tumor suppressor activity of PML.

Reaserch Paper III

Nováková Z, Janderová-Rossmeislová L, **Dobrovolná J**, Vašicová P, Hořejší Z, Bártek J, Hozák P, Hodný Z: Sustained activation of Jak/STAT signaling pathway and induction of interferon simulated genes in 5-bromo-2'-deoxyuridine and distamycin A-induced senescence (submitted manuscript)

Cellular senescence is characterized as a persistent block of proliferation, which is proposed to protect an organism from the unrestricted growth of cells with damaged genome. Several studies implicated PML to play substantial role in replicative and oncogene-induced senescence[225, 283, 301] (Research Paper II). PML expression is upregulated in senescent cells[225, 283] and PML IV isoform is sufficient to induce premature senescence[301]. However, events that initiate PML expression in replicatively or prematurely aging cells remained unclear.

In this project we attempted to examine changes in PML expression and PML NBs formation in senescent cells and to study mechanisms contributing to induction of PML in senescent cell. For this purpose we established in vitro model of drug-induced senescence using 5-bromo-2'-deoxyuridine and/or distamycin A that promptly provoke senescence even in tumor cells. Already after six days of 100 μM BrdU (in the text below referred as BrdU) or 10 μM BrdU/10 μM DMA (BrdU/DMA) treatment we have observed morphological changes characteristic for senescent cells and positivity for SA-β-gal staining, at the same time immunofluorescence revealed four- or five-fold elevation of PML NB number, respectively. DMA strongly synergized the effect of BrdU as 10 μM BrdU caused only less than two-fold increase of number of PML NBs. Western blot and quantitative RT-PCR analysis confirmed that PML expression was markedly elevated in several human cell lines senesced by BrdU or BrdU/DMA treatment. Numerous studies have reported dramatic changes in protein turnover in senescent cells (reviewed in[440]). To exclude possibility that observed increase of PML protein levels is not caused by changed protein stability, we followed levels of PML protein in presence cycloheximide that blocks translation. PML protein appeared to be stable for at least 24 hours and no alteration of its stability was observed in senescent cells.

We concentrated further on the exploration of the mechanism leading to up-regulation of PML in senescent cells. As the most potent inducers of PML expression are interferons, we checked whether Jak-STAT signaling pathway is activated. Indeed, we have found out that levels of STAT1 protein, a key mediator of IFN signal, are significantly elevated and STAT1 was in its active state (phosphorylated at Tyr701 and Ser727) in drug-senescent cells. As the medium conditioned by senescent cells was able to induce PML up-regulation in untreated cells *per se*, we started to search for activator of Jak-STAT pathway secreted into medium by senescent cells. Enzyme-linked immunosorbent assay revealed elevated levels of IFNβ in medium collected from senescent cells. Moreover, senescent cells exhibited increased levels of IFNβ mRNA as was detected by quantitative RT-PCR. IFN response is a complex mechanism influencing expression of about 300 of genes[97]. We examined changes in expression of 84 ISGs using RT^2 Profiler PCR Array System that showed that many of them (between mostly induced were e.g. Mx1, ISG15, 2',5'-oligoadenylate synthetase 1, IRF1, IRF7) are upregulated in drug-induced senescent cells. By quantitative RT-PCR and western

blot analysis we verified that mRNA and protein levels of IRF1 and IRF7 significantly increased after BrdU or BrdU/DMA treatment. These two proteins are involved in delayed IFN response and their elevation could explain prolonged activation of Jak-STAT pathway.

In summary, we have shown that drug-induced senescent cells produce IFNβ and other cytokines (our unpublished data), which then through auto/paracrine mechanism activates Jak-STAT pathway that mediates upregulation of several ISGs. Among these genes belongs PML, which upregulation resulted in elevation of PML NBs numbers. This phenomenon is thus common to replicative, oncogene- and drug-induced senescence that further supports the role of PML in onset and/or maintenance of senescence. Furthermore, observed upregulation of IRF1 and IRF7, which could be an induced response to stress, e.g. DNA damage that is currently thought as major cause for senescence onset, can initiate expression of IFNβ and/or take part in next rounds of transcriptional activation of IFNβ and other ISGs. The positive feedback loop of IFNβ-IRF1/IRF7-IFNβ might be responsible for sustained Jak-STAT signalling. Based on our results, known antiproliferative activity of IFNβ and the observation that IFNβ can induce senescence in several cell lines[242], we hypothesized that secretion of IFNβ could substantially contribute to maintenance of senescence state. The detailed analysis of the cytokine production by senescent cells is important for medical applications because premature senescence is considered as a potential outcome of the cancer treatment and it is supposed to be a critical effector program triggered in the response to DNA damaging chemotherapeutics[178]. Therefore, it is important to consider the risk that senescent cells may have detrimental effect on neighboring cells exposing them to the pro-inflammatory cytokines and initiate secondary tumor development. The determination of the senescence-associated cytokine production may also contribute to our knowledge about the mechanisms responsible for significant changes in gene expression during cell senescence.

5. CONCLUSIONS

Major results of this PhD thesis can be summarized as follows:

1. For full transcriptional activation of ISG (PML, Sp100, IRF1) by interferon α the deacetylation of unknown factor(s) is necessary. IFNα-induced expression of PML at mRNA and protein level and subsequent increase of PML NBs are suppressed by all tested HDACIs independently on cellular origin. Importantly, basal (i.e. IFNα-nonstimulated) expression of PML is not influenced by HDACIs. The classical Jak-STAT pathway mediating transition of IFNα signal remains unaffected by HDACIs and the target of HDACIs lies downstream of ISGF3 binding to PML promoter. Adverse affects of HDACIs used in clinical praxis are predicted.

2. Human mesenchymal stem cells (growing, confluent or terminally differentiated) express PML protein, form PML nuclear bodies and respond to IFNα treatment by dramatic elevation of PML mRNA and protein levels and moderate increase of PML NBs. Number of PML NBs per cell nucleus is dependent on the culture proliferative age. In normal diploid but not in immortal cells PML forms various nucleoli-associated structures. The localization of PML into nucleoli is reestablished in senescent cells. The importance of PML association with nucleoli for its tumor suppressor activities is hypothesized.

3. Drug-induced senescence in several human cell lines leads to increased PML expression and formation of PML NBs. The senescent cells have changed sercetory phenotype and exhibited elevated expression of IFNβ and other cytokines. These cytokines probably through auto/paracrine mechanism activate Jak-STAT signaling pathways resulting in up-regulation of ISGs including PML, STAT1, IRF1, IRF7, Mx1, ISG15, 2',5'-oligoadenylate synthetase 1, and others. Observed activation of IRF1 and IRF7 suggests that senescent phenotype, can be sustained in positive feedback loop.

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7. LIST OF PRESENTED PUBLICATIONS

Research Paper I
Vlasáková J, Nováková Z, Rossmeislová L, Kahle M, Hozák P, Hodný Z: Histone deacetylase inhibitors suppress IFNalpha-induced up-regulation of promyelocytic leukemia protein. Blood 2007;109(4):1373-80

Blood IF 2006: 10,37

Research Paper II
Janderová-Rossmeislová L, Nováková Z, **Vlasáková J**, Philimonenko V, Hozák P, Hodný Z: PML protein association with specific nucleolar structures differs in normal, tumor and senescent human cells. J Struct Biol. 2007 Jul;159(1):56-70

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Research Paper III
Nováková Z, Janderová-Rossmeislová L, **Dobrovolná J**, Vašicová P, Hořejší Z, Bártek J, Hozák P, Hodný Z: Sustained activation of Jak/STAT signaling pathway and induction of interferon simulated genes in 5-bromo-2'deoxyuridine and distamycin A-induced senescence (submitted manuscript)

Please, note that in two publications the author of this PhD thesis Jana Dobrovolná is referred by maiden name – Vlasáková.