

**REGULATION OF TUMOR SUPPRESSOR PROTEIN p53 IN
CELLULAR STRESS AND TUMORIGENESIS**

SARI TOJKANDER

MOLECULAR AND CANCER BIOLOGY PROGRAM, BIOMEDICUM HELSINKI,
DEPARTMENT OF VIROLOGY, HAARTMAN INSTITUTE
AND
HELSINKI GRADUATE SCHOOL OF BIOTECHNOLOGY
AND MOLECULAR BIOLOGY, UNIVERSITY OF HELSINKI,
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Faculty of Medicine of the University of Helsinki,
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Thesis supervisor

Professor Marikki Laiho
Molecular and Cancer Biology Program
Biomedicum Helsinki and
Haartman Institute
University of Helsinki
Helsinki, Finland

Thesis reviewers

Docent Ismo Ulmanen
Department of Molecular Medicine
National Public Health Institute
Helsinki, Finland

Professor Kirsi Vähäkangas
Department of Pharmacology and Toxicology
University of Kuopio
Kuopio, Finland

Thesis opponent

Associate Professor Mats Ljungman
Department of Radiation Oncology
Division of Cancer Biology
University of Michigan
Michigan, USA

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ABSTRACT

Functional loss of tumor suppressor protein p53 is a common feature in diverse human cancers. The ability of this protein to sense cellular damage and halt the progression of the cell cycle or direct the cells to apoptosis is essential in preventing tumorigenesis. Tumors having wild-type p53 also respond better to current chemotherapies. The loss of p53 function may arise from *TP53* mutations or dysregulation of factors controlling its levels and activity. Probably the most significant inhibitor of p53 function is Mdm2, a protein mediating its degradation and inactivation. Clearly, the maintenance of a strictly controlled p53-Mdm2 route is of great importance in preventing neoplastic transformation. Moreover, impairing Mdm2 function could be a nongenotoxic way to increase p53 levels and activity. Understanding the precise molecular mechanisms behind p53-Mdm2 relationship is thus essential from a therapeutic point of view.

The aim of this thesis study was to discover factors affecting the negative regulation of p53 by Mdm2, causing activation of p53 in stressed cells. As a model of cellular damage, we used UVC radiation, inducing a complex cellular stress pathway. Exposure to UVC, as well as to several chemotherapeutic drugs, causes robust transcriptional stress in the cells and leads to activation of p53. By using this model of cellular stress, our goal was to understand how and by which proteins p53 is regulated. Furthermore, we wanted to address whether these pathways affecting p53 function could be altered in human cancers.

In the study, two different p53 pathway proteins, nucleophosmin (NPM) and promyelocytic leukemia protein (PML), were found to participate in the p53 stress response following UV stress. Subcellular translocations of these proteins were discovered rapidly after exposure to UV. The alterations in the cellular localizations were connected to transient interactions with p53 and Mdm2, implicating their significance in the regulation of p53 stress response. NPM was shown to control Mdm2-p53 interface and mediate p53 stabilization by blocking the ability of Mdm2 to promote p53 degradation. Furthermore, NPM mediated p53 stabilization upon viral insult. We further detected a connection between cellular pathways of NPM and PML, as PML was found to associate with NPM in UV-radiated cells. The observed temporal UV-induced interactions strongly imply existence of a multiprotein complex participating in the p53 response. In addition, PML controlled the UV response of NPM, its localization and complex formation with chromatin associated factors.

The relevance of the UV-promoted interactions was demonstrated in studies in a human leukemia cell line, being under abnormal transcriptional repression due to expression of oncogenic PML-RAR α fusion protein. Reversing the leukemic phenotype with a therapeutically significant drug was associated with similar complex formation between p53 and its partners as following UV. In conclusion, this thesis study identifies novel p53 pathway interactions associated with the recovery from UV-promoted as well as oncogenic transcriptional repression.

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ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

- I Kurki, S., Latonen, L. and Laiho M. Cellular stress and DNA damage invoke temporally distinct Mdm2, p53 and PML complexes and damage-specific nuclear relocalization. *J. Cell Sci.* **116**: 3917-3925 (2003).
- II Kurki, S., Peltonen, K., Latonen, L., Kiviharju, M., Ojala, P. M., Meek D. and Laiho, M. Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. *Cancer Cell.* **5**: 465-475 (2004).
- III Kurki, S., Syrjäkari, H. and Laiho, M. NPM and PML interact and cooperate in the regulation of p53 pathway in cell stress and tumorigenesis. Manuscript, submitted.

ABBREVIATIONS

6-4PP	(6-4)-photoproduct
ALCL	Anaplastic large cell lymphoma
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ARF	alternative reading frame
ATM	Ataxia telangiectasia mutated
ATO	arsenic trioxide, As ₂ O ₃
ATR	Ataxia telangiectasia-related
Bcl-2	B-cell lymphoma 2
BER	base excision repair
BLM	Bloom syndrome
C	carboxy
CDK	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
CPD	cyclobutane-type pyrimidine dimer
CS	Cockayne syndrome
DBD	DNA binding domain
DFC	dense fibrillar component
DRB	5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole
DSB	double strand break
FC	fibrillar center
FRAP	fluorescence recovery after photobleaching
GC	granular component
GGR	global genomic repair
HAT	histone acetyl-transferase
HDAC	histone deacetylase
Hdm2	human Mdm2
HIPK2	homeodomain-interacting protein kinase-2
HR	homologous recombination
IFN	interferon
IR	ionizing radiation
JNK	c-Jun N-terminal kinase
KSHV	Kaposi's sarcoma associated herpesvirus
MAP	mitogen-activated protein
Mdm2	murine double minute 2
MDS	Myelodysplastic syndrome
MEF	mouse embryo fibroblast
MMR	mismatch repair
N	amino
NB	nuclear body
NER	nucleotide excision repair

NES	nuclear export signal
NHEJ	non-homologous end-joining
NLS	nuclear localization signal
NoLS	nucleolar localization signal
NPM	nucleophosmin
NPMc ⁺	cytoplasmic NPM mutant
PCNA	proliferating cell nuclear antigen
PI-3-K	phosphoinositide-3-kinase
PML	Promyelocytic leukemia
PRD	proline-rich domain
RA	retinoic acid
RAR	retinoic acid receptor
Rb	retinoblastoma
RPA	replication protein A
RXR	retinoic-X receptor
SUMO	small ubiquitin-related modifier
TAD	transactivation domain
TCR	transcription-coupled repair
TET	tetramerization domain
TSA	trichostatin A
UV	ultraviolet
UVC	ultraviolet C radiation
Wt	wild type
XP	Xeroderma pigmentosum

INTRODUCTION

Cancer, a disease defined as abnormal proliferation and invasion of the cells, is one of the major causes of death in the western societies. The cellular changes leading to this disease may take several years to develop and due to the longer lifespan of the population, the frequency of cancer has increased dramatically.

The multistep process of cancer development requires several genetic changes over a long period of time. Each cell contains the genetic information that has to be replicated and passed to the next progeny in the process of cell cycle. This hereditary code is, however, altered constantly due to external pressure and the DNA in most of the cells experience numerous mutations every day. To support the precise genetic code from one cell generation to the next one, the cells have developed a number of regulatory pathways to monitor the entire process. Despite the high fidelity of this machinery, some occasional mistakes can be passed by this system and be further transferred to the progeny. Errors in the control of the damage response may lead to the accumulation of genetic lesions and multiple phases of clonal selection eventually results in uncontrolled growth and predisposition to cancer.

One of the key proteins in the regulation of the genomic integrity is tumor suppressor protein p53. p53 can prevent accumulation of harmful mutations, inhibiting tumor-promotion. Upon exposure to various kind of damage, p53 protein is activated and halts the cell cycle to give the repair machinery some time to solve the errors in the hereditary material. In case of excessive damage, however, the DNA may be in an unrepairable condition and the cell may have to choose a cell death pathway instead of the growth arrest to insure maintenance of the genome. The ability of p53 to induce this programmed cell death, apoptosis, is probably its major function in preventing the neoplastic transformation.

The early events leading to cellular p53 response are not totally understood, even though they have been studied extensively over the past two decades. Inactivation of the p53 pathway is very common in cancers and reactivation a potential key factor in killing tumor cells. Although preventing the incidence of cancer by eliminating the risk factors would probably be the most effective way of reducing the number of cancer cases, new therapeutic possibilities are required. Activation of the p53 pathway may have an important role in this process. Thus, knowing the factors that affect the function of p53 are critical to understand in detail. This study has concentrated on exploring the proteins that regulate p53 stability and functional activity in DNA-damaged cells. In addition, the aim was to find how these regulatory steps could be defective in human cancers.

REVIEW OF THE LITERATURE

CONTROL OF CELL PROLIFERATION

Formation of a multi-cellular organism requires proper regulation of the cell division and death in the developmental stage as well as in the renewal and maintenance of the functions of different tissues and organs in the adult body. Cells reproduce themselves by transmitting their genetic information to their daughter cells in a strictly controlled process of cell cycle. Any mistakes in the network of cell cycle-regulatory proteins may lead to aberrations of cellular functions in the next progeny and alterations in these key proteins of the cycle is thus a very common feature for a number of cancers.

The cell cycle

The eukaryotic cell cycle is divided into four phases (Figure 1) (reviewed in Nurse, 2000). The duplication of DNA takes place during the synthesis or S phase of the cycle. This phase of the cycle is the most time consuming, requiring usually about half of the cell cycle time. The segregation of the newly synthesized chromosomes to new daughter cells occurs in the mitosis, M phase of the cycle. Two gap phases, G1 and G2, are inserted between the synthesis and mitosis phases of the cycle. G1 and G2 phases provide the cell more time to grow and produce proteins required for DNA synthesis and cell division. During these periods, the cell can also monitor outside and inside signalling to ascertain that the conditions are appropriate for proceeding further in the cycle. Cells are also able to exit the division cycle to stay in a quiescent state, G0.

The cell cycle control system is conserved in all eukaryotes. The central regulatory proteins responsible for this system are the cyclins and their partners, cyclin dependent kinases, CDKs (Nurse, 2000). Sequential activation and inactivation of these protein complexes allows progression through the cycle (Figure 1). During G1, CDK4 and its homologue CDK6 are activated by complex formation with D-type cyclins. The expression of cyclin D is controlled by the mitogen-activated protein kinase (MAPK) pathway, playing a major role in entry to G1 phase (Lavoie et al., 1996). CDK4/6 phosphorylate and inactivate retinoblastoma protein (pRb), a tumor suppressor protein responsible for normal cell cycle progression (Weinberg, 1995; Classon and Harlow, 2002). In early G1, pRB is in its hypophosphorylated form, blocking the synthesis of DNA through inactivation of transcription factor E2F. When CDK4/6-cyclin D complex becomes active, it allows phosphorylation of pRB in late G1, leading to release of E2F and transcription of genes involved in DNA synthesis (Weinberg, 1995; Classon and Harlow, 2002). At the end of G1, CDK2 complexes with cyclin E and commits the cell to DNA replication phase (Tsai et al., 1993). CDK2 further phosphorylates pRb, resulting in complete inactivation of pRb. In S phase cyclin A replaces cyclin E from the complex and regulates DNA replication (Pagano et

al., 1992). The same cyclin can bind mitotic CDK1 and participate in the G2/M transition, although the key mitotic regulator is cyclin B in complex with CDK1 (Smits and Medema, 2001).

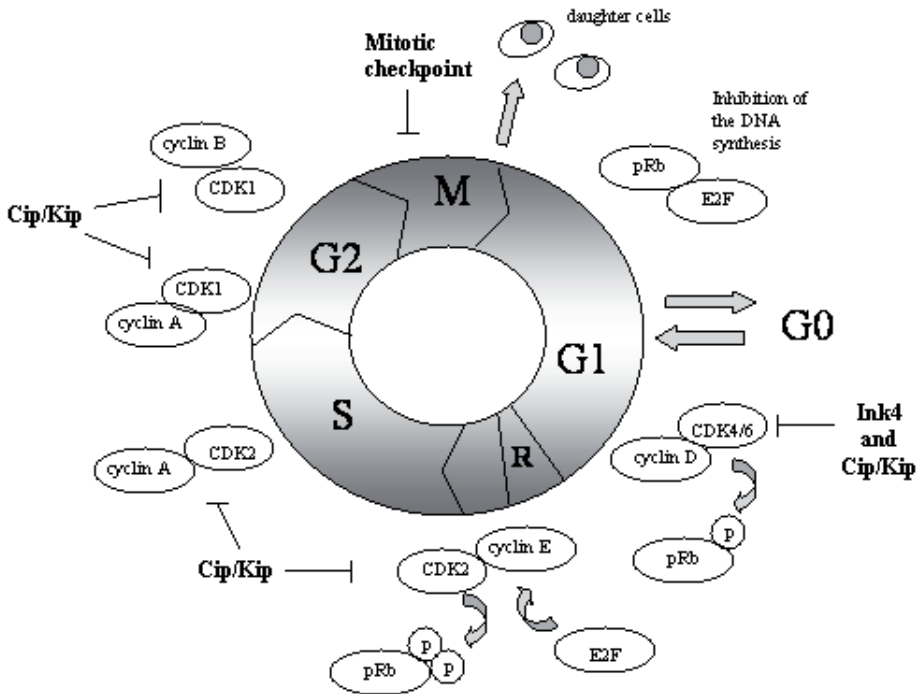


Figure 1. Regulation of the cell cycle. Progression through the cell cycle is precisely controlled by fluctuating activities of the cyclin-CDK complexes and CDK target pRb. See text for further details. CDK, cyclin-dependent kinase; P, phosphorylated; R, restriction point.

The oscillations in the activities of different CDK-cyclin complexes is influenced by several different factors, including rise and fall in the levels of cyclins through proteolytic degradation, inhibitory phosphorylations of the CDKs and binding of CDKs by their specific inhibitory proteins, cyclin dependent kinase inhibitors, CDKIs (Nurse, 2000). The CDKIs are further divided into two families: Ink4- and Cip/Kip-families, of which the Ink4 family members, p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d} prevent the activity of CDK4/6 by utilizing the same binding domain as D-type cyclins and Cip/Kip family proteins, p21^{Cip1}, p27^{Kip2} and p57^{Kip2} which bind and inactivate CDK1, CDK2 as well as CDK4/6 (Pines, 1997; Pavletich, 1999) (Figure 1).

Cell cycle checkpoints

Cells are in a continuous pressure on facing endogenous damage and stress due to changes in their environment and alterations in the growth conditions. Preserving genetic stability in the next generation of cells requires that cells are able to sensor and respond to extra- and intracellular signalling and that events in the cell cycle are properly timed and occur in an exact order. In addition, these events can occur only once in a cycle. To secure normal functions, cells have a specific control system that monitors the condition of the cell and is able to delay the progression of the cycle subsequent to damage, contact inhibition, senescence or growth inhibitory signals from other cells. These control points, referred to as checkpoints, screen and holdup the cell cycle at specific phases of the cycle and gain time for the cell to respond to the situation (Hartwell and Weinert 1989; Bartek and Lukas 2001b; Lukas et al., 2004).

The cell cycle checkpoints operate in late G1, S-phase and G2/M transition of the cycle and are controlled by the activation of pathways involving Cip/Kip and Ink4 family members (Figure 1). Transition from G1 phase to S phase requires the presence of growth factors. During mid and late G1, cells sensor outside and inside signalling for favourable conditions and request for a license to continue in the cycle. Loss in mitogenic signalling leads to rapid degradation of cyclin D and inhibits the cells from entering the S phase (Matsushime et al., 1991). In the presence of mitogenic signalling, active CDK4/6-cyclin D complex drives the cells into S phase through a point after which there is no return. If cells are allowed to bypass this restriction point (R) they start to synthesize DNA and the division is completed without extracellular signals, unless the conditions for some reason turn unfavourable (Pardee 1989; Bartek and Lukas 2001a & b) (Figure 1). Loss in the control of this restriction point appears to be a universal feature in the development of tumors, leading to aberrant mitosis. The S phase checkpoint insures proper replication of DNA and that the genetic material has been duplicated only once per cycle before cell division. In addition, this checkpoint monitors correct duplication of the centrosomes. After progression to G2, the cells can still assess the condition of the replicated DNA and halt the cycle if required. Lastly, the mitosis checkpoint, also referred to as spindle point checkpoint, monitors the attachment of chromosomes to the mitotic spindle. Any negative signal from an unattached kinetochore blocks progression to anaphase and delays the cycle.

DNA damage checkpoints

Genotoxic stress and errors in the DNA replication processes challenge the damage sensing system continuously. Depending on the damaging source, genetic material is subjected to different kind of mutations. Ionizing and ultraviolet radiation, mutagenic compounds and reactive oxygen species from metabolic pathways of the cell each cause particular type of lesions, triggering specific signalling cascades. The DNA damage checkpoints are evolution-

ary conserved and they largely overlap with the cell cycle checkpoints (Zhou and Elledge, 2000) (Figure 2). G1/S checkpoint delays entry into S phase if damaged DNA is discovered. In S phase, the replication-independent intra-S checkpoint slows down the DNA replication in damaged cells. G2/M checkpoint inhibits cells from going into mitosis if cells are exposed to DNA damage during G2 or they have unreparable damage left from the preceding phases of the cycle.

The DNA damage checkpoint system composes of multiple factors forming a complex signalling pathway. Although the checkpoints are in the different phases of the cycle, many of their signalling components are common and share the same upstream events (Bartek and Lukas 2001b; Lukas et al., 2004). Proteins of the checkpoint pathways can be divided into three groups: sensing factors that initially recognize the damaged site, signal transducers that mediate the signal further in the cascade and effector proteins, which induce the final response in the pathway (Iliakis et al., 2003). One of the most prominent sensors and activators of the checkpoint responses are the phosphoinositide-3-kinases (PI-3-Ks), Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia-related (ATR) kinases (Abraham, 2001; Shiloh, 2003). Activation of these primary sensors of lesions is followed by DNA damage in G1/S, S and G2/M phases of the cycle. Despite partly overlapping functions of these kinases, ATM seems to be more important in double strand breaks (DSBs) induced by ionizing radiation, while ATR responds to broader range of damage types, including UV radiation. ATM and ATR work through a signalling cascade involving checkpoint kinases, Chk1 and Chk2. In the activation of G1/S checkpoint, Chk1/Chk2 kinases mediate the degradation of Cdc25A phosphatase, holding cyclin E/CDK2 complexes inactive through an inhibitory phosphorylation (Mailand et al., 2000; Bartek and Lukas, 2001a). This response is acute, leading to a transient cell cycle arrest (Lukas et al., 2004). A slower and more sustained response is mediated by the p53 protein through its key downstream effector p21, resulting in delayed inhibition of the cyclin E/CDK2 complex (Sherr and Roberts, 1999; Bartek and Lukas, 2001b; Wahl and Carr, 2001). The intra-S-phase checkpoint is able to delay the progression of the cycle in a p53-independent manner, operating mainly through Cdc25A degradation pathway and cyclin E/CDK2, cyclin A/CDK2 or alternatively through Nbs1/SMC1 pathway (Falck et al., 2002; Yazdi et al., 2002). Activation of both of these pathways cause a transient delay of the cycle, rather than a proper cell cycle arrest. In G2/M phase of the cycle the ATM/ATR and Chk1/Chk2 signalling cascades lead to inactivation of cyclin B/CDK1 complexes through inhibition of the Cdc25c (Abraham, 2001; Nyberg et al., 2002) (Figure 2). Additionally, p53 operates through p21, GADD45 and 14-3-3 σ proteins for long term silencing of cyclin B/CDK1 (Taylor and Stark, 2001), although this p53-induced pathway is probably not essential for the sustained G2 arrest. Even though the upstream signalling is overlapping in most of the checkpoint pathways, the downstream signalling in response to various kinds of damage follow separate routes and may have distinct outcomes (reviewed in Lukas et al., 2004). Despite the final response of each route, the common task of

these pathways is to ultimately eliminate the DNA lesions and secure the genetic stability of the cells.

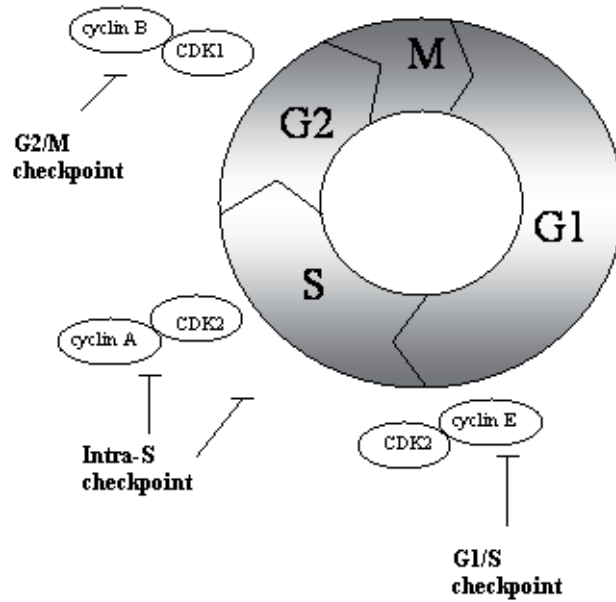


Figure 2. DNA damage checkpoints. The cells respond to DNA lesions by halting progression of the cycle through several independent pathways leading to inhibition of CDK-cyclin complexes. See text for details.

DNA DAMAGE RESPONSES

Repair of the damaged DNA

During the cell cycle arrest the cells have time to deal with the damage they have experienced. Human cells have several, partly overlapping, mechanisms for repairing different kind of damage lesions. Plenty over 100 genes are involved in the different repair pathways (Wood et al., 2001), summarized in table 1.

Direct reversal is probably the simplest repair pathway. This single enzyme reaction is responsible for the removal of DNA adducts, like miscoding methylated bases, caused by DNA alkylating agents or endogenous catabolites. Several DNA methyltransferases are involved in this process (Mishina et al., 2006). The most common repair pathways are base excision repair (BER) and nucleotide excision repair (NER). BER pathway utilizes a group of specific DNA glycosylases for excision of the altered bases and is mainly induced by cellular metabolites (Lindahl and Wood, 1999). NER is a versatile repair pathway, managing several types of lesions and will be later discussed in more detail in the context of its relevance in the UV-induced damage repair. Mismatch repair (MMR) corrects noncomplementary base pairs and other DNA structure-distorting loops during replication as well as in damaged cells (Jiricny, 2000). MMR is a multistep process, consisting of recognition and excision of the incorrect site, resynthesis and ligation of the newly synthesized strand and involves numerous different proteins, including human mutS homolog (MSH) family proteins and mutL homolog 1 (MLH1) forming mismatch recognition complexes, proliferating cell nuclear antigen (PCNA) and replication protein A (RPA) (Jiricny, 2000). The removal of double-strand breaks, induced by ionizing radiation, chemical agents or cellular dysfunctions, occurs through two major pathways. Non-homologous end-joining (NHEJ) is the main DSB-repair pathway in humans, while the second pathway, homologous recombination (HR) is preferred in S and G2 phases of the cell cycle (Haber, 2000). The initiating signal in NHEJ pathway is by the DNA-PK kinase, while the ATM kinase is the major coordinator of the damage response in the HR pathway. Both of these pathways eventually lead to activation of the Mre11-Rad50-Nbs1 complex, involved in sensing and repairing the damaged site (Petrini & Stracker, 2003). In addition to activation of several other repair proteins, these kinase pathways also activate many downstream substrates, like Chk2 and p53, involved in halting the cell cycle. A variety of human diseases are associated with defects in the DNA repair capacity of the cells. Many of these diseases are inherited conditions, leading to a higher mutation rate and predisposition to cancer in the carriers of these defective DNA repair genes, as discussed later.

Repair mechanism	Activating event and special features
Nucleotide excision repair, NER (Hanawalt et al., 2003; Peterson and Côté, 2004)	Several types of bulky lesions induced by UV radiation, chemicals and DNA crosslinking agents
Base excision repair, BER (Lindahl and Wood, 1999)	Altered bases and damage induced mainly by cellular metabolites
Mismatch repair, MMR (Jiricny, 2000)	Noncomplementary base pairs, causing distortion in the DNA helix during replication or DNA damage
Non-homologous end-joining, NHEJ (Haber, 2000; Petrini & Stracker, 2003)	DSBs induced by ionizing radiation and other DNA damaging agents; Main pathway for DSB repair in humans.
Homologous recombination, HR (Haber, 2000; Petrini & Stracker, 2003)	DSBs induced by ionizing radiation and other DNA damaging agents; Activated mainly in S/G2 phases.
Direct reversal (Mishina et al., 2006)	Methylated base-adducts in DNA, caused by DNA methylating agents or cellular catabolites

Table 1. Summary of the main repair pathways in human cells. Several DNA repair systems have evolved for the repair of the damaged DNA caused by cellular processes and metabolic byproducts. These same repair pathways are utilized for the correction of lesions, induced by extracellular agents, which could contribute to accumulation of genetic instability, carcinogenesis as well as lethality due to malfunction in essential cellular pathways.

Apoptosis

Besides the strict control of cell division, equally important for the interests of functional organisms is to control the number of cells by cell death. By inducing an intracellular death program, programmed cell death or apoptosis, cells that are superfluous or cells that could be of threat to the organism, are destroyed. Triggering this suicide pathway may be the only alternative in cells exposed to excessive DNA damage.

The evolutionary conserved program of apoptosis is mainly affected by a family of proteases, caspases, which mediate the cleavage of their specific target proteins. Caspase pathway is activated by intracellular or extracellular stimuli. External activation can occur through the death receptors by ligand binding, which induce a signalling pathway leading to activation of the caspase pathway (Muppidi et al., 2004). Internal signalling requires release of mitochondrial cytochrome c. B-cell lymphoma 2 (Bcl-2) family members are the key regulators of caspases and apoptosis, directly impacting the permeabilization of the outer-membrane of mitochondria and cytochrome c release (Spierings et al., 2005).

This family contains both apoptotic and anti-apoptotic factors and their significance is underlined by the knowledge that lack of the apoptosis inducing factors of this group makes the cells extremely resistant to programmed cell death. On the other hand, the anti-apoptotic factors of this family, like Bcl-2, are overexpressed in several cancer types (Willis and Dyer, 2000).

During the apoptotic response, the release of cytochrome c activates Apaf-1, apoptotic protease-activating factor, triggering caspase pathway and simultaneously blocking other anti-apoptotic factors, like IAPs, inhibitor of apoptosis proteins (Li P. et al., 1997; Zou et al., 1997). The final outcome of this process is associated with specific cellular features including shrinkage of the cell size and disruption of the cytoskeletal structure, breakage of the nuclear envelope as well as fragmentation of the DNA. The remains of the apoptotic cell are rapidly phagocytosed, causing no damage to the neighbouring cells.

UV DAMAGE RESPONSES

UV radiation-induced DNA damage

UV radiation of the sun is associated with skin cancers, including basal cell carcinoma, squamous cell carcinoma and malignant melanoma (de Gruijl, 1999). It is invisible electromagnetic radiation that can be divided into three wavelength areas: UVA 315-380 nm, UVB 280-315 nm and UVC 190-280 nm, the shorter wavelength radiation being the most harmful (Tyrrell, 1994). Most of the radiation reaching the ground of earth is UVA, but the proportion of the shorter wavelength light is unfortunately increasing due to a decline in thickness of the ozone layer.

UV radiation induces bulky DNA-lesions, cyclobutane-type pyrimidine dimers (CPDs) and structurally more distorting (6-4)-photoproducts (6-4PPs), which cross-link DNA bases, inhibiting transcription (Ravanat et al., 2001; Thoma et al., 1999; Tornaletti et al., 1999; Mitchell et al., 2003). UVA and UVB radiation are somewhat more environmentally relevant as most of the shorter wavelength radiation is still absorbed by the ozone layer. However, the UV-induced transcriptional stress is more efficiently triggered by UVC due to higher energy absorbance by DNA from this type of light (Ravanat et al., 2001) and most of the studies have for this reason used UVC as a model. As the wavelength increases, UV radiation-induced oxidative stress becomes more likely (Kielbassa et al., 1997). UVB causes different proportion of 6-4PPs than does UVC and additionally it causes the oxidative lesions and interacts also with other molecules than DNA. In addition, some DNA strand-breaks and protein-DNA cross-links can be detected after exposure to longer wavelengths of UV radiation.

Cellular responses induced by UV radiation

UV radiation-induced DNA damage evokes a set of cellular responses, including transcriptional inhibition, damage recognition and activation of several signalling pathways (de Gruijl et al., 2001). The early response in UV-damaged cells is provoked by ATR-Chk1 kinase pathway, leading to phosphorylation of several downstream targets. In addition to this pathway, the MAP kinases, Erk, JNK and p38 are essential and activated upon UV damage in a dose dependent manner (Davis, 2000; Bode and Dong, 2003). The changes in the cell surface receptors trigger these intracellular signalling pathways and their activation plays a role in the control of cell growth, changes in the chromatin structure and apoptotic responses (Bode and Dong, 2003). Depending on the damage-induced cascade, different target genes are activate through activation of UV-induced transcription factors, including p53, antiapoptotic factor nuclear factor κ B (NF κ B) and activating protein 1 (AP-1) (Ryan et al., 2000; Shaulian and Karin, 2002; Chen and Greene, 2004). Activation of a certain cascade is dependent on the amount of UV dose and affects eventually the final transcriptional response, leading to either cell cycle arrest and DNA repair or to apoptosis, if cells are subjected to

excessive amounts of lesions (Gentile et al., 2003). Transcriptional responses differ also depending on the cell type (Valery et al., 2001; Sesto et al., 2002; Gentile et al., 2003). In addition to growth arrest and apoptotic response, exposure to UV light provokes immunosuppression, possibly contributing to neoplastic transformation (Clydesdale et al., 2001).

Nucleotide excision repair

Nucleotide excision repair, NER, is probably the most comprehensive repair pathway, facilitating the repair of a variety of dissimilar lesions in DNA, including UV-induced CPDs and 6-4PPs (Peterson and Côté, 2004). The complex NER pathway is well conserved in bacteria, yeast and mammals (Eisen and Hanawalt, 1999; Petit and Sancar, 1999) and the mammalian NER function requires nearly 30 different factors for full activity (Lindahl and Wood, 1999; Volker et al., 2001). NER is divided into two different subpathways, transcription-coupled repair (TCR) and global genomic repair (GGR) (reviewed in Hanawalt et al., 2003; Peterson and Côté, 2004). TCR is less well understood and repairs damage sites found in transcribed DNA strands of genes, while GGR is a more general repair machinery, correcting lesions throughout the whole genome.

The action of NER involves the following steps: lesion detection in DNA and chromatin remodelling, removal of the lesion and resynthesis of the nucleotide sequence and ligation of the newly-synthesized strand to the pre-existing one (Figure 3). The initial lesion detection in TCR and GGR is done by separate proteins, although many of the other enzymatic processes in GGR and TCR have overlapping factors. The GGR proteins are, however, usually kept at low levels until the cells are exposed to DNA damage. Several of the proteins in Xeroderma pigmentosum (XP) complementation group play a role in both subpathways (Friedberg et al., 2004). For these repair pathways to work efficiently the chromatin structure has to be altered (Smerdon and Lieberman, 1978). In TCR the chromatin accessibility appears to be ensured by the presence of the transcription machinery itself (Friedberg, 2001), while in GGR the chromatin accessibility has to be achieved by other factors (Tijsterman et al., 1999; Friedman, 2001). Initial recognition of lesions by GGR include the mammalian XPC-HR23B-centrin2 complex and XPA protein (Sugasawa et al., 1998; Volker et al., 2001). Additionally, UV-DDB, UV DNA damage binding protein is required in some damage types like CPDs (Tang and Chu, 2002; Wakasugi et al., 2002). These factors can also recruit histone acetyltransferase (HAT) activities to further increase the access to these sites. In TCR, the Cockayne syndrome proteins, CSA and CSB as well as XAB2 protein, initially target NER to stalled RNA pol II on DNA strands (Tornaletti and Hanawalt, 1999; Mitchell et al., 2003). CSB/Rad26 remodelling complex may also facilitate increased accessibility to the damage sites during TCR. In addition, the UVSS, ultraviolet-sensitive syndrome protein has been shown to be essential for properly functioning TCR (Spivak et al., 2002). Further verification and direction of unwinding in the damaged area is performed in both subpathways by a multisubunit transcription factor/repair

protein TFIIH and its partners XPB and XPD (Giglia-Mari et al., 2004). This is followed by incision of the lesion area by XPF and XPG proteins with the help of XPA and RPA proteins, stabilizing the formation of the repair complex. The formed gap is filled when synthesis of a new DNA strand by DNA pol δ/ϵ and PCNA takes place (de Laat et al., 1999; Peterson and Côté, 2004) (Figure 3).

Failure in NER function leads to increased cancer incidence, as observed in the hereditary Xeroderma pigmentosum in humans (Friedberg, 2004). XP is defined as a group of recessive disorders caused by defects in the nucleotide excision repair genes. The GGR deficient cells of these patients have weaker apoptotic signalling resulting in higher mutation rates and transformation of the surviving cells. Due to this XP patients are very prone to sunlight-induced diseases, like skin cancers, developing both benign and malignant neoplasms (Bootsma, 1993). Cockayne's syndrome and UV-sensitive syndrome of humans are both diseases associated with defective TCR DNA repair of the cells (Spivak, 2005). However, these syndromes are not associated with increased cancer risk as cells deficient in TCR are even more prone to UV-induced apoptosis (Ljungman and Zhang, 1996). Cell survival as well as cancer incidence so appears to be more dependent on functional GGR than TCR and cellular damage left after deficient TCR function can be still rescued by GGR machinery.

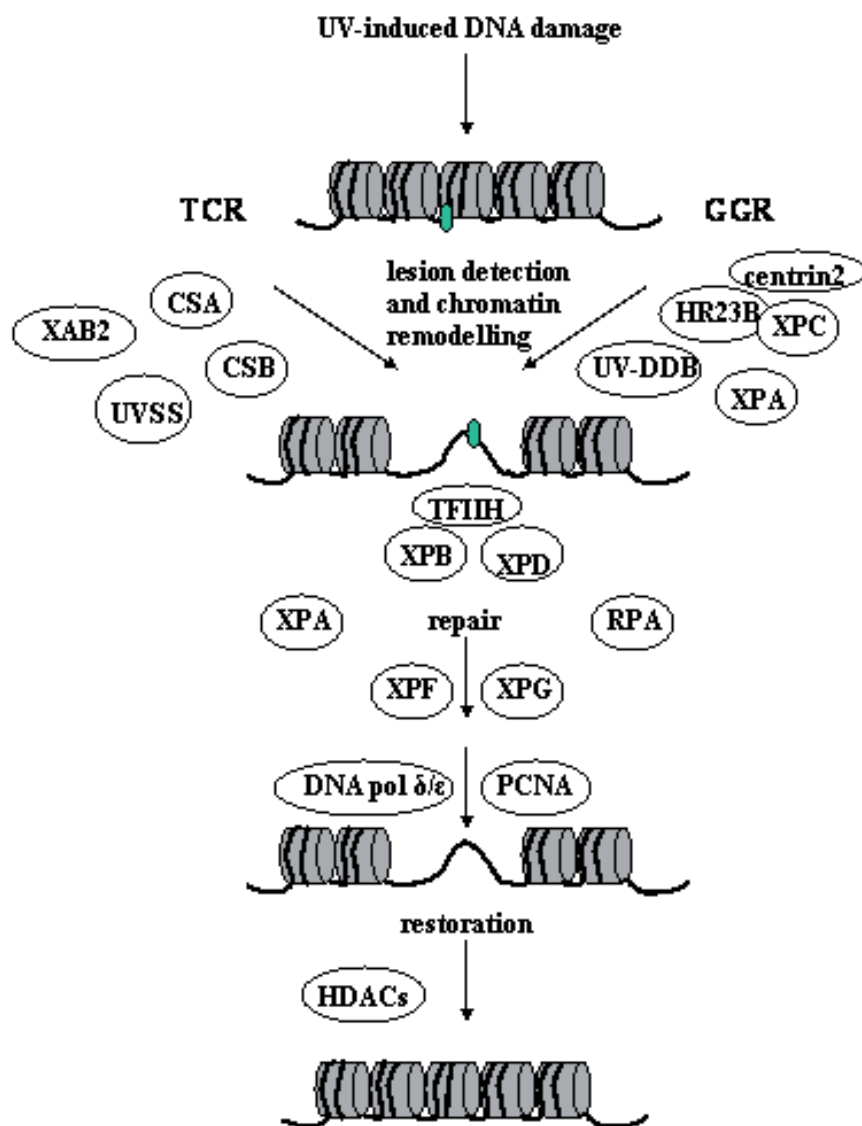


Figure 3. Nucleotide excision repair pathway. NER is composed of two subpathways, TCR and GGR, specified by their differences in the initial lesion detection factors. The repair of the damage site is overlapping for both pathways, including 1) unwinding of the lesion surroundings by TFIIH and its partners XPB and XBD, 2) incision of the damaged area by XPF and XPG and 3) synthesis of a new DNA strand by DNA pol δ/ϵ and PCNA. Finally the newly synthesized strand is ligated to the pre-existing one and the structure is restored by chromatin modifiers.

Nucleolar stress response

Nucleoli are specific subcompartments of the nucleus, clearly visible dense structures under the microscope. The main function of these dynamic compartments is to act as ribosome factory. Nucleoli orchestrate the synthesis and processing of ribosomal RNAs (rRNAs) and their assembly to pre-ribosomal particles in specific compartments of the nucleolus (Carmo-Fonseca et al., 2000; Olson & Dundr, 2005). The nucleolus is formed of small fibrillar centers (FCs), which are responsible for the initiation of the rRNA transcription. These structures are surrounded by dense fibrillar component (DFC), processing the nascent rRNA transcripts. Finally the further processing occurs in the granular component (GC), surrounding FC and DFC structures. The rate and efficiency of this process reflects the transcriptional activity of the cell, being high in rapidly proliferating cells. Cancer cells often have very prominent nucleoli (Derenzin et al., 2000). In addition to its traditional role in ribosome biogenesis, nucleolus has lately been connected to several other functions due to its protein composition (Andersen et al., 2005; Pendle et al., 2005; Leung et al., 2006). Many of the nucleolus-associated proteins have roles in the cell cycle control, aging, viral replication, nuclear export and telomerase activity, reflecting the versatility of the functions of nucleolar compartment (Carmo-Fonseca et al., 2000; Olson et al., 2002). Furthermore, recent results show the importance of nucleolus as a stress sensor, responding to various kind of cellular damage and mediating p53 stabilization (Rubbi and Milner, 2003b; Olson et al., 2004; Mayer et al., 2005).

Exposure of the cells to external and internal stress, including UV radiation, hypoxia, heat shock and nucleotide depletion, causes so called “nucleolar stress” impairing the function of these sub-nuclear compartments (Rubbi and Milner 2003b; Olson et al., 2004; Mayer and Grummt, 2005). All of these stress inducers are basically inhibitors of the transcription and disruption of this key function of the nucleolus leads to reorganization of its structure and several nucleolar proteins are released to nucleoplasm (Olson et al., 2004; Shav-Tal et al., 2005) (Figure 4). For instance, Ki-67, nucleolin, fibrillarin, p120 and Hrad17 have been shown to relocate from the nucleoli upon UV-induced stress (Chang et al., 1999; Daniely et al., 2002; Rubbi and Milner, 2003; Al-Baker et al., 2004). The structure of the nucleoli reorganizes rapidly upon transcriptional inhibition and the FC compartments move to the perinucleolar area (Panse et al., 1999). These kind of dotted compartments called “nucleolar necklaces” were already described in the 1970’s (Granick & Granick, 1971; Granick, 1975). The function of these necklaces is still not clear, even though several proteins have been reported to colocalize with them upon cellular stress (Fuchsová et al., 2002; Hoogstraten et al., 2002).

The mechanism leading to reorganization in the nucleolar structure and translocations of different proteins to the perinucleolar area is not totally understood, although it is possible that the initiating signal comes from the RNA pol II inhibition by UV radiation. One of the key factors regulating the nucleolar structure could be transcription factor TIFIA, which regulates the activity

of RNA polII (Schnapp et al., 1990). In stressed cells TIFIA is phosphorylated by c-Jun N-terminal kinases (JNK2), disrupting the TIFIA-RNA polII connections and leading to nucleoplasmic TIFIA upon reorganization of the nucleolus (Mayer et al., 2005). The importance of this pathway is highlighted by the fact that inactivation of TIFIA phosphorylation by JNK results in stress-resistance of PolII transcription and rRNA synthesis. The recent reports show the versatility of the nucleolar functions and underline its importance as a stress sensor in damaged cells.



Figure 4. Reorganization of the nucleoli upon transcriptional inhibition. The nucleoli are damage sensors, undergoing rapid morphological changes in stressed cells. The fibrillar centers (FC) relocate to the perinucleolar area and several nucleolar proteins are released to the nucleoplasm (Olson et al., 2004; Shav-Tal et al., 2005), and may thus affect the stress response through specific targets in the nucleoplasmic compartment.

UNCONTROLLED GROWTH AND CANCER

Every day one single human cell has to deal with thousands of errors in its genome due to endogenous and exogenous damaging agents (Friedberg, 2001). Even though the cells have efficient and overlapping machineries for repairing the altered sites, sometimes the systems and their backups fail and cells with mutated genomes continue multiplying. In the worst situation, the mutations may give the cell selective advantage, allowing it divide more efficiently than the neighbouring ones. Over time the cells may also acquire more genetic alterations that lead to tumorigenesis, driving the cells from normal human cells into cancerous derivatives. As normal cells act in the benefit of the whole organism, by either resting, dividing, differentiating or dying, the cancerous cells have forgotten about these normal rules of cell behaviour leading to uncontrolled growth of the cells at the expense of the whole cell community.

The development of malignant tumors in a long period of time requires sequential steps of mutations contributing to loss of tumor suppressor gene functions and gain of function with oncogenes, as well as epigenetic changes. Bypassing the phenomenon of replicative senescence leads to immortalization of the cells and is prerequisite for the malignant transformation. Basically, six different alterations in the normal cell functions have been suggested to lead to tumorigenesis in most of the cancer types. These features include: Self-sufficiency in maintaining growth signals, unresponsiveness to growth-inhibiting signals, inhibition of the apoptotic pathways, unlimited replication potential, angiogenic signalling and potential to metastasize (Hanahan and Weinberg, 2000). Transforming cells are capable of generating their own growth-inducing signals by producing growth-factors of their own, by inducing their neighbours to release these signals or by switching on the downstream signalling of the growth factors inside the cell. Many of the oncogenes can as well mimic the players in the signalling pathway and promote transfer from the quiescent state to a proliferative one. For example, about a quarter of the human cancers have upregulation of the Ras-signalling pathway, leading to mitogenic signals inside the cell (Medema and Bos, 1993). The insensitivity to growth-inhibiting signals results often from deregulation of the TGF- β pathway and its intracellular targets (Levy and Hill, 2006). TGF- β inhibits cyclin D/CDK4/6 complex, prerequisite for pRb phosphorylation and progression into S phase (Hannon and Beach, 1994; Weinberg, 1995). pRB and its regulatory pathway is one of the main targets in tumorigenesis, in addition to the p53 pathway. Furthermore, unresponsiveness to TGF- β leads to upregulation of growth promoting c-Myc (Adhikary and Eilers, 2005). Features of the transformed cells also include amplification of the centrosomes, contributing to chromosome instability, further giving the tumor cells a more malignant potential (Brinkley and Goepfert, 1998; D'Assoro et al., 2002).

Cancer incidence is increasing, mostly due to longer life time expectancy and living habits. In addition to spontaneous mutations affecting cancer development, some germline mutations, linked to inherited susceptibility to certain cancers, have been found. These mutated genes, usually associated with DNA

damage checkpoints, repair functions and apoptosis, can be either recessively or dominantly inherited and can cause a specific cancer phenotype or just general increased risk of cancer incidence. In addition, phenotypically similar cancers can result from a single gene or a group of genes acting in the same cellular pathway. Usually the cancer-linked genes have several different mutations affecting the activity of its respective protein product, leading to either low-or high-risk predisposition for certain cancers, the high-penetrance mutations affecting its carriers with relatively early age. The most well-studied inherited forms of cancer involve breast and ovarian cancer, which have been linked to mutations in checkpoint proteins BRCA1 and BRCA2 genes (Easton et al., 1993; Miki et al., 1994; Wooster et al., 1995). Other well-known dominantly inherited forms of cancer involve mutations of the Rb gene, causing retinoblastoma of the eye (Ward et al., 1984), APC tumor suppressor gene, mutations of which cause familial adenomatous polyposis (FAP) and high susceptibility to colorectal cancers (Bodmer et al., 1987) and some forms of melanoma, which have been linked to mutations in the CDKN2A gene, encoding p14ARF and p16 proteins (Cannon-Albright et al., 1994). Li-Fraumeni syndrome, causing susceptibility to several kinds of cancers, including breast cancers, soft tissue sarcomas, brain tumors, leukemia, osteosarcoma and adrenocortical carcinoma, has been linked to both tumor suppressor protein p53 as well as DNA damage kinase Chk2 (Li and Fraumeni, 1969; Malkin et al., 1990; Bell et al., 1999). Most studied recessively inherited forms of cancers are probably Xeroderma pigmentosum (XP), described earlier in the nucleotide excision repair chapter as well as Bloom syndrome and Ataxia-Telangiectasia, caused by mutations in the BLM and ATM genes respectively (Ellis et al., 1995; Savitsky et al., 1995). These genes are involved in DNA checkpoint functions and repair, and mutations in both cases cause abnormalities in the development as well as increased cancer risk, especially for leukemias and lymphomas (German et al., 1997). Despite these cancer associated genetic disorders, the inherited genetic susceptibility for cancer is still quite rare and most of the cancers are sporadic and occur due to risks caused by the individual itself or the living environment.

TUMOR SUPPRESSOR PROTEIN p53

p53 tumor suppressor protein, also known as “the guardian of the genome” was initially identified as an oncogenic protein, in complex with viral proteins (DeLeo et al., 1979; Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979). Later, this transcription factor was found to be essential for the prevention of tumor formation, dependent on its ability to induce apoptosis. Despite its essential role in inhibition of neoplastic transformation, p53 expression is not essential during the development of mice in utero (Donehower, 1996).

Somatic mutations in p53 are found in approximately 50% of the cancers, positioning p53 as the most frequently mutated gene in human malignancies (Hollstein et al., 1991; Levine et al., 1996). Even one mutant p53 allele can result in a gain-of-function phenotype with acquired new oncogenic properties and inactivation of the wt p53 allele (Lang et al., 2004; Olive et al., 2004; Chan et al., 2004). As mentioned in the previous chapter, p53 is also mutated in Li-Fraumeni syndrome, a rare inherited syndrome predisposing the carriers of p53 germline mutations to early-onset tumors (Varley, 2003). Further support for the essential role of p53 in prevention of cancer formation comes from the mouse models, which show highly increased predisposition to malignancies in p53 null mice and mice with mutated p53 (Donehower et al., 1992; Jacks et al., 1994). The tumor spectrum of p53 null mice include lymphomas, soft tissue sarcomas, brain and lung tumors, while the heterozygous mice have a more variable tumor spectrum corresponding better to tumor incidence in Li-Fraumeni syndrome (Donehower, 1996).

Structure

Human p53 protein, encoded by the *TP53* gene in chromosome 17, is constructed of 393 amino acids and contains several different functional domains (figure 5). The amino-terminus of p53 forms its transactivation domain (TAD) and is heavily modified in response to cellular stress (Appella and Anderson, 2001). TAD participates in the transcriptional regulation and binds several factors required for p53-mediated response (Lin et al., 1994; Zhu et al., 1998). In addition, regulation of p53 occurs through TAD via binding to Mdm2 protein, which blocks the transactivation activity of p53. The crystal structure of p53-Mdm2 interface has been solved (Kussie et al., 1996). Mdm2 contains a hydrophobic pocket in which the hydrophobic site of the amphipathic p53 α -helix, amino acids 19-26 respectively, are inserted (Chen et al., 1993; Kussie et al., 1996; Bottger et al., 1997). This tight configuration of the binding cleft probably hinders p53 interactions with the transcriptional machinery. The proline-rich domain (PRD) follows the TAD and is presumably involved in the apoptotic response of p53 (Zhu et al., 2000; Baptiste et al., 2002; Edwards et al., 2003). It also mediates the co-factor binding through interaction with acetyltransferase p300 (Dornan et al., 2003).

The central domain of p53 contains its highly conserved DNA binding domain (DBD) required for its transcriptional properties and for identifying the p53 DNA consensus recognition elements on its target promoters (Kern et al., 1991). DBD, composed of a β -sandwich and three loop-based structures, binds different p53 target sequences with variable affinities, resulting in great variations in the transactivation potential (Inga et al., 2002). Most of the tumor-associated p53 mutations occur in this DNA-binding domain of the protein, leading to inactivation of p53 functions through disruption of its sequence-specific binding or through destabilization of its tertiary structure (Cho et al., 1994; Bhullock et al., 1997; Royds and Iacopetta, 2006). The structure is stabilized by a zinc atom, connecting the residues C176 and H179 of the second loop and C238 and C242 of the third loop of DBD (Cho et al., 1994). Interference of this structure disturbs DNA-binding of p53 as well as its tumor suppressive properties.

Carboxy-terminus of p53 is composed of a linker region, oligomerization/tetramerization (TET) site and a basic C-terminal DNA-binding domain (CTD). The oligomerization site is composed of a β -sheet-turn- α -helix structure (Clore et al., 1995), essential for p53 ability to form tetramers. The tetramers are also the most active forms of this protein (Jeffrey et al., 1995; Arrowsmith and Morin, 1996; McLure and Lee, 1998). CTD contains a number of phosphorylation, acetylation, sumoylation and ubiquitination sites associated with the regulation of p53 functions (Appella and Anderson, 2001). While the DBD recognizes specific target sequences, CTD binds DNA without any sequence specificity (Kim and Deppert, 2006; Liu and Kulesz-Martin, 2006). As CTD is also capable of binding various lesions, it has been associated with the DNA damage recognition (Bakalkin, 1995; Lee et al., 1995; Reed et al., 1995).

p53 function can also be influenced via its cellular localization, regulated by the nuclear localization signal (NLS) and nuclear export signal (NES) of the protein (Shaulsky et al., 1991) as well as interactions with some of its partner proteins. p53 has a major NLS in its linker region and two other NLS sequences in its very C-terminal end (Dang and Lee, 1989; Shaulsky et al., 1990). The nuclear export signal (NES) of p53 lies within the tetramerization domain and is possibly masked by the formation of oligomeric forms (Stommel et al., 1999). Another NES has been found in p53 N-terminus, in the Mdm2 binding domain (Zhang and Xiong 2001).

p53 protein is very well conserved (Soussi et al., 1990) and it belongs to a family consisting of two other proteins, p63 and p73 (Yang et al., 2002). These proteins are structurally very similar and have several overlapping duties with p53 in cellular stress response (Yang et al., 2002) besides their specific roles in the development (Irwin and Kaelin, 2001). p63 and p73 isoforms lacking their TAD domain may inhibit p53 function through oligomer formation, while some p53 mutants are able to attenuate the function of p63 and p73 and contribute to oncogenesis in this way (Yang et al., 2002; Olive et al., 2004). In addition to the p53 family members, multiple splice variants from an alternative promoter of p53 are expressed in a tissue-dependent manner and may influence the activity of the full length protein (Bourdon, 2005). These forms can regulate p53

transcriptional activity by enhancing gene expression from specific promoters or by blocking the activity of the full length p53. Furthermore, distinct expression patterns of these isoforms have been discovered in human tumors, possibly affecting the response to therapeutic drugs and general biological features of different cancer types.

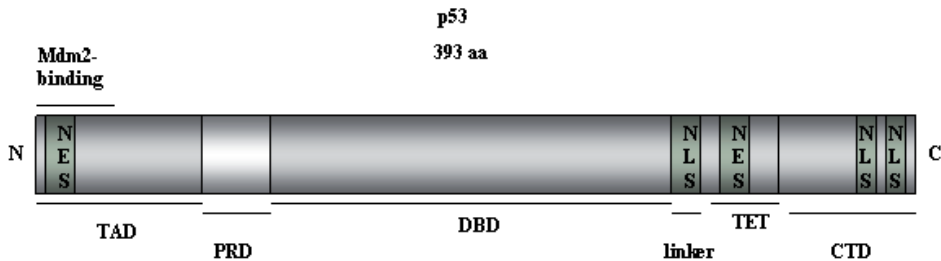


Figure 5. Organization of p53 functional domains. TAD, transactivation domain (aa 1-42); PRD, proline-rich domain (aa 63-97); DBD, DNA binding domain (aa 102-292); linker region (aa 300-318); TET, tetramerization domain (aa 323-356); CTD, C-terminal DNA binding domain (aa 363-393); NES, nuclear export signal; NLS, nuclear localization signal.

Regulation of p53 stability and activity

p53 stability and activity are tightly controlled and the protein levels are kept low and in latent form in unstressed cells. However, in response to various kind of cellular damage, p53 is stabilized and its transcriptional activity is rapidly enhanced through several existing mechanisms. The stabilization of p53 is not required for its transactivation activity, suggesting that these events are at least partly independent of each other (Hupp, 1999).

p53 protein is constantly synthesized and its accumulation in response to stress has been thought to be mainly based on inhibition of its degradation, not on de novo gene transcription and translation. A recent paper has, however, shown that increased translation of p53 mRNA is also an important step in the induction of p53 protein in DNA-damaged cells (Takagi et al., 2005). Despite extensive studies on the stabilization mechanisms of this protein, these stress-induced pathways leading to stable p53 are not yet fully understood. Yet, both the activation and stabilization events of p53 have been proposed to include a number of site- and time-specific post-translational modifications, like phosphorylations, acetylations and sumoylation, as well as interactions with other activators. Alterations in the localization pattern of p53 may as well play a role in its function, as nuclear localization of p53 has been shown to be critical for its full activity (Shaulsky et al., 1991).

The major regulation of p53 occurs via its degradation. p53 is degraded through the ubiquitin-proteasome pathway (Maki et al., 1996), which was for the first time discovered from papilloma virus-infected cells (Scheffner et al.,

1990 & 1993). The papilloma virus protein E6 was shown to mediate the degradation of p53 and play a role in this way in the oncogenesis of the infected cells (Scheffner et al., 1990 & 1993). Later, Mdm2 (murine double minute 2) protein was found to be the major mediator of the ubiquitination and proteasomal degradation of p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). The ubiquitination of p53 C-terminal residues competes for its acetylation on the very same sites (Ito et al., 2002; Li et al., 2002). Acetylation by p300/CBP blocks p53 degradation (Ito et al., 2001) and may contribute to p53 activation upon various cellular stress situations (Gu and Roeder, 1997; Lill et al., 1997; Sakaguchi et al., 1998; Liu et al., 1999). In addition to acetylation, several phosphorylations of p53 N-terminus and C-terminus have been proposed to play a key role in controlling p53 stability and activity as well as target gene selection (Siliciano et al., 1997; Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998; reviewed in Xu, 2003). Phosphorylations on p53 N-terminus, especially residues serine 15 and serine 20 have been thought to block the interaction between p53 and Mdm2 in DNA damaged cells and in this way lead to elevation in p53 levels (Shieh et al., 1997; Craig et al., 1999; Prives and Hall, 1999; Unger et al., 1999; Kapoor et al., 2000; Zhang and Xiong, 2001). Later, phosphorylation of threonine 18 was shown to be the only critical residue, affecting Mdm2-p53 interface (Lai et al., 2000; Schon et al., 2002).

Although these phosphorylations in many models contribute to p53-Mdm2 interactions, contrasting reports also exist (Ashcroft et al., 1999) and the *in vivo* data has showed no evidence of any p53 phosphorylations being critical for its stabilization or activation (Blattner et al., 1999; Xu et al., 2003). Similarly, the *in vivo* results about the effect of p53 acetylation on the protein stability are conflicting with the previous studies (Feng et al., 2005; Krummel et al., 2005). According to Feng et al., acetylation plays a role in p53 transactivation activity, while the other study proposed that this modification only appears to have a slight effect in fine-tuning the p53 response (Krummel et al., 2005). p53 has also been shown to be modified by SUMO, a small ubiquitin like protein, on its C-terminal Lys386. This modification was proposed to enhance its transcriptional activity (Gostissa et al., 1999; Rodriguez et al., 1999; Melchior and Hengst, 2002), although contrasting results again exist (Kwek et al., 2001). Additional modifications, including neddylation and methylation of p53 have also been discovered under certain stress situations, but their influence on p53 regulation still remains rather unknown.

The C-terminal domain of p53 has been proposed to influence its activity, by mediating the conversion from the latent form to an active DNA-binding protein (Hupp et al., 1992). The latent form of p53 can also be in tetrameric form (Hupp and Lane, 1994), but the regulation of the conversion to active p53 has been proposed to involve allosteric transition through the C-terminal site of p53 in DNA-damaged cells (Hupp and Lane, 1994; Waterman et al., 1995). According to this model, the stress-induced p53 modifications in its CTD would affect positively the ability of DBD to bind its target sequences (Gu and Roeder, 1997; Sakaguchi et al., 1998; Luo et al., 2004). Later, this model has been questioned

by other studies (Ayed et al., 2001; Krummel et al., 2005) and several different hypothesis about the role of CTD in the activation of p53 has emerged. Some studies propose that CTD acts as a negative regulator for DBD by binding DNA non-specifically and that the post-translational modifications of this domain upon stress blocks this function of CTD, allowing sequence-specific binding of DBD (Anderson et al., 1997; Friedler et al., 2005). C-terminus has also been suggested to play a role in enhancing the recognition of specific p53-response elements through the central domain (Ahn and Prives, 2001; McKinney et al., 2004). The study of McKinney et al. (2004) showed the ability of CTD to diffuse linearly on DNA, acting as a positive regulator for the sequence-specific binding, independently of the modification status of CTD. Moreover two studies have shown the requirement for intact C-terminus for the efficient promoter activation of p53 in vivo (Liu et al., 2004; McKinney et al., 2004). In all, the network regulating p53 stability and activity seems to be very complex, showing no simple on-off features.

Mdm2

Mdm2 (murine double minute 2) was first identified from transformed murine 3T3 fibroblasts (BALB/c), amplified in small extrachromosomal nuclear bodies (Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991). It was later shown to decrease p53 activity, suggesting that this function of Mdm2 was responsible for its oncogenic potential (Momand et al., 1992; Oliner et al., 1992). Mdm2 can inhibit p53 transactivation and act as its E3-ligase, mediating the degradation of p53 through the proteasome pathway (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). The degradation through the proteasome pathway requires the polyubiquitination of the target protein (Thrower et al., 2000) and is controlled by three enzymes: ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3. Mdm2 acts as the E3-ligase enzyme towards p53 and determines its fate to be degraded through this pathway. The importance of Mdm2 as a p53 regulator was proven by mice knock-out studies, where loss of p53 rescued the embryonic lethality of Mdm2 null mice (Jones et al., 1995; Montes De Oca Luna et al., 1995). Mdm2 itself is a p53 target gene, induced by cellular stress (Perry et al., 1993; Saucedo et al., 1999), creating a negative feedback loop between these proteins (Wu et al., 1993).

Mdm2 gene is composed of 12 exons, which locate under two different promoters, the other one being p53 responsive. The two promoters result in two different Mdm2 forms, p90 and p76, of which p76 does not bind p53 and acts as a dominant negative inhibitor of the full length form (Perry et al., 2000). In addition, a number of different *Mdm2* splice variants exists and some of them also control the activity of the full length form (Bartel et al., 2002). *Mdm2* gene is amplified in one third of human sarcomas and approximately 7% of all human cancers (Oliner et al., 1992; Bond et al., 2004). Polymorphisms in its promoter region in a subgroup of human population may also lead to enhanced Mdm2 expression and downregulation of p53 (Bond et al., 2004).

The N-terminal site of the full length 491-amino acid Mdm2 is required for binding the p53 transactivation domain and repression of its activity (Chen et al., 1993, Oliner et al., 1993) (Figure 6). The amino acids 25-109 of Mdm2 form a hydrophobic pocket, which bind the N-terminus of p53 and hide it from the transcriptional machinery (Chen et al., 1993; Kussie et al., 1996). Recently, another binding site between the DBD of p53 and acidic-domain of Mdm2 has been reported (Shimizu et al., 2002; Yu GW et al., 2005). This interaction could possibly stabilize the Mdm2-p53 complex and modulate p53 degradation. Besides mediating p53 degradation and transactivation, Mdm2 may use other means in regulating p53 functions. Mdm2 controls the location of p53 protein, targeting it either to cytoplasm or nucleus (Roth et al., 1998; Freedman et al., 1998), possibly sequestering it from its target genes. The shuttling of Mdm2 requires its nuclear localization (NLS) and nuclear export signals (NES) (Figure 6.). The C-terminus of Mdm2 contains its RING-domain, coordinating the E3-ligase activity and ubiquitination of p53 C-terminal lysines (Nakamura et al., 2000; Rodriguez et al., 2000). Additionally, Mdm2 is able to mediate its own degradation through the same domain (Fang et al., 2000; Honda et al., 2000).

For a long time it was thought that Mdm2 alone promotes p53 polyubiquitination. More recent data suggests that it actually mediates monoubiquitination of p53 on several lysine residues and participates in polyubiquitination in cooperation with other factors (Lai et al., 2001). Because the degradation through the proteasome pathway requires polyubiquitination, other proteins besides Mdm2 must be required for efficient degradation of p53. One of these proteins is p300/CBP (Grossman et al., 1998; Zhu et al., 2001; Grossman et al., 2003). In unstressed cells Mdm2-p300-p53 form complexes, in which p53 is not modified by p300 and stays transcriptionally inactive (Kobet et al., 2000; Ito et al., 2001). The degradation of p53 can take place in both cytoplasm and nucleus (Xirodimas et al., 2001; Joseph et al., 2003), although the cytoplasmic translocation was at first suggested to be prerequisite for p53 degradation (Freedman and Levine, 1998; Tao and Levine, 1999a). The nuclear degradation function appears to be critical for shutting off the p53 activity in later stages of the damage response (Shirangi et al., 2002), while low levels of Mdm2 in unstressed cells induce p53 monoubiquitination and subsequent translocation to the cytoplasm (Geyer et al., 2000; Li et al., 2003). Other factors besides Mdm2 could be responsible for degrading p53 in unstressed cells possessing low Mdm2 levels. Whether the monoubiquitination of p53 by Mdm2 triggers some other unknown p53 functions, still remains to be solved.

In addition to p53 modifications in the Mdm2-p53 interface, Mdm2 protein is also heavily modified in response to cellular stress, possibly affecting their interactions (Meek and Knippschild, 2003; Moll and Petrenko, 2003). Mdm2 is acetylated in its RING domain, inactivating it and leading to p53 transactivation (Wang et al., 2004). Several phosphorylations/dephosphorylations on Mdm2 have also been shown to modify its effect on p53 degradation and inhibition of its transactivation activity in stressed cells (Maya et al., 2001; Okamoto et al., 2002; Blattner et al., 2002). ATM, for instance, phosphorylates Mdm2 on Ser

395 and inhibits p53 degradation (Khosravi et al., 1999; de Toledo et al., 2000; Maya et al., 2001). Mdm2 is also a target of the AKT-kinase pathway, which phosphorylates Mdm2 and targets it to nucleus where it is able to ubiquitinate p53 (Mayo and Donner, 2000). Tumor suppressor protein PTEN on the other hand is able to reverse this action of AKT and protect p53 from the Mdm2-mediated degradation (Mayo et al., 2002; Freeman et al., 2003). DNA damage also promotes new interactions with Mdm2 and its partner proteins, possibly contributing to p53 activation and stabilization. In addition to the control of p53-Mdm2 interaction, the levels of Mdm2 could be critical in regulating p53 stability and activity and Mdm2 protein and mRNA levels have been shown to decrease upon various treatments that lead to elevated levels of p53 (Wu and Levine, 1997; Arriola et al., 1999; Ashcroft et al., 2000; Inoue et al., 2001; Wang et al., 2002).

Besides its function as a regulator of p53 activity, Mdm2 is capable of affecting the cell cycle, DNA repair, basal transcription, differentiation and cell fate determination independently of p53 (reviewed in Ganguli and Wasylyk, 2003). Splice variants of Mdm2 having no p53 binding domain clearly operate in p53-independent functions. Mdm2 binds DNA pol ϵ (Vlatkovic et al., 2000) and stimulates its activity (Asahara et al., 2003). DNA pol ϵ has roles in DNA repair, recombination, replication, damage sensing and chromatin remodelling, linking Mdm2 to regulation of these functions. Possible role in ribosome biosynthesis and in translational regulation comes from Mdm2 interaction with L5 (Marcehal et al., 1994). Mdm2 could also affect transcription as it interacts with general transcription factors (Ganguli and Wasylyk, 2003). The cell cycle regulatory role of Mdm2 derives from its ability to bind Rb and perturb Rb-mediated G1-arrest (Xiao et al., 1995) and cooperate with E2F, stimulating E2F-dependent activation of some promoters involved in DNA synthesis (Martin et al., 1995). Interestingly, Mdm2 has also two cell cycle arrest-inducing domains (ID1 and ID2), which do not overlap with p53 interaction domain (Brown et al., 1998). These domains could be lost during the tumorigenesis, as Mdm2 is mostly associated with transformation of the cells. Overexpression of the entire Mdm2 gene predisposes to spontaneous tumor formation in a cell type-dependent manner (Jones et al., 1998). Mdm2 also contributes to the transformed phenotype in the absence of p53 and confers to a growth advantage in cells that lack p53 and Rb and can overcome the cell cycle arrest induced by p107 (Dubs-Poterszman et al., 1995). Tumors with both p53 mutation and Mdm2 amplification are rare but lead to poorer prognosis, further underlining the p53-independent role of Mdm2 in cellular transformation (Cordon-Cardo et al., 1994). Mdm2 promoter is also a target of Ras/MAPK pathway and activation of this pathway can increase Mdm2 levels during neoplastic transformation, giving a growth advantage for the cells (Ries et al., 2000).

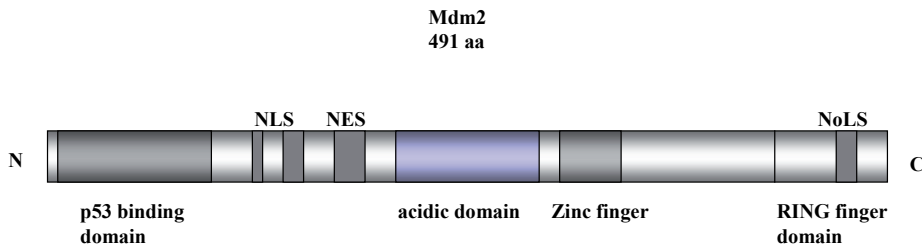


Figure 6. Structure of Mdm2 protein. N, N-terminal domain; C, C-terminal domain; NLS; nuclear localization signal; NES, nuclear export signal; NoLS, nucleolar localization signal.

MdmX

MdmX (Mdm4), a Mdm2 homologue, is also an important negative regulator of p53 activity, as the embryonic lethal phenotype of MdmX null mice is rescued by p53 knock-out (Parant et al., 2001; Finch et al., 2002; Migliorini et al., 2002) and amplification of MdmX directly affects tumor formation by inhibiting the tumor suppressor activity of p53 (Danovi et al., 2004). Several studies on this Mdm2 homologue has been published, but the relevance of MdmX in the regulation of p53 is not fully understood and many contradictory results exist (reviewed in Marine and Jochemsen, 2005).

MdmX is able to bind p53 and structurally resembles Mdm2, but does not contain the C-terminal domain responsible for the E3-ligase activity. It seems to play a dual role in the regulation of p53 stability and activity, as it can inhibit Mdm2 and stabilize p53 when overexpressed, still keeping p53 in an inactive form (Jackson and Berberich, 2000). However, when the MdmX protein remains at low physiological levels, it co-operates with Mdm2 in p53 downregulation (Gu et al., 2002). One of the mechanisms in regulation of p53 activity could be the inhibitory effect of MdmX on the acetylation of p53 C-terminus (Sabbatini and McCormick, 2002). A recent paper by Toledo et al. (2006) showed a mouse model expressing p53 mutant lacking the proline-rich domain (p53DeltaP) and with reduced p53 apoptotic response. Expression of this mutant rescued the lethal phenotype of MdmX deficiency, but not Mdm2 deficiency. Furthermore, decreasing Mdm2 levels increased p53DeltaP levels without altering its transactivation, suggesting that MdmX mainly regulates p53 activity while Mdm2 controls p53 stability. Interestingly, MdmX is a target for Mdm2-mediated ubiquitination and proteasomal degradation in DNA-damaged cells, indicating that Mdm2 can also insure proper p53 transactivation activity (de Graaf et al., 2003; Kawai et al., 2003). The ubiquitination of MdmX by Mdm2 is also enhanced by tumor suppressor protein ARF, correlating with the ability of ARF to bind Mdm2 (Pan and Chen, 2003). Regardless of many unknown aspects in p53-MdmX-Mdm2 relationship, MdmX probably has as important biological impact on p53 function as its homologue, Mdm2

Other regulators of p53 stability

Mdm2 was for long thought to be the single E3-ligase for p53. Nowadays a few other proteins accomplishing this same duty have been discovered. Two of these factors are Pirh2 and Cop1, also E3-ligases for p53 and able to trigger p53 degradation in a Mdm2-independent manner (Leng et al., 2003; Dornan et al., 2004). Similarly to Mdm2, both of these genes are p53 targets, upregulated in cellular stress and forming an autoregulatory negative feedback loop with p53. Recently, another new E3-ligase, ARF-BP1, was found to be a mediator of p53 degradation (Chen et al., 2005). This protein is a major binding-partner of ARF tumor suppressor protein and its negative effect on p53 levels is prevented by this complex formation. As ARF-BP1 is not a p53 target gene in cellular stress, it could be responsible for maintaining the basal levels of p53, while Mdm2, together with Pirh2 and Cop1, could be the key regulators of p53 in stressed cells as at least Mdm2 levels are regulated by p53 only in response to cellular stress (Figure 7).

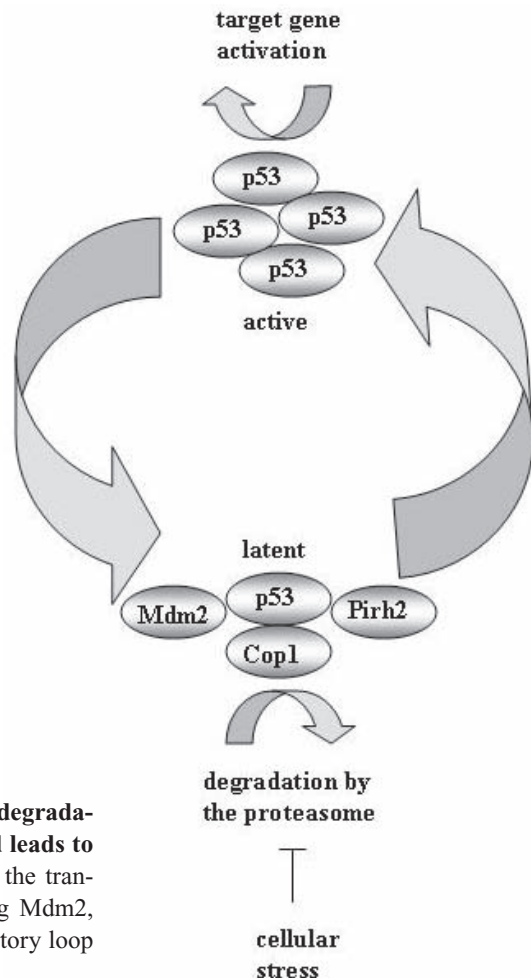


Figure 7. Cellular stress blocks p53 degradation by the proteasome pathway and leads to stable and active p53. p53 regulates the transcription of its target genes, including Mdm2, Pirh2 and Cop1 creating an autoregulatory loop in the control of p53 levels.

p53-mediated stress responses

p53 is a versatile, stress-induced protein, responding to a variety of cellular stress (Duthu et al., 1985; Fritsche et al., 1993; Hall et al., 1993; Lu and Lane, 1993; Graeber et al., 1994; Yamaizumi et al., 1994) (Figure 8). Most of the p53 functions coordinating cellular responses to stress are exerted by transcriptional activation of p53 target genes involved in cell cycle arrest, apoptosis or DNA repair (Ko & Prives, 1996). p53 acts as a transcription factor, recruiting a number of other transcriptional regulators and chromatin modifying proteins, like TFIID and p300/CBP to promote the transcription of its target genes through DNA-binding upon stress (Xiao et al., 1994; Espinosa and Emerson, 2001). The functional activation of p53 leads to either up-regulation (Harms et al., 2004; Yu and Zhang et al., 2005) or down-regulation (Mirza et al., 2003) of numerous different p53 targets. Depending on the severity of the damage, p53 is able to either promote cell cycle arrest and DNA repair or in the case of excessive damage, lead the cells to apoptosis (Vousden and Lu, 2002). How p53 determines the destiny of the cell is not totally clear. It may, however, be dependent on the levels of p53 (Chen et al., 1996), p53 modifications and interactions or interplay with other regulatory pathways.

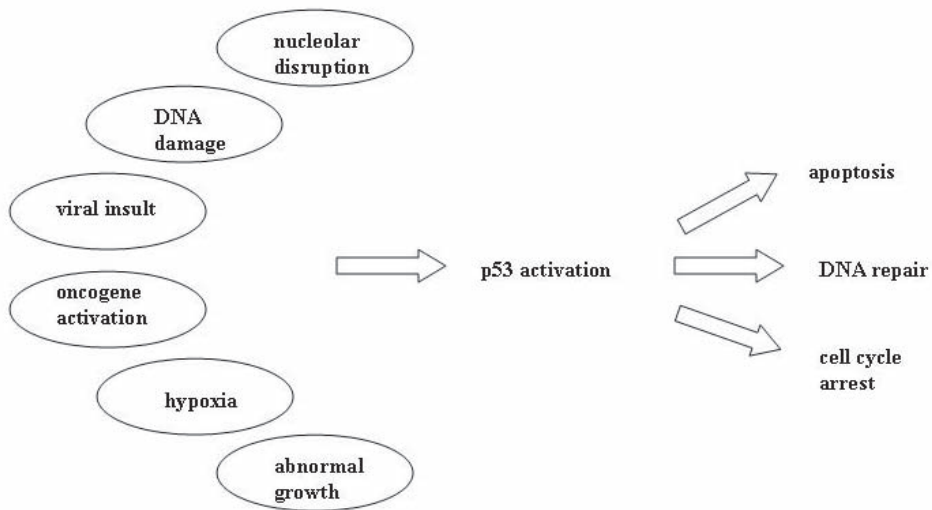


Figure 8. p53 responds to a variety of cellular stress and regulates apoptosis, cell cycle arrest and DNA repair by transcription-dependent and -independent means.

Cell cycle arrest

Following DNA damage, activation of p53 leads to halting the cell cycle progression. The p53-dependent arrest in G1 phase of the cycle mainly relies on its target gene p21^{WAF1/CIP1} (El-Deiry et al., 1993; Dulick et al., 1994; El-Deiry et al., 1994), which inhibits CDKs and concomitantly entry to S-phase (Harper et al., 1993; Xiong et al., 1993). Although p53 is not required for delaying the cell cycle progression in S phase upon DNA damage, it is directly involved in the control of centrosome duplication (Tarapore et al., 2001). In undamaged cells cyclin E/CDK2 complex triggers DNA synthesis as well as centrosome duplication. However, in the presence of impaired DNA synthesis p21-mediated inhibition of the cyclin E/CDK2 complex stops the centrosome cycle and protects the cells from centrosome amplification (Hinchcliffe et al., 1999; Lacey et al., 1999). This control of the centrosome duplication cycle is maintained in a p53-dependent manner (Tarapore et al., 2001). In G2 phase, the p53-dependent response is controlled by its targets 14-3-3 σ and GADD45, in addition to its major transactivation target p21 (Hermeking et al., 1997; Taylor and Stark, 2001). However, p53 seems not to be essential for this checkpoint as many cell types deficient for p53 still accumulate in G2 upon DNA damage (Lukas et al., 2004). In addition, p53 participates in the spindle checkpoint, ensuring proper chromatin segregation and maintenance of ploidy in the daughter cells (Cross et al., 1995).

DNA repair

p53 is a major player in the DNA damage-induced pathways and has an essential role in the maintenance of intact genome (Lane, 1992; Levine, 1997). Its role in several repair networks either directly or indirectly was discovered quite early and evidence for p53 involvement in NER, BER, MMR and the repair of DSBs has come from numerous studies (Reviewed in Sengupta and Harris, 2005; Gatz and Wiesmuller, 2006). p53 regulates the damage repair by either inducing the transcription of repair proteins or through interactions with the repair machinery (Gatz and Wiesmuller, 2006 and references therein) or by recognizing and associating with the damage sites themselves (Bakalkin et al., 1995; Lee et al., 1995; Reed et al., 1995). p53 is also capable of catalyzing the reannealing of the DNA strands (Oberosler et al., 1993; Brain and Jenkins, 1994) and has 3'-5' exonuclease activity (Mummenbrauer et al., 1996; Huang, 1998; Janus et al., 1999; Skalski et al., 2000).

The evidence for the involvement of p53 in mismatch repair comes mainly from its ability to transactivate some MMR genes, such as MSH2, MLH1 and PMS2 (Scherer et al., 2000; Warmick et al., 2001; Chen and Sadowski, 2005). The role of p53 in BER can be either direct or indirect, and a few BER genes are under the transcriptional regulation of p53 (Offer et al., 1999; Offer et al, 2001; Seo et al., 2002; Zurer et al., 2004; Lu et al., 2004). The regulation of the DSB repair by p53 seems not to require p53 transcriptional activation. Several studies have suggested a direct role for p53 in the repair of DSBs: p53 can bind many

central DSB repair proteins, like Rad51, RPA, BRCA1 and BRCA2, Bloom's syndrome protein and Werner's syndrome protein. It also represses HR upon DSBs and stalling of the replication fork, independently of its transactivation activity (Gatz and Wiesmuller, 2006 and references therein). p53 function may thus be required for inhibiting error-prone DSB repair and for halting replication until the damage has been repaired. Similarly p53 can probably contribute to the nonhomologous end-joining of DSBs to secure error-free NHEJ (Bill et al., 1997; Dahm-Daphi et al., 2005).

p53 in nucleotide excision repair

UV radiation and some DNA-damaging agents induce DNA lesions that block the transcription by RNA pol II and trigger nucleotide excision repair (Mello et al., 1995; Selby et al., 1997; Culliane et al., 1999). The formation of DNA damage-induced lesions and transcriptional inhibition acts as a signal for p53 induction (Yamaizumi et al., 1994; Ljungman and Zhang, 1996; Dumaz et al., 1997; Ljungman et al., 1999; Ljungman et al., 2001). p53 seems to have a transcription-dependent and -independent role in NER network. Its function in NER is independent of its stabilization, induced already by smaller amounts of damage. Cells expressing very low levels of wt p53 have been reported to have defective NER (Ford and Hanawalt, 1995; Smith et al., 1995; Wang et al., 1995) and the cells derived from Li-Fraumeni patients or cells infected with HPV-E6 to have defective GGR (Ford and Hanawalt, 1995; Zhu Q et al., 2000). p53's involvement in GGR has been widely accepted, while its effect on TCR is somewhat contradictory, some papers supporting the association of p53 in TCR (Wang et al., 1995; Mirzayans et al., 1996; McKay et al., 1999; Therrien et al., 1999; Mathonnet et al., 2003) whereas others do not (Ford and Hanawalt, 1995 & 1997; Ford et al., 1998; Wani et al., 2000). The differences in these results may, however, partly result from variable experimental setups as well as different wavelengths used in the UV studies (Therrien et al., 1999; Mathonnet et al., 2003).

p53 participates in the repair of both UV-induced 6-4PPs and CPDs and its activity is essential for CPD repair (Ford and Hanawalt, 1995; Ford and Hanawalt, 1997; Ford et al., 1998; Bowman et al., 2000). The presence of p53 is required for the repair of other types of DNA adducts as well, induced by exposure to environmental carcinogens, benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE) or benzo(g)chrysene (B(g)CDE) (Lloyd and Hanawalt, 2000; Lloyd and Hanawalt, 2002). p53's ability to participate in NER comes from its direct interactions with the repair factors of this pathway, like TFIIH (Wang et al., 1995; Gatz and Wiesmuller, 2006 and references therein) and it also controls the expression of XPC, XPE, a GGR repair protein p48 and Gadd45, a factor involved in growth arrest as well as NER (Hwang et al., 1999; Smith et al., 2000; Amundson et al., 2002; Adimoolam et al., 2002; Adimoolam and Ford, 2002; Tan and Chu, 2002). Additionally, p53 may have a role in mediating histone modifications and act as a chromatin accessibility factor to assist in the GGR pathway of the nucleotide excision repair (Rubbi and Milner, 2003a).

p53-induced apoptosis

The apoptosis-inducing activity of p53 is probably its major function in preventing tumor formation (Symonds et al., 1994; Schmitt et al., 2002). p53 coordinates apoptosis by either inducing several pro-apoptotic genes or by repressing anti-apoptotic factors. The most studied and relevant apoptotic p53 targets are Bax, Noxa, PUMA, Apaf-1 and p53AIP1 (Miyshita and Reed, 1995; Oda E. et al., 2000; Oda K. et al., 2000; Moroni et al., 2001; Nakano and Vousden, 2001). These targets play a role in the mitochondrial apoptotic pathway (Chipuk and Green, 2006) and deletion of any of them results in resistance to p53-mediated apoptosis, depending on the stress stimulus and cell type (Jeffers et al., 2003; Shibue et al., 2003; Villunger et al., 2003). p53-mediated gene repression also takes place under some stress conditions. For example, in hypoxic cells p53 suppresses the anti-apoptotic survivin to promote activation of the caspase pathway (Hoffman et al., 2002; Hammond and Giaccia, 2005). Additionally, p53 contributes to the external signalling pathway of apoptosis by inducing several different death receptors, like Fas/APO1, PERP and KILLER/DR5 (Muller et al., 1998; Benchimol, 2001) and by participating in their intracellular transport (Bennett et al., 1998).

In addition to the transcriptional regulation of apoptosis, p53 also contributes to the death pathway by directly associating with the Bcl-2 family members in the cytoplasm. p53 is rapidly translocated to the mitochondria in response to multiple death stimuli and binds anti-apoptotic factors Bcl-2 and Bcl-xL as well as apoptotic Bak and Bax to release cytochrome c from the mitochondria (Marchenko et al., 2000; Mihara et al., 2003; Chipuk et al., 2004; Leu et al., 2004). This rapid mitochondrial response inducing apoptosis may represent the immediate death signal, the transcriptional activation being the second-wave response to death stimuli (Erster et al., 2004). The p53 DNA-binding domain probably plays an important role in mediating this first-wave response through its interactions with Bcl-2 family proteins (Petros et al., 2004), again underlining the importance of this domain in tumor-suppression.

The apoptotic function of p53 is controlled by several p53-binding and modulating proteins, like the ASPP family proteins (Samuels-Lev et al., 2001). The ASPP family consists of ASSP1, ASPP2 and iASPP proteins, regulating p53-induced apoptosis. iASPP is likely to be an oncoprotein and acts as inhibitor of p53-mediated apoptosis by binding to p53 C-terminus (Bergamaschi et al., 2003). ASPP1 and ASPP2 are activated by DNA damage or oncogenic stress and bind to p53 and other p53 family members and stimulate their ability to induce apoptosis through proapoptotic genes, like Bax, PUMA and PIG3 (Samuels-Lev et al., 2001). The importance of the ASPP proteins is underscored by the finding that the ASPP contacting residues in p53 are mutated with high frequency in human cancers and that either overexpression of iASPP or downregulation of the ASPP1 and 2 are common in human tumors (Gorina and Pavletich, 1996; Samuels-Lev et al., 2001; Bergamaschi et al., 2003). Expression of E2F proteins also regulates p53-mediated apoptosis and E2Fs are induced by several types of

DNA-damaging agents that activate p53 (O'Connor and Lu, 2000). Increased E2F-levels can enhance p53 activity through induction of ASPPs (Fogal et al., 2005b) or through complex formation between p53 and E2F that enhance the apoptotic activity of p53 in damaged cells (Hsieh et al., 2002). In addition to ASPPs and E2Fs, other factors not reviewed in here, may modulate p53 response in the decision to choose the cell death pathway.

Senescence

The phenomenon of senescence prevents normal human fibroblasts from dividing indefinitely in cell cultures (Hayflick, 1965). Senescence is associated with shortening of telomeres in the ends of chromosomes during each cell cycle (Harley et al., 1990) and this shortening can be reversed by the action of telomerase enzyme, promoting the replicative potential of the cells (Bodnar et al., 1998).

In addition to tumor suppressor protein Rb, p53 is the major controller of cellular senescence (Ithana et al., 2001; Schmitt et al., 2002b; Beausejour, 2003) and can also induce premature senescence upon oncogenic signaling (Serrano et al., 1997). Cellular senescence is associated with changes in p53 modifications and enhanced p53 transcriptional activity (Atadja et al., 1995; Bond et al., 1996; Vaziri et al., 1997; Webley et al., 2000). ATM and Chk-kinases are recruited to the shortened telomeres and may promote a DNA-damage signalling cascade, leading to phosphorylation of p53 (d'Adda di Fagagna et al., 2003; Gire et al., 2004, Herbig et al., 2004). Subsequently, p53 can be regulated by p300-mediated acetylation, which can be controlled for example by the ING-family protein ING2 (Pedeux et al., 2005). Upon activation, p53 helps to maintain a nonproliferative state in the late passage cells mainly by upregulating p21 expression (Beausejour, 2003). Moreover, it can participate in the cellular senescence by decreasing the expression of the catalytic subunit of telomerase, hTERT (Xu et al., 2000). As cells need to bypass the senescence to become transformed (Shay and Roninson, 2004), this may be an essential function for p53's tumor suppressive properties.

p53 response to UV radiation

p53 is stabilized and activated by UV radiation and has an essential role in the protective UV response (Maltzman and Czyzyk, 1984; Ziegler et al., 1994). It is the key player in inducing UV radiation-promoted cell cycle arrest or apoptosis upon higher exposure to UV (Decraene et al., 2001). Mutations disturbing these functions are also frequently associated with skin cancers (Ziegler et al., 1994; Hartmann et al., 1996; Haapajarvi et al., 1999). The extent of p53-mediated cellular responses in the skin keratinocytes depend on the age and differentiation state of the cells, being more prominent in differentiated keratinocytes and

impaired in the aged ones (Latonen and Laiho, 2005 and references therein). Additionally, p53 has a protective role in the skin through participation to the melanin production and tanning process (Nylander et al., 2000).

Most of the studies on p53 responses to DNA damage have been performed with ionizing radiation or cytotoxic damage. Despite some overlapping features of the p53 response to DSBs, many of the UV-induced events in p53 activation are distinct (Reviewed in Latonen and Laiho, 2005). p53 modifications and stabilization for example occur with slower kinetics than with IR (Saito et al., 2003). p53 can be phosphorylated on its N-terminus by the ATM/ATR-pathway (Canman et al., 1998; Khanna et al., 1998; Khosravi et al., 1999; Chebab et al., 2000). Upon UV radiation, p53 is phosphorylated primarily by ATR and its downstream kinase Chk1. UV damage-induced kinases, DNA-PK, p38, HIPK2, JNK, TAFII250 and CK2 also phosphorylate p53 on its N-or C-terminal sites (Xu, 2003 and references therein). In addition, p53 is modified by acetylations and sumoylation upon UV radiation of the cells (Sakaguchi et al., 1998; Ljungman et al., 2001; Melchior and Hengst, 2002). As previously discussed, these modifications can possibly affect p53 stress response by finetuning its transcriptional activity or by enhancing its stability and interactions with other proteins, although contrasting results exist. Some p53 modifications, like Ser 392 phosphorylation, are associated only with UV damage (Latonen and Laiho., 2005 and references therein) and many of the modifications occur in a dose-dependent manner (Reinke and Lozano, 1997; Latonen et al., 2001). For example, p53 phosphorylation on Ser 46 after higher doses of UV by homeodomain-interacting protein kinase-2 (HIPK2), has been linked to the apoptotic p53 response (D'Orazi et al., 2002).

Low doses of UVC radiation induce a transient p53 activation and cell cycle arrest, while higher doses, leading to persistent transcription blockage, induce slower and more prominent induction of p53. The apoptotic response is associated with the activation of apoptotic p53 target genes and downregulation of anti-apoptotic p53 targets (Cotton and Spandau, 1997; Reinke and Lozano, 1997; Wu and Levine, 1997; Latonen et al., 2001). The transient activation of p53, leading to transactivation of cell cycle-regulatory genes, also leads to induction of Mdm2 levels and is associated with the feedback loop that is lacking from the apoptotic cells (Perry et al., 1993; Latonen et al., 2001).

In addition to the posttranslational modifications, p53 function is modulated by several protein-protein interactions upon UV damage. Prolyl isomerase (Pin1) controls p53 stability upon various kind of stress and regulates p53 activation by modulating its interactions with DNA and some cofactors (Wulf et al., 2002; Zacchi et al., 2002; Mantovani et al., 2004). In UV-damaged cells Pin1 is required for the Chk2 phosphorylation of p53 on Ser 20, leading to dissociation from Mdm2 and p53 stabilization (Zacchi et al., 2002; Berger et al., 2005). Several of the known stress-induced protein-protein associations and effects occur in a UV dose-dependent manner. For example p33ING1b and ASPP1 and ASPP2 proteins contribute to p53-induced apoptosis upon higher doses of UV radiation (Samuels-Lev et al., 2001; Cheung and Li, 2002).

As discussed above, p53 also participates in NER pathway in UV-damaged cells. While p53 participates in the repair of DNA lesions, it is also capable of protecting the cells against UV- or cisplatin-induced apoptosis in a TCR- and transcriptional recovery-dependent manner (McKay and Ljungman, 1999; McKay et al., 1999, 2000 and 2001). TCR-deficient cells undergo massive apoptosis upon UV radiation. Even though the induction of apoptosis correlates with p53 stability, it is not p53-dependent and p53 actually contributes to inhibition of apoptosis in TCR-proficient fibroblasts (Ljungman and Zhang, 1996; McKay et al., 1998; Ljungman et al., 1999; McKay et al., 2001).

p53-PATHWAY PROTEINS

This chapter introduces the most relevant p53-pathway proteins with respect of this study.

ARF

Alternative reading frame (ARF) protein is encoded by the Ink4a/ARF locus, producing both p16^{INK4a} and p19^{ARF} proteins (Quelle et al., 1995). p19^{ARF} (mouse ARF) and p14^{ARF} (human ARF) are nucleolar proteins involved in the p53 pathway (Quelle et al., 1995; Pomerantz et al., 1998; Weber et al., 1999; Zhang and Xiong, 1999). The proteins of the Ink4a/ARF locus control the progression of the cell cycle mainly by regulating the activities of Rb and p53 (Sharpless and DePinho, 1999). The gene products of Ink4a/ARF locus are also commonly inactivated in human cancers.

ARF is activated upon oncogene expression, like Ras, c-Myc, v-Abl and adenovirus E1A, leading to downstream activation of p53 (De Stanchina et al., 1998; Palmero et al., 1998; Radfar et al., 1998; Zindy et al., 1998; Sherr, 2001). ARF may also perform its growth suppressive activities in p53-independent manner (Sugimoto et al., 2003). The activation of p53 downstream of ARF is independent of p53 modifications (de Stanchina et al., 1998) as ARF exerts its p53-inducing functions by binding to Mdm2 RING domain and inhibiting the Mdm2-mediated degradation of p53 (Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998; Honda and Yasuda, 1999). Several mechanisms have been proposed for the ARF-mediated activation of p53, including degradation of Mdm2 by ARF (Zhang et al., 1998), and ARF-mediated relocalization of Mdm2 to nucleoli (Tao and Levine, 1999; Weber et al., 1999), which requires nucleolar localization signal (NoLS) of both proteins (Honda and Yasuda, 1999; Lohrum et al., 2000). This could possibly enable p53 accumulation in the nucleoplasm, although contrasting reports on the importance of this nucleolar Mdm2 relocalization exist (Llanos et al., 2001). Another model has proposed that Mdm2-p53 complex could exit from the nucleus to cytoplasm via nucleoli and that ARF-Mdm2 interaction could interfere with this function (Tao and Levine, 1999). ARF, Mdm2 and p53 have also been detected in the nuclear bodies in the nucleoplasm and this has been linked to ARF's capability of stabilizing p53 (Xiang and Xiong, 1999). Recently, a new p53 E3-ligase, ARF-BP1, was discovered (Chen et al., 2005). Blockage of the function of ARF-BP1 by ARF may be one way to stabilize p53.

Nucleophosmin

Nucleophosmin, NPM, (also referred to as B23, numatrin or NO38) is an abundant nucleolar phosphoprotein, shuttling constantly between the nucleoli and cytoplasm (Schmidt-Zachmann et al., 1987; Schmidt-Zachmann and Franke, 1988; Borer et al., 1989). Npm is essential for the development and its loss in germ-line

leads to embryonic lethality (Colombo et al., 2005; Grisendi et al., 2005). The first identified functions for this protein involved ribosome biogenesis and transport of the pre-ribosomal particles (Prestayko et al., 1974; Spector et al., 1984; Olson et al., 1986; Herrera et al., 1995; Savkur and Olson, 1998). Depletion of NPM modifies pre-RNA processing and alters maturation of the ribosomes (Ithana et al., 2003; Grisendi et al., 2005). The survival of Npm^{-/-} mouse embryos to mid-gestation does not suggest that NPM is an essential factor in the biogenesis of ribosomes (Colombo et al., 2005; Grisendi et al., 2005). NPM has also been linked to various other cellular processes, including stress response, DNA-repair and maintenance of the genomic integrity (Grisendi et al., 2006).

NPM belongs to a nuclear chaperone family of nucleoplasmins and can act as a chaperone for nucleic acids and proteins (Szebeni and Olson, 1999; Okuwaki et al., 2001). The chaperone activity of NPM has been mapped to its N-terminal domain (Hingorani et al., 2000) (Figure 9). NPM can also act as a histone chaperone and this may reflect its importance in the regulation of chromatin structure and transcription (Okuwaki et al., 2001; Swaminathan et al., 2005). In addition, NPM seems to be involved in the centrosome cycle (Okuda et al., 2002). It associates with unduplicated centrosomes during G1 and is dissociated from the centrosomes by phosphorylation on Thr 199 by cyclin E/CDK2 complex (Okuda et al., 2000; Tokuyama et al., 2001; Okuda et al., 2002; Tarapore et al., 2002). This allows the duplication of centrosomes during S phase of the cycle. Recent results also show that Ran-CRM1 complex, involved in the nucleo-cytoplasmic transport of NPM, is involved in controlling the NPM localization at the centrosomes (Wang et al., 2005). During mitosis, NPM re-associates with centrosomes and colocalizes with NUMA and BRCA1-BARD1 proteins (Zatsepina et al., 1999; Sato et al., 2004). The importance of controlled regulation of NPM during the centrosome cycle is underlined by the finding that centrosome amplification has been detected in the presence of excessively phosphorylated NPM (Saavedra et al., 2003; Zhang et al., 2004) and that its functional loss also leads to centrosome amplification and aneuploidy (Grisendi et al., 2005).

NPM protein exists in two different isoforms, B23.1 and B23.2, due to alternative splicing (Chang and Olson, 1989; Chang and Olson, 1990; Wang et al., 1993). Of these forms B23.1 is the major form and localizes predominantly in the nucleoli and functions in ribosome biogenesis. B23.2, lacking the DNA and RNA binding domain of NPM C-terminus localizes to nucleoplasm and also nucleoli through its interactions with the full length form (Wang et al., 1994; Okuwaki et al., 2002; Wang et al., 1993). In native conditions, NPM exists in an oligomeric form, as a hexamer (Herrera et al., 1996) and the splice variants are able to form multimers as well (Chang and Olson, 1989; Umekawa et al., 1993).

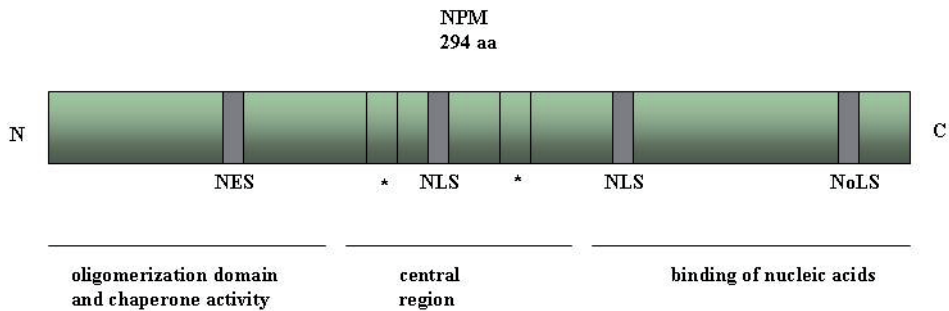


Fig 9. Functional domains of NPM (B23.1). The hydrophobic N-terminus of NPM contains its chaperone activity as well as the domain required for oligomer formation. The central region contains two acidic domains (*), responsible for histone binding. Binding of DNA and RNA occurs through the C-terminal domain, of which 35 last residues are lacking in the spliced form, B23.2. Ribonuclease activity requires both the C-terminal domain and the region between the two acidic domains. Additionally, NPM contains nuclear export signal (NES), two nuclear localization signals (NLS) and a nucleolar localization signal (NoLS).

NPM and cancer

In addition to its role in various cellular functions, NPM has been linked to neoplastic transformation (see Grisendi et al., 2006 for review). NPM is a transcriptional target of Myc-oncogene and may support cell proliferation in transformed cells through enhanced ribosome biosynthesis (Boon et al., 2001; Zeller et al., 2001). Its expression is enhanced in response to mitogenic signals and its protein level is often high in rapidly proliferating and malignant cells (Feuerstein et al., 1988; Chan et al., 1989; Gubin et al., 1999; Dergunova et al., 2002). Overexpression of NPM is detected in several cancer types, including melanoma, prostate, gastric, colon and ovarian carcinomas, possibly reflecting the high translational activity of these cells (Tanaka et al., 1992; Nozawa et al., 1996; Shields et al., 1997; Subong et al., Skaar et al., 1998; Bernard et al., 2003; 1999; Tsui et al., 2004). Opposite to highly proliferating cells, NPM levels in apoptotic or quiescent cells are decreased (Jiang and Yung, 1999; Wu et al., 1999; You et al., 1999). In addition to a supportive role in ribosome biogenesis, high levels of NPM may enhance proliferation by inhibiting apoptotic pathways (Ye, 2005). NPM may for instance inhibit apoptosis by blocking the activity of transcription factor IRF-1 (Kondo et al., 1997) and eukaryotic initiation factor 2 kinase PKR (Pang et al., 2003) or by mediating the anti-apoptotic activity of nerve growth factor, NGF (Ahn et al., 2005).

Interestingly, recent knock-out studies have shown that NPM has also growth-suppressive properties and an important role in the cellular stress response. NPM protein has for long been known to react to various kind of stress and several DNA-damaging or cytotoxic drugs. These cause NPM translocation from the nucleoli to the nucleoplasm (Yung et al., 1985; Chan et al., 1987; Yung et al.,

1990; Bor et al., 1992; Chan, 1992; Wu and Yung, 2002). In DNA-damaged cells NPM can promote repair of lesions by upregulating PCNA protein (Wu et al., 2002) and through regulating the localization of GADD45, a protein involved in DNA repair and chromatin remodelling (Gao et al., 2005). Moreover, *Npm*^{-/-} cells show increased γ -H2AX-ATM DNA damage foci formation (Colombo et al., 2005) and *Npm*^{-/-} or hypomorphic MEFs show genomic instability (Grisendi et al., 2005), indicating an essential role for NPM in the maintenance of the integrity of the genome.

NPM binds ARF in the nucleoli in a quantitative manner (Bertwistle et al., 2004). The complex relationship between these proteins has been studied extensively over the last few years and is still not fully understood. ARF has been found to inhibit the growth-promoting effect of NPM by blocking its function in rRNA processing and the transport of pre-ribosomal particles (Savkur et al., 1998; Ithana et al., 2003; Sugimoto et al., 2003; Brady et al., 2004) and by mediating NPM degradation (Ithana et al., 2003). NPM, on the other hand, stabilizes ARF, and ARF mutants lacking NPM binding domain have been shown to be more unstable (Kuo et al., 2004). Moreover, in MEFs lacking both p53 and NPM, ARF is relocalized to nucleoplasm and is found in lower protein levels (Colombo et al., 2005). Such cells are also more prone to transformation. Thus NPM's potential tumor-suppressive functions could occur through ARF pathway upon oncogenic stress. An opposite study has, however, suggested that ARF function could be inhibited through its nucleolar sequestration by NPM (Korgaonkar et al., 2005).

NPM may also affect p53 pathway by directly associating with p53 or through controlling p53 pathway proteins. The indirect control of p53 pathway can occur for example through GADD45 nuclear localization. In this way NPM could participate in the p53-mediated growth arrest (Wang et al., 1999; Gao et al., 2005). Several studies suggest that NPM acts as a negative regulator of p53 (Li et al., 2004; Chan et al., 2005; Li et al., 2005). NPM has been proposed to inhibit p53-induced apoptosis in hypoxic cells through reduced p53 Ser 15 phosphorylation by competing for the same kinase (Li et al., 2004; Li et al., 2005). NPM has also been found to affect p53 stability and activity in a positive way upon DNA damage (Colombo et al., 2002). Deletion of *NPM* gene, however, results in p53 activation. This occurs probably through an indirect mechanism due to DNA damage (Colombo et al., 2005) and aneuploidy of these cells (Grisendi et al., 2005).

NPM is associated with several hematopoietic malignancies, including acute myeloid leukemia (AML), anaplastic large cell lymphoma (ALCL) and acute promyelocytic leukemia (APL), through chromosomal translocations forming oncogenic fusion proteins (Raimondi et al., 1989; Morris et al., 1994; Yoneda-Kato et al., 1996; Redner et al., 2002; Chiarle et al., 2003) (Table 2). In addition, NPM is often deleted in myelodysplastic syndrome (MDS), and mice heterozygous for *Npm* develop hematological abnormalities, resembling this human syndrome (Olney and Le Beau, 2002; Grisendi et al., 2005, Berger et al., 2006). Mutations causing cytoplasmic NPM (NPMc⁺) have been detected in AML (Al-

calay et al., 2005; Falini et al., 2005; Falini et al., 2006). The cytoplasmic localization of NPM probably alters its normal nucleolar functions, but it also affects other NPM-binding proteins, including ARF which is relocalized to cytoplasm in NPMc+ -expressing cells (den Besten et al., 2005; Colombo et al., 2006).

The complex nature of NPM has led to several contrasting reports on its role as either an oncogene or tumor suppressor. Most likely NPM has both tumor-suppressive and -promoting functions through its role in ribosome biogenesis and interactions with important tumor suppressor proteins, these functions being dependent on its levels and localization as well as the genetic background of the cell. The aberrant overexpression of NPM could then lead to neoplastic transformation and too low levels again to genomic instability, pointing out the importance of the strict regulation of NPM levels and localization.

hematological malignancy	associated genetic alterations
acute promyelocytic leukemia, APL (Redner et al., 2002)	NPM-RAR α fusion due to t(5;17)(q35;q12) translocation
acute myeloid leukemia, AML (Raimondi et al., 1989; Yoneda-Kato et al., 1996; Alcalay et al., 2005; Falini et al., 2005)	NPMc+ mutations; NPM-MLF1 fusion due to t(3;5)(q25;q35) translocation; deletion (-5q35, -5)
anaplastic large cell lymphoma, ALCL (Morris et al., 1994)	NPM-ALK1 fusion due to t(2;5)(p23;q35)
myelodysplastic syndrome, MDS (Yoneda-Kato et al., 1996; Olney and Le Beau, 2002; Berger et al., 2006)	NPM-MLF1 fusion due to t(3;5)(q25;q35) translocation; deletion (-5q35, -5)

Table 2. Genetic alterations of NPM in human hematological malignancies.

Promyelocytic leukemia protein

Promyelocytic leukemia protein, PML, was initially found as a fusion protein with retinoic acid receptor α in patients with specific types of leukemia (de The et al., 1990 & 1991). PML protein is responsible for the formation of so-called PML or nuclear bodies, NBs (Also previously referred to as ND10 or promyelocytic oncogenic domains, PODs). These nuclear matrix-associated structures are usually about 0.2- 0.5 μ m in diameter and their number in the nucleus varies between 5-20 (Ascoli and Maul, 1991; Stuurman et al., 1992). The PML bodies were described already in 1984 by Bernstein et al. and unlike the cytoplasmic organelles, these subnuclear compartments are not surrounded by a lipid bilayer. Several regulatory factors, including Sp100, Daxx, SUMO-1, CBP, p53, HIPK2 and some DNA damage repair proteins are localized to these sites in a PML-

dependent manner (Szostecki et al., 1990; Boddy et al., 1996; Kamitani et al., 1998a; Ishov et al., 1999; Kim et al., 1999; Fogal et al., 2000; Li et al., 2000a; Lombard and Guarente, 2000; Zhong et al., 2000a; Boisvert et al., 2001). PML in NB structures is sumoylated (Sternsdorf et al., 1997; Kamitani et al., 1998a & 1998b; Muller et al., 1998). Several studies have suggested that sumoylation of PML is prerequisite for the recruitment of other proteins to these sites (Ishov et al., 1999; Lallemand-Breitenbach et al., 2001; Li & Chen, 2000; Maul et al., 2000; Zhong et al., 2000a), whereas sumoylation of p53 was found to be dispensable for its relocation to these structures (Fogal et al., 2000). PML itself has three main sumoylation sites, lysines 65, 160 and 490 (Figure 10), of which lysine 160 conjugation by SUMO-1 appears to be the most critical one for PML body formation. Removal of main sumoylation sites from PML protein prevents formation of NBs (Muller et al., 1998; Kamitani et al., 1998, Zhong et al., 2000a), although contrasting reports exist as well (Ishov et al., 1999; Lallemand-Breitenbach et al., 2001). Only the nuclear PML seems to be sumoylated, as colocalization with SUMO was not detected with cytoplasmic PML body aggregates (Ishov et al., 1999).

PML belongs to a family of nuclear proteins, containing one RBCC motif (Borden et al., 1995; Jensen et al., 2001) (Figure 10). PML protein exists as seven different, equally expressed isoforms due to alternative splicing, ranging from 48-97 kDs (de The et al., 1991; Goddard et al., 1991; Kakizuka et al., 1991; Fagioli et al., 1992; Kastner et al., 1992; Jensen et al., 2001). The N-terminal site is identical in all isoforms, differing only in their C-terminus or the length of the central region (Fagioli et al., 1992; Jensen et al., 2001). The exact functions of all the different isoforms is still not fully understood, even though some isoforms with specified functions exist. For instance, one isoform with cytoplasmic localization and association with TGF- β pathway has been characterized lately (Lin et al., 2004).

The number of PML bodies is known to vary depending on the cell type and condition, stress responses and cell cycle, being highest in G2 phase and then dispersing in the M phase (Koken et al., 1995; Maul et al., 1995; Terris et al., 1995; Everett et al., 1999). The number of NBs starts to increase already in G1 and peaks in G2 due to several fission and fusion events of these structures in S phase (Dellaire et al., 2006b). During mitosis, PML is partly found in so called "Mitotic accumulations of PML protein" (MAPPs) some of which are in physical contact with mitotic chromosomes (Dellaire et al., 2006a). These particles, which no longer contain SUMO, Sp100 or Daxx, may explain the partitioning of PML in dividing cells and contribute to the reformation of PML NBs in new daughter cells. The regulation of PML NB integrity during cell cycle is most likely controlled through its contacts with chromatin (Eskiw et al., 2003; Eskiw et al., 2004).

Multiple functions of PML

Several functions have been suggested for PML and PML NBs. The NBs may act as storage compartments for several proteins, which can be released upon need to other cellular compartments (Everett et al., 1999; Negorev et al., 2001; Borden, 2002). In addition, a role in cell cycle and growth control, apoptosis, viral infections, DNA repair and transcriptional regulation have been proposed for PML and NBs containing several different regulatory factors.

The levels of PML and the number of NBs are induced by interferon, reflecting their role in response to viral infection (Lavau et al. 1995; Stadler et al., 1995; Grotzinger et al., 1996; Gaboli et al., 1998). PML NBs attract several viral proteins, including herpes simplex virus type 1, cytomegalovirus (CMV), adenovirus 5, Epstein-Barr virus (EBV) and Simian virus 40 (SV40) proteins (Doucas et al., 1996; Ishov & Maul 1996; Maul et al., 1996; Szekely et al., 1996; Ishov et al., 1997; Everett 2001). Some of these proteins are able to disperse the structural integrity of PML NBs, and start the degradation of specific PML body associated proteins (Maul et al., 1993; Everett & Maul, 1994; Maul & Everett 1994; Ahn and Hayward, 1997; Everett et al., 1998; Chelbi-Alix & de The, 1999). The viruses may also utilize PML body associated proteins in their lifecycle by recruiting them for viral replication (Doucas et al., 1996). Alternatively, the PML NB itself could act as the site for viral DNA replication and transcription.

PML protein has growth suppressive properties (Mu et al., 1994; Ahn et al., 1995; Koken et al., 1995; Le et al., 1996; Quignon et al., 1998) and has been accepted as tumor suppressor (Salomoni and Pandolfi, 2002). The expression of PML is lost in several human cancer types of multiple histological origins and its expression status often correlates with the grade and progression of these cancers (Gurrieri et al., 2004a). It also plays a major role in the pathogenesis of acute promyelocytic leukemia, which will be described in more detail in the following chapter. PML overexpression leads to either growth arrest or apoptosis (Le et al., 1998; Pearson and Pelicci, 2001), and together with its partner Daxx, PML participates in nuclear apoptotic pathways (Torii et al., 1999; Zhong et al., 2000c). PML can inhibit the transformation induced by neu (c-erbB2, ERBB2), Ha-Ras and c-Myc as well as mutant p53 (Mu et al., 1994; Liu et al., 1995; Mu et al., 1996). It is also able to modulate the cell cycle progression by affecting several key proteins involved in the G1/S transition. Stable overexpression of PML alters the progression of the cycle and induces growth arrest by lengthening G1 (Mu et al., 1997). The apoptotic function of PML is probably one of its main growth suppressive properties, as PML is required for the induction of several apoptotic pathways, including Fas, tumor necrosis factor (TNF), ceramide, IR and interferons (Quignon et al., 1998; Wang et al., 1998). Pml null mice are protected against several of these pathways. These mouse cells have increased proportion of cells in S phase (Wang et al., 1998a) and they are less sensitive to lethal doses of γ radiation or Fas antibody treatment, supporting the pro-apoptotic role for PML protein (Wang et al., 1998b). Pml^{-/-} mice do not

develop spontaneous tumors, but they are subjected to a greater number of skin papillomas and B- and T- cell lymphomas when exposed to DMBA (dimethylbenzanthracene) and TPA (12-O-tetradecanoylphorbol-13-acetate) (Wang et al., 1998a and 1998b).

In addition, PML is able to recruit several other apoptotic proteins into PML NBs (Quignon et al., 1998). PML IV isoform for example regulates p53 localization to PML NBs through binding p53 central domain and affecting its transcriptional activity (Fogal et al., 2000). Several studies have suggested that PML plays a role in potentiating p53-mediated apoptosis (Fogal et al., 2000; Guo et al., 2000; D'Orazi et al., 2002). PML may for example enhance p53 transcriptional activity towards PIG3 promoter, a gene induced in apoptosis (Fogal et al., 2000), and mediate HIPK2 phosphorylation of p53 upon UV radiation (D'Orazi et al., 2002). In addition to apoptotic control, PML is induced by oncogenic Ras and regulates p53-dependent senescence in response to oncogenic signalling (Ferbeyre et al., 2000; Pearson et al., 2000; Pearson and Pelicci, 2001). Ras has been shown to induce PML-p53-CBP complex formation and p53 acetylation in a PML-dependent manner in vivo (Pearson et al., 2000).

PML has been suggested to participate in RNA synthesis and processing as well as replication and modification of the chromatin structure (de Jong et al., 1996). The control of chromatin structure by PML is likely as PML bodies include transcription coactivator and histone acetyltransferase CBP as well as chromatin modifying proteins, histone deacetylases (HDACs) (LaMorte et al., 1998; Doucas et al., 1999; Von Mikecz et al., 2000; Bandobashi et al., 2001; Boisvert et al., 2001; Wu et al., 2001). PML NBs associate with sites of active transcription (Wang, J. et al., 2004). Nascent transcripts have been shown to accumulate on the surface of PML bodies, although the bodies themselves do not contain RNA or DNA (Boisvert et al., 2000). PML and its most studied partner Sp100 have been linked to transcriptional control, mainly to repression. PML IV interacts with the nonphosphorylated form of Rb and has been shown to be required for the transcriptional repression by Rb and Mad (Mu et al., 1994; Seeler et al., 1998; Vallian et al., 1998; Li et al., 2000b Alcalay et al., 1998; Khan et al., 2001a; Khan et al., 2001b). PML can also control Daxx by sequestering it and inhibiting Daxx-mediated transcriptional repression (Li et al., 2000a). In addition, PML may regulate the expression of particular genes or gene families at specific gene loci, like MHC class I gene family and TP53 gene locus, found in the vicinity of PML bodies (Shiels et al., 2001; Sun et al., 2003).

PML may participate in the recognition and repair of DNA lesions. Several repair factors, including Nbs1, Rad50, Mre11 and Chk2 are localized to these sites and controlled in a damage-dependent manner (reviewed in Dellaire and Bazett-Jones, 2004). PML may also affect genomic stability through Bloom syndrome protein (BLM). *BLM* gene encodes a RecQ DNA helicase, whose loss in Bloom syndrome patients leads to genomic instability and predisposition to cancer due to higher levels of sister-chromatid exchange (SCE) (Ellis et al., 1995). BLM protein colocalizes with PML NBs and cells lacking PML or expressing PML-RAR α fusion protein, have abnormal BLM localization. Interest-

ingly, these cells have also higher frequency of SCE, mimicking the phenotype of Bloom syndrome cells (Zhong et al., 1999). The control of genomic stability by PML may also come from its centrosome association as PML IV isoform has been linked to the control of proper centrosome cycle (Xu et al., 2005).

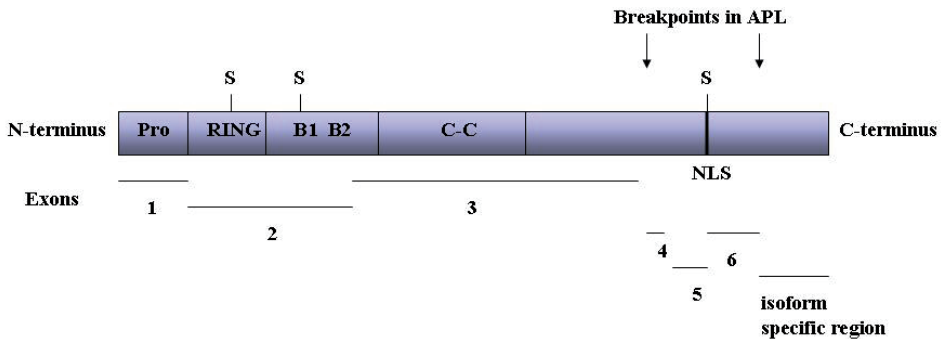


Figure 10. Structure of PML protein. PML exists in seven different cellular isoforms due to alternative splicing. All of the isoforms share common N-terminal region including a proline rich region, followed by a RING finger (C_3HC_4 zinc finger), two cys-rich B-boxes and a α -helical coiled-coil domain, together forming the RBCC-domain. The coiled-coil region can mediate the formation of PML homodimers (Perez et al., 1993). Three major sumoylation site at positions 65, 160 and 490 are indicated with S and breakpoints in APL at positions 394 and 552 at the C-terminal site.

Acute promyelocytic leukemia

PML was indicated as a tumor suppressor protein already a decade ago (Mu et al., 1994). As mentioned above, the protein was initially discovered in acute promyelocytic leukemia, APL patients, where PML was found to be fused to retinoic acid receptor α due to a reciprocal translocation event (de The et al., 1990 & 1991; Goddard et al., 1991; Kakizuka et al., 1991; Pandolfi et al., 1991; Kalantry et al., 1997). APL is a specific subtype of acute myeloid leukemia, AML, accounting for about 10 % of all AML cases. In APL leukemic cells, blocked at the promyelocytic stage, start to accumulate in the bone marrow. Cytogenetically, a translocation between chromosomes 15 and 17, PML and RAR α genes (t(15;17)(q22;q21)) respectively, is found in most of the APL cases (de The et al., 1990; Goddard et al., 1991; Kakizuka et al., 1991; Pandolfi et al., 1991). Two major breakpoints of PML have been described in APLs (de The et al., 1991; Goddard et al., 1991; Kakizuka et al., 1991; Pandolfi et al., 1991; Kastner et al., 1992). Depending on the breakpoint either between exons 3 and 4 or downstream of exon 6, the generated PML-RAR α form can be *bcr3* (short) or *bcr1* (long) (Huang et al., 1993; Vahdat et al., 1994) (Figure 10). The shorter form localizes to cytoplasmic bodies, CBs, and the patients carrying this *bcr3*

form have generally poorer prognosis than the ones with *bcr1* (Vahdat et al., 1994; Bellodi et al., 2006). While the other allele of PML is lost due to this chromosomal translocation, the remaining one may be wt or mutated. Total loss of functional PML in APL is associated with poor prognosis due to resistance to therapeutic agents (Gurrieri et al., 2004b).

Expression of PML-RAR α increases the survival of hematopoietic cell lines and causes resistance to apoptosis (Grignani et al., 1993; Wang et al., 1998). The fusion protein acts as an oncogenic transcription factor, promoting relocalization of PML from NBs through heterodimer formation between PML and PML-RAR α coiled-coil domains (Perez et al., 1993). The loss of normal functions of these structures inhibits the tumor suppressive properties of PML and other NB proteins, giving the cells growth advantage (Melnick and Licht, 1999) (Figure 11). The PML NB structure can be reinduced by treatments providing clinical remission (Daniel et al., 1993; Dyck et al., 1994; Weis et al., 1994; Koken et al., 1994). The development of APL in transgenic mice expressing PML-RAR α fusion protein has also been described extensively and was shown to occur with incomplete penetrance (Brown et al., 1997; Grisolan et al., 1997; He et al., 1997; He et al., 1998; Kogan et al., 2000; Kogan et al., 2001, Pandolfi et al., 2001). In addition, reduction in the levels of the normal PML form, by crossing Pml^{-/-} mice with PML-RAR α transgenic mice, led to an increase and earlier onset in the incidence of leukemia (Rego et al., 2001).

Expression of PML-RAR α also leads to dysregulation of the retinoic acid pathway (Kigan et al., 2000). The fusion protein competes with RAR α for binding to RA-response elements of RAR α target genes and abnormal binding to co-repressor complexes leads to changes in transcriptional regulation under physiological concentrations of RA. PML-RAR α is also a more potent transcriptional repressor and has abnormal associations with corepressor-histone deacetylase complex, remodeling the general chromatin structure to a more condensed configuration. Additionally, it is capable of repressing transcription through a pathway independent of HDACs and corepressors (Segalla et al., 2003). Combination of PML-RAR α homodimerization, enhanced corepressor binding and inhibition of the RA-pathway have been proposed to contribute to the hematopoietic differentiation block, accumulation of promyelocytic blasts and the development of APL (Grignani et al., 1993; Grignani et al., 1998; He et al., 1998; Lin & Evans, 2000; Lin et al., 1998; Minucci et al., 2000). PML-RAR α can in this way act as a double dominant-negative fusion protein, affecting both the normal PML and RAR α functions (Kastner et al., 1992; Perez et al., 1993).

The oncogenic activity of PML-RAR α has been associated with its N-terminal C-C domain, which is required for its sumoylation, microspeckle formation and for the inhibitory effect on RA-signalling pathway (Kim et al., 2005). A recent report showed that a sumoylation site, K160, in the N-terminus of PML is essential for the ability of PML-RAR α to block differentiation and immortalize primary hematopoietic precursor cells (Zhu et al., 2005). This transcriptional repression was due to Daxx binding, which was abolished by mutating the K160 residue.

Pharmacological concentrations of RA can release the repressor complexes from PML-RAR α and recruit activator complexes, normalizing the function of the cells through differentiation of the leukemic blasts, degradation of the fusion protein and restoration of normal PML NBs (Daniel et al., 1993; Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994; He et al., 1999). The transcriptional response by RA is largely PML-RAR α -dependent (Meani et al., 2005). cDNA microarrays of an APL cell line, NB4, has revealed that over 1100 transcripts may be regulated in APL cells in response to RA. Genes involved in the regulation of hematopoietic differentiation cofactors and chromatin modifiers are early targets of RA treatment (Meani et al., 2005). Other targets include factors associated with calcium signalling and IFN-signalling pathways (Zheng et al., 2005).

In addition to RA, arsenic trioxide (ATO, As₂O₃) has proven to be an effective inducer of remission in APL patients (Chen et al., 1997; Zhu et al., 1997). Over 90 % of the patients benefit from a high-dose all-*trans*-retinoic acid (ATRA) or arsenic trioxide (ATO) therapy, which induce a complete remission in most of the cases (Warrell et al., 1991; Chen et al., 1997; Shen et al., 1997; Shao et al., 1998; Shen et al., 2004). Patients treated with RA as a first choice usually relapse at some point, after which they may be switched to arsenic trioxide therapy. ATO has several ways of affecting the differentiation and apoptosis in APL cells. Lower concentrations of ATO (0.5 μ M) induce differentiation of the hematopoietic cells, while the higher concentration (2 μ M) is apoptotic. Like RA, ATO treatment also leads to the degradation of the fusion protein (Zhu et al., 1999; Lallemand-Breitenbach et al., 2001). In addition, PML protein is degraded upon this treatment (Lallemand-Breitenbach et al., 2001). Compared to RA, ATO also induces changes in less number of regulated genes and they do not involve the RA-pathway. The array study of ATO-treated NB4 cells showed the downregulation of β 1 integrins, upregulation of genes involved in the ubiquitin-proteasome pathway and downregulation of genes involved in the RNA processing and protein synthesis (Wang et al., 2003). In general, ATO may exert its effects more at the proteome level, affecting posttranslational and translational modifications. ATO and RA may also have additive effects together, as some genes involved in differentiation, cell cycle and growth control as well as apoptosis regulators have been found to be synergistically modified (Zhen et al., 2005). Moreover, in clinical studies targeting of PML-RAR α oncoprotein by combined RA and ATO treatment has led to high-quality disease-free survival (Shen et al., 2004). Basically, the more PML-RAR α is degraded, the better the recovery is in APL patients (Shen et al., 2004).

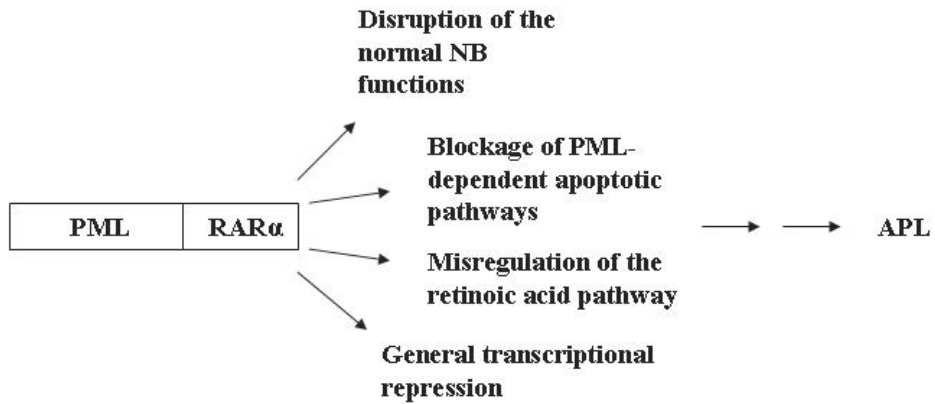


Figure 11. Cellular events leading to development of acute promyelocytic leukemia. Expression of PML-RAR α fusion protein disturbs several cellular pathways, including disruption of the normal functions of NB-associated proteins and PML-dependent apoptotic pathways. Blockage of normal RAR α and PML functions also inhibits the RA-response and prevents differentiation of the hematopoietic cells. PML-RAR α fusion protein may also have a general impact on chromatin structure and transcriptional regulation through HDACs and corepressor recruitment.

AIMS OF THE STUDY

Functional loss of p53 is a common feature for most of the cancers. In addition to p53 mutations, overexpression of its negative regulator Mdm2 may be responsible for the inactivation of this essential pathway in significant proportion of human cancers. Clearly, the maintenance of a strictly controlled p53-Mdm2 circuit is of great importance in controlling p53 functions and preventing tumorigenesis. Understanding the exact molecular mechanisms in p53 pathway is thus essential from therapeutic point of view.

The early events leading to p53 stabilization and activation have been studied extensively over the last decade. Most of the studies have concentrated on the relevance of p53 modifications, occurring in response to various kind of DNA damage. In addition to the posttranslational modifications of p53, its cellular localization and complex formation with other proteins may be critical in the alteration of its function.

In our studies we have used UV radiation as a model of DNA damage. UV damage activates a complex cellular stress response in the cells, leading to transcriptional inhibition and activation of p53. The main aims of this research were:

- 1) to study the early events in damaged cells leading to release of p53 from the negative pressure of Mdm2
- 2) compare the cellular localizations of p53 pathway proteins in stressed and unstressed cells
- 3) find out which proteins could regulate p53 activity and stability in UV-damaged cells and unravel the molecular mechanisms behind them
- 4) address whether these particular p53 pathways are altered in human cancers

MATERIALS AND METHODS

Cells

The following cell lines were used in the study:

Cell line	cell type and description	source
A375	human malignant melanoma	ATCC, CRL 1619
HL-60	myelocytic leukemia	A.Vaheri (*)
NB4	acute promyelocytic leukemia, APL (Lanotte et al., 1991)	A. Vaheri
p53 ^{-/-} -mdm2 ^{-/-} MEF	mouse embryonic fibroblasts (Montes de Oca Luna et al., 1995)	G. Lozano (**)
Pml ^{-/-} MEF	mouse embryonic fibroblast (Wang et al., 1998)	P.P. Pandolfi (***)
SaOS-2	p53-null human osteosarcoma	ATCC, HTB 85
U2OS	human osteosarcoma	ATCC, HTB 96
U937	promonocytic leukemia cell line	A. Vaheri
WS1	human skin fibroblast	ATCC, CRL 1502

(*) Haartman Institute, University of Helsinki, Helsinki, Finland

(**) University of Texas, M.D. Anderson Cancer Center, Houston, Texas, USA

(***) Memorial Sloan-Kettering Cancer Center, New York, New York, USA

Cell culture

Cells were cultured in a humidified atmosphere at 5% CO₂ at 37°C in Dulbecco's modified Eagle medium, DMEM supplemented with 10% fetal calf serum (FCS) (GIBCO or PromoCell) (A375 and mouse embryonic fibroblasts), 10% FCS + non-essential amino acids (NAA) (WS1 human fibroblasts) or 15% FCS (SaOS-2, U2OS cells). Suspension cells (HL-60, NB4, U937) were maintained in RPMI, supplemented with 10% FCS. Mononuclear cells were isolated by purification through Ficoll gradients from fresh peripheral blood samples, using established protocols and the lysates were used for studying p53 and NPM levels (III, Fig. 5A).

Treatment of the cell cultures

UV treatments of the cells were carried out with Stratalinker 2400 (Stratagene, La Jolla, CA) and the cells were exposed to either 10 or 35 J/m² of UVC (254 nm) (I, II, III). For the inhibition of the proteasome activity, the cells were treated with 10 μ M MG132 (Affinity Research Products) (I, II). Histone deacetylase inhibitor experiments were carried out by treating the cells with 100 ng/ml Trichostatin A, TSA (Sigma) (III). Arsenic trioxide (ATO, As₂O₃) (Sigma) was used at 1 μ M (I) or 2 μ M (III) concentrations. retinoic acid (RA) (Sigma) was used at 2 μ M concentration (III).

Mutagenesis

Mdm2 deletion mutants Δ 89-222 and Δ NoLS (Δ 464-471, nucleolar localization-defective mutant Mdm2) were constructed by site-directed mutagenesis (QuickChange Site-directed Mutagenesis Kit, Stratagene) and the products were verified by DNA sequencing.

Transfections

Mouse embryonic fibroblasts were transfected by electroporation (Gene Pulser II, Bio-Rad) with 280 V and 975 μ F in Optimem (GIBCO) (I,II). U2OS cells were transfected by lipofection (Lipofectamine 2000, Invitrogen) (II,III) and NB4-suspension cells by Amaxa nucleofector, Kit T, program X-001 (III). The following plasmids were used in transfections: PML III (PML-L) in pSG5, PML IV (PML-3) and PML IV-3K (sumoylation deficient triple mutant PML IV) in pCDNA3 and PML-RAR α in pSG5 (obtained from G. del Sal) (I, III); wt Mdm2 and Mdm2 Δ NoLS (Δ 464-471, nucleolar localization-defective mutant Mdm2) (I); B231.1-pCHA and B231.2-pCHA (obtained from Dr. Kyosuke Nagata, Okuwaki et al., 2001) (II), SUMO-1 expression vector (obtained from Dr. Jorma Palvimo) (II); Myc-tagged K-cyclin expression vector (originally obtained from Dr. Sibylle Mitnacht, Ellis et al., 1999) (II); Myc-tagged Xenopus NPM (NO38) (obtained from Dr. Marion Schmidt-Zachmann, Zirwes et al., 1997); NPM-ECGFP (described in Kurki et al., 2006) (III); p53-pCDNA3 (III); PG13xRE-luciferase reporter vector (obtained from Dr. Bert Vogelstein) and pRLSV40 Renilla Luciferase control vector (III).

siRNA

siRNA was used to deplete NPM and Mdm2 from U2OS cells (III). The duplex sequences for NPM siRNA were as described in Colombo et al., 2002 (purchased from Dharmacon Research, Inc.). Mdm2 RNAi duplexes containing the sequence 5'UGGUUGCA UUGUCCAUGGC3' targeting Mdm2 mRNA and SMARTpool Mdm2 siRNA mix were purchased from Dharmacon Research, Inc. The duplexes were transfected into cells by lipofection (Oligofectamine,

Invitrogen). The cells were incubated for 1 (Mdm2 siRNA) or 3 days (NPM siRNA) posttransfection.

Luciferase reporter activity assays

For p53 activity assays, NB4 cells were treated with either ATO or RA 24 hours prior the transfection (III). p53-pDNA3, PG13xRE luciferase reporter vector and Renilla luciferase control vector, pRLSV40, were transfected by using Nucleofector Kit T (Amaxa). Luciferase activities were measured by Dual-Luciferase Reporter Assay System (Promega) and luminometer (DCR-1, Digene Diagnostics) five hours post-transfection. Renilla activity was used to normalize the transfection efficiencies. Fold induction of p53 activity was calculated as a mean value of at least two separate experiments.

Preparation of cellular extracts

Monolayer cells were washed with Tris-buffered saline (TBS). EBC lysis buffer containing 25 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 4 mM NaF, 100 μ M Na_3VO_4 , 1mM phenylmethylsulfonyl fluoride, 100 KIU/ml aprotinin and 10 μ g/ml leupeptin was added on the plates and cells were scraped into eppendorf tubes and incubated on ice for 20 minutes. The insoluble fraction was separated from the soluble one by centrifuging the cells with 14 000 rpm for 15 min. The pellet of the insoluble fraction was boiled in Laemmli sample buffer (LSB), containing dithiothreitol (DTT) (100mM) (I, II, III). Suspension cells were pelleted prior to washing with TBS. Cells were suspended into lysis buffer and treated as above to separate the soluble and insoluble fraction (III).

The obtain total cellular lysates, cells were resuspended into urea-Tris buffer containing 9 M urea, 75 mM Tris-HCl (pH 7.0) and 0.15 M 2-mercaptoethanol (III). The suspension was sonicated briefly and protein concentrations were determined by Bio-Rad D_c protein assay kit (Bio-Rad, Hercules, CA). The samples were boiled in LSB-DTT for 5 min. Alternatively, total cell lysates were extracted in LSB-DTT and sonicated briefly before boiling the samples (I).

Immunoprecipitation

After normalization of protein concentrations, cellular lysates were immunoprecipitated with specific antibodies and the samples were collected on GammaBind-G Sepharose beads (Pharmacia Biotech). The beads were washed four times with TBS. Immunocomplexes were boiled in LSB-DTT prior to analysis. The following antibodies were used in immunoprecipitations: anti-acetyl-Histone H3 (06-599, Upstate) (III), anti-Mdm2 mix (SMP14, Santa Cruz Biotechnology; 2A10; IF2, Oncogene Sciences) (I, II), anti-c-Myc 9E10 (Biosite) (II), anti-NPM (Zymed) (II, III), anti-p300 (N-15, Santa Cruz) (III), anti-p53 mix (DO-1, PAb1801, PAb421) (I, II), anti-p53 (FL393, Santa Cruz Biotechnology) (III), anti-PML (PG-M3, Santa Cruz Biotechnology) (I, III), anti-PML (H-238,

Santa Cruz Biotechnology) (III). To exclude unspecific binding, mouse or rabbit IgG (Dako Cytomation, Denmark) were used as negative controls (II, III).

Immunoblotting

Lysates and immunoprecipitates were separated by 7.5%, 9%, 10% or 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were transferred to nitrocellulose membrane (Trans-Blot, Transfer Medium, Bio-Rad) and the membranes were blocked with either 5% milk in TBS or 3% bovine serum albumin (BSA). Immunoblotting was carried out by using specific antibody dilutions in 1% BSA. The following antibodies were used in this study: anti-acetyl-Histone H3 (06-599 and 06-942, Upstate) (III), anti-GAPDH (Europa Bioproducts, Cambridge, UK) (II, III), anti-GST (clone BC8E8) (II, III), anti-Histone H3 (FL-136, Santa Cruz Biotechnology) (III), anti-Mdm2 mix (SMP14, Santa Cruz Biotechnology; 2A10; IF2, Oncogene Sciences) (I, II), anti-NPM (Zymed) (II, III), anti-p53 (FL393, Santa Cruz Biotechnology) (I, II, III), anti-p53 (DO-1) (III), anti-PML (PG-M3, Santa Cruz Biotechnology) (I, III), anti-PML (H-238, Santa Cruz Biotechnology) (I, III), anti-SUMO-1 (GMP-1, Zymed) (II) and anti-PCNA (Santa Cruz Biotechnology) (III). The primary antibodies were followed by secondary antibodies coupled to horseradish peroxidase, HRP (Dako Cytomation, Denmark). The washes of the membranes between primary and secondary antibodies were done in TBS containing 0.05% Tween 20 (Amersham Biosciences). The proteins were detected with enhanced chemiluminescence, ECL (Amersham Life Sciences or Millipore).

Immunofluorescence analysis

Monolayer cells were fixed for 20 minutes with 3.5% paraformaldehyde, PFA, followed by permeabilization with 0.5% NP-40 lysis buffer and blocking with 3% BSA (I, II, III). Suspension cells were centrifuged for 3 minutes 600 rpm on glass slides prior to fixation with PFA (Shandon cytospin II cytocentrifuge, Thermo Electron Corporation) (III). The following primary antibodies were used in the study: anti-Mdm2 mix (SMP14, Santa Cruz Biotechnology; 2A10; IF2, Oncogene Sciences) (I, II), anti-c-Myc 9E10 (Biosite) (II, III), anti-NPM (Zymed) (II, III), anti-NPM (C-19, Santa Cruz Biotechnology) (II), anti-p53 (FL393, Santa Cruz Biotechnology) (I, II, III), anti-p53 (DO-1, PAb421, Pab1801) (I), anti-PML (PG-M3, Santa Cruz Biotechnology) (I, III), anti-PML (H-238, Santa Cruz Biotechnology) (I, III) or anti-PML antibody mix (A-20 and N-19, Santa Cruz Biotechnology). Specific antibodies were detected by secondary antibodies conjugated to fluorochromes. The following secondary antibodies were used: swine anti-rabbit or rabbit anti-goat FITC (I, II), rabbit anti-mouse TRITC (DAKO) (II), goat anti-mouse, goat anti-rabbit or donkey anti-goat antibody conjugated Alexa fluorochromes 488 and 594 (Molecular Probes) (I, II, III). The absence of crossreactivity was verified in separate experiments. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) (I, II, III) and RNA

with Syto 12 green fluorescent nucleic acid stain (Molecular Probes) (II). The fluorochromes were visualized with the Zeiss Axioplan 2 Imaging MOT (Jena, Germany) equipped with appropriate filters (Chroma). Images were captured with Zeiss AxioCam CCD-videocamera and image processing and analysis was performed with AxioVision programs, versions 3.0 (I,II) or 4.4 (III). Confocal images in the study I were made with Bio-Rad MRC1024. Staining intensities in the study II were quantified by KS Run 3.0 analysis program (KS 400, Zeiss) from 100 nuclei per each time point.

Fluorescence Recovery After Photobleaching (FRAP) and image analysis

U2OS cells were cultured on LabTek II chambered coverglass (Nalge Nunc International), and transfected with NPM-EGFP (III). The cells were treated with 35 J/m² UVC 24 hours post-transfection. For imaging, the medium was changed to DMEM without phenol red, supplemented with 25 mM Hepes (PromoCell). Zeiss 510 META confocal laser scanning microscope (LSM, Zeiss) with heating stage and Plan-Neofluar 40x oil objective with 1.3 NA was used for photobleaching and imaging of the samples. For imaging, the Argon laser line (458 nm) was set at 2% and for bleaching at 100% with 85% output. The size and shape of each nucleolus was defined with region of interest (ROI) and the ROI was bleached after three scans with 30 iterations. 97 post-bleach images were collected every second.

The image analysis and quantification of the fluorescent intensities were calculated from at least two separate experiments and 8-10 cells. LSM 510 Physiology Software was used for measuring the fluorescent intensities. The method of Rabut and Ellenberg (2005) was used for the analysis of mobile fractions and recovery halftimes. Statistical significance of the results were evaluated as p-values by using Student's t-test.

In vitro translation

In vitro translation of Mdm2 (I, II), p53 (I, II), NPM (II, III) and different PML isoforms (I, III) were performed with TNT Coupled Reticulocyte Lysate System (Promega) from T7 promoter containing expression vectors of each gene, either in the presence of 20 μ Ci of ³⁵S-methionine (specific activity 1000 Ci/mmmol, Amersham) (II, III) or unlabeled methionine (I). Translation products were immunoprecipitated as described above. The samples were separated by SDS-PAGE and the proteins were analyzed by autoradiography (II, III) or immunoblotting (I).

GST pulldown assays

Mdm2 GST-fusion proteins (II) (obtained from Dr. David Meek), PML IV-GST (III), GST-NPM (III) and GST-protein control (GST-CRP1) (II) were produced in BL-21 Escherichia coli cells following induction with IPTG. The fusion proteins were captured on glutathione-Sepharose 4B beads (Amersham) for the pull-down experiments. Binding of the specific ³⁵S-methionine labeled partner protein, ³⁵S-NPM (II, III) or ³⁵S-PML (III), was performed in 140 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mM PMSF (TNE-buffer) overnight at +4 °C under rotation, after which the beads were washed ten times with TNE-buffer and finally with PBS. Washed beads were boiled in LSB containing dithiothreitol (10mM) for 5 minutes and the supernatant was loaded to SDS-PAGE gels. The proteins were analyzed by autoradiography.

Chromatin isolation

Chromatin preparations and nucleoplasmic fractions from U2OS cells and MEFs were performed as described by Mendez and Stillman (2000) with slight modifications (III). The samples were separated by SDS-PAGE and analyzed by immunoblotting as described above.

RESULTS AND DISCUSSION

UV radiation induces stabilization of the tumor suppressor protein p53 (Maltzman and Czyzyk, 1984). The stabilization is associated with posttranslational modifications of both p53 and its negative regulator Mdm2 (Meek and Knippschild, 2003; Xu, 2003) as well as decreased interaction between them (Latonen et al., 2001). Release of p53 from the negative pressure of Mdm2 promotes transactivation of specific p53 target genes in a dose-dependent manner, leading to either growth arrest and p53-assisted NER pathway or to apoptosis upon excessive damage (Latonen et al., 2001; Gentile et al., 2003; Gatz and Wiesmuller, 2006). In addition to the posttranslational modifications, the complex network resulting in functional p53 requires multiple stress-induced protein-protein interactions, each contributing to p53 activation (Lavin and Gueven, 2006).

Regulation of the Mdm2-p53 interface has a great potential in the development of new therapeutics targeting p53 pathway in cancer cells. Particularly potential factors reinducing the p53 pathway in cancer cells could be proteins interacting with either Mdm2 or p53, uncoupling the degradation pathway and leading to active p53 upon cellular stress. A couple of promising pharmacological inhibitors of this interaction have recently been described (Issaeva et al., 2004; Vassilev et al., 2004; Yang et al., 2005). During our studies of the impact of cellular stress on p53 and Mdm2 interaction, we observed associations of Mdm2 with promyelocytic leukemia protein as well as with nucleolar protein nucleophosmin. The similar localization patterns detected upon certain stress situations as well as the damage-induced relocalizations of these particular proteins led us to study the possible association of PML and NPM in the regulation of p53 pathway through its negative inhibitor Mdm2.

Cellular stress and DNA damage evoke subnuclear translocations of p53 pathway proteins (I, II, III)

PML exists in the nucleus mostly attached to PML NBs, in a detergent-insoluble form (Muller et al., 1998; Lallemand-Breitenbach et al., 2001). The size and number of these bodies is affected by the phase of the cell cycle as well as exposure to cellular stress (Koken et al., 1995; Maul et al., 1995; Terris et al., 1995; Everett et al., 1999). γ radiation for example is known to increase the number and size of NBs and attract p53 to these suborganelles (Pearson and Pelicci, 2001). In contrast to the γ radiation-induced effect on PML NB structure, we found that PML bodies lost their nuclear architecture upon UV radiation of the cells (I, Figure 1A and 6). The NBs were dispersed to smaller microstructures and nucleoplasmic PML staining increased rapidly, starting from one hour after UV exposure. Similar effect on PML NB structures has been earlier described to occur upon heat shock and exposure to heavy metals, like Cd^{2+} (Maul et al., 1995; Ishov et al., 1999; Eskiw et al., 2003; Nefkens et al., 2003). As the level

of PML was not upregulated during the early timepoints (I, Figure 5C) and we detected an increase in the soluble form and decrease in the insoluble form of PML (I, Figure 4A), the evident enhanced nucleoplasmic PML staining was due to release of PML from the NBs to a more soluble form. Similar observations about the disruption of PML body structures were published by Seker et al., 2003 and later by Salomoni et al., 2005. The mechanism for this UV-induced PML NB dispersion is not known, although DNA damage-induced kinase pathways may be involved. UV radiation activates the p38 MAPK and ERK1/2 kinase pathways, which are regulating PML release from the NBs upon Cd²⁺-exposure (Nefkens et al., 2003). Other suggested mechanisms for PML release include changes in its sumoylation status, which has been proposed to control the formation of PML NBs. Exposure to heat shock, heavy metals or adenovirus E1A expression leads to dispersal of PML bodies through a desumoylation event (Eskiw et al., 2003). Also transcriptional activity of the cells may play a role as inhibition of either RNA pol I (Kiesslich et al., 2002) or RNA pol II by DRB (Eskiw et al., 2004; our unpublished results) leads to scattering of PML and its associated proteins to the nucleoplasm, suggesting that any stress leading to inhibition of the transcription may interfere the integrity of these structures. The reason for PML body dispersal upon transcriptional inhibition may result from the alterations in the chromatin structure as only a slight change in the conformation of the surrounding DNA is enough to obstruct the maintenance of intact PML NBs in a SUMO-independent manner (Eskiw et al., 2004). PML NBs themselves do not contain nucleic acids in their core (Boisvert et al., 2000), but they are surrounded by and in extensive contact with chromatin, which maintains the structural integrity of NBs in interphase cells (Eskiw et al., 2003; Eskiw et al., 2004). This could also mean that DNA damage, causing reorganization of the chromatin, may be the only required signal for the instability of PML NBs.

PML translocation also involved the perinucleolar area, which started to show positivity of PML staining in the nucleolar necklace structures, also referred to as nucleolar caps, in UV-radiated cells (Figure 12 & I, Figure 1B). Interestingly, Mdm2 was also detected in these insoluble structures, partly colocalizing with PML (I, Figure 1B, C & D), while p53 remained in the nucleoplasmic fraction (I, data not shown). The significance of this localization pattern of PML and Mdm2 is presently not clear, although it could be indicative of their role in rRNA transcription. PML bodies are known to contain several factors associated with transcription (reviewed by Zhong et al., 2000b). They contain both CBP/p300 and RNA pol II (Von Mikecz et al., 2000) and associate with RNA pol II at the sites of active transcription (Kiesslich et al., 2002). DNA helicase II also associates with intact PML NBs and inhibition of transcription leads to translocation of this protein to the perinucleolar area (Fuchsová et al., 2002). Furthermore, Mdm2 could play a role in transcription through its interaction with RNA and ribosomal protein L5 (Marechal et al., 1994; Elenbaas et al., 1996). Another study, with similar observations on PML and Mdm2 translocation to the perinucleolar area upon DNA-damage caused by cytotoxic drugs, suggested that

the nucleolar localization of these proteins affects p53 stabilization (Bernardi et al., 2004). According to this model PML would potentiate p53 stabilization in stressed cells by sequestering Mdm2 to nucleolus. This hypothesis, however, is unlikely due to the slow kinetics of these events and because only a small fraction of the total Mdm2 is being translocated to nucleoli in stressed cells.

Similarly to UV-induced translocation of PML, we also detected that UV exposure induced translocation of the nucleolar protein NPM to the nucleoplasmic compartment (II, 1A & B, 3A) as well as to the perinucleolar area around to nucleoli (II, data not shown). This kind of translocation has been shown before to occur upon some DNA damaging and cytotoxic drugs (Yung et al., 1985; Chan et al., 1987; Yung et al., 1990; Bor et al., 1992; Chan, 1992; Wu and Yung, 2002), and the relocalization upon UV was recently also reported by Rubbi and Milner (2003b). The translocation was detected in p53 and ARF null cell lines, indicating that it did not require a functional p53 pathway (II, Figure 1A and data not shown). The relocalization was also evident in analysis of the insoluble and soluble fractions of the cells, which showed a decrease of NPM in the nucleoli containing insoluble compartment, associated with an increase in the free, soluble nucleoplasmic fraction (II, Figure ID). A slight increase in the total levels was also detected, as has been earlier shown also by Wu and Yung (2002) (II, 1D). Translocation of nucleophosmin from nucleoli to nucleoplasm is known to require ATP (Wu et al., 1995; Finch and Chan, 1996). The activating signal for this UV-induced effect on NPM distribution is not yet understood. Although the nucleolar structures undergo morphological changes upon transcriptional inhibition, the nucleoli themselves are not completely disrupted. Therefore, nucleolar disruption may not fully explain the release, as observed by staining of the ribosomal RNA in the nucleoli (II, Figure 1C). As NPM is a heavily modified phosphoprotein and its cellular localization is known to be affected at least during the cell cycle by several phosphorylation events, the mechanism could include UV-induced modifications, like phosphorylations (Grisendi et al. 2006, and references therein). Although many of the kinase pathways known to phosphorylate NPM are activated upon UV, we could not find any evidence that they would play a role in UV-induced translocation of NPM (unpublished results). Other posttranslational modifications can be involved as well. An obvious inducer of NPM relocalization could be a direct signal from stalled RNA pol II by UV-induced lesions, causing ribosomal stress and release of NPM upon disturbance of its main functions in the nucleoli.

As both NPM and PML were found in the perinucleolar area of the UV-treated cells, we also studied the colocalization of these proteins. We observed a fraction of NPM and PML in necklace structures, the majority still colocalizing in the nucleoplasmic fraction (III, Figure 1B). Additionally, we found that NPM and PML colocalized in mature PML NBs (III, Figure 1C) as well as at centrosomes of the metaphase cells (unpublished data). The detection of these colocalizations with endogenous proteins and in different compartments, depending on the cell phase and stress, suggests that the association of NPM and PML is physiologically relevant and function in both stressed and unstressed cells.

In addition to the observed colocalizations in UV-treated cells, we detected similar cellular localization patterns of the p53 pathway proteins Mdm2, PML and NPM upon exposure of the cells to other kind of cellular stress as well. Proteasome inhibitor MG132 and arsenic trioxide both induced the number of PML NBs (I, Figure 1A & C). MG132 induces accumulation of several proteins, including many PML NB-associated factors, into the nucleoli (Klibanov et al., 2001; Mattsson et al., 2001; Latonen et al., 2003). The colocalization of PML and Mdm2 was also detected in this compartment as well as in the PML NBs of the proteasome-inhibited cells (I, Figure 1A). Mdm2 was also found to colocalize with NPM upon same conditions in the nucleoli (II, Figure 2A). ATO, on the other hand, induced recruitment of Mdm2 to larger PML NB aggregates (I, Figure 1C), while NPM was not detected in these structures (unpublished data). The relocalization of Mdm2 to these larger NBs upon ATO treatment occurred with slower kinetics than seen with other cellular stress inducers, emphasizing the differences in the timing and spatial distribution of these associations upon exposure to various kind of cellular stress.

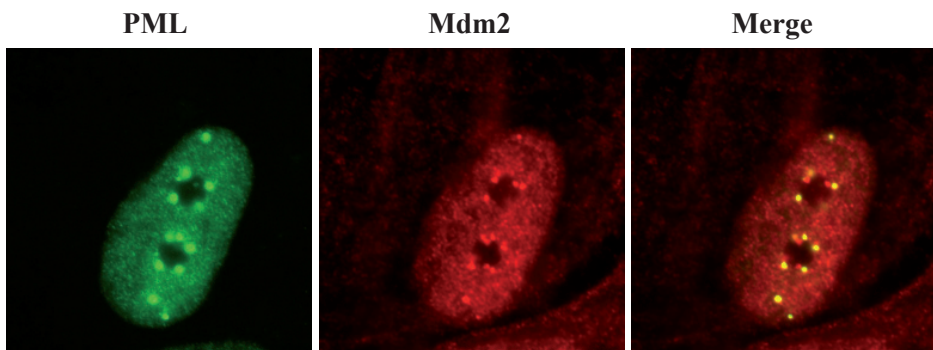


Figure 12. UV-induced nucleolar structures in WS1 human fibroblasts.

UV radiation induces rapid and transient complex formation between p53, Mdm2, PML and NPM (I, II, III)

The ability of PML to affect cellular stress responses of p53 has been amply demonstrated by many studies (Fogal et al., 2000; Guo et al., 2000; D’Orazi et al., 2002; Ferbeyre et al., 2000; Pearson et al., 2000; Pearson and Pelicci, 2001). In addition, the positive effect of NPM on p53 function upon DNA damage of the cells was shown by Colombo et al. (2002). As we had detected colocalizations between these p53 pathway proteins and Mdm2, we started to study their possible effect on p53 function through Mdm2. Moreover, the impact of PML on p53 function upon exposure to UV radiation was largely unknown at the time this study was started.

Studies were performed using several cell lines, WS1, SaOS-2 and U2OS, to detail their dependency on p53 or ARF. The cells were exposed to 35 J/m² of UVC radiation to induce UV stress and p53 stabilization and activation. As expected, p53 protein in normal WS1 human fibroblasts accumulated clearly upon this treatment, starting from three hours postradiation (I, Figure 5A & C). As a sign of a transient p53 induction, the feedback loop with Mdm2 was also activated and the levels of Mdm2 protein were increased at later time points leading to enhanced p53-Mdm2 complex formation (I, Figure 5A & C). Interestingly, we also observed complex formation between PML and p53 as well as PML and Mdm2 (I, Figure 5A). These interactions took place early after radiation, prior to p53 stabilization, suggesting a role in the regulation of p53 function in UV-treated cells. To study whether the novel interaction between Mdm2 and PML could be mediated through p53, we performed similar assays in a p53-negative cell line, SaOS-2. As Mdm2-PML interaction was also observed in this cell, in a dose-dependent manner, the respective complex formation was clearly not p53-dependent (I, Figure 4B). In addition, we observed the interaction between these proteins upon MG132-treatment, blocking the proteasomal degradation of both Mdm2 and PML (I, Figure 4B).

To address whether the interactions of the proteins are direct we performed *in vitro* interaction assays. These studies showed that PML binding to Mdm2 was significantly weakened by using Mdm2-deletion constructs lacking the C-terminal domain of Mdm2 (I, Figure 3B). This domain also contains the Mdm2 RING domain responsible for its E3-ligase activity. As none of the used Mdm2-deletion mutants were fully devoid of interaction between these proteins, Mdm2 may have more than one PML binding site. Mdm2, on the other hand, preferred binding to PML isoform IV, independently of its sumoylation status (I, Figure 3A). Interestingly, p53 has earlier been shown to interact with only this PML isoform, and require PML C-terminus and p53 DBD (Fogal et al., 2000). Considering these results, we performed *in vitro* competition assays to study whether a shared binding site for p53 and Mdm2 is localized in the PML C-terminus (I, Figure 7). Higher amounts of PML in the *in vitro* assay increased the association of p53 with Mdm2 and vice versa, the elevation of Mdm2 levels increased p53-PML complex formation. The results suggested that rather than competing for the same binding site on PML, p53, Mdm2 and PML can form trimeric complexes and that this is promoted by the PML-Mdm2 interaction. As both p53 and Mdm2 were found to transiently interact with PML rapidly after UV stress, it is possible that a trimeric complex is formed, playing a role in the UV response of the tumor suppressor protein p53.

Similarly to PML, we found that NPM forms kinetically rapid and transient complexes with Mdm2 early after UV exposure (II, Figure 3B). Moreover, this interaction was independent of the p53 status of the cells (II, Figure 3C). A recent paper on UV-induced responses of NPM and ARF, showed similar association between NPM and Mdm2 following UV-exposure, although with a little bit delayed kinetics (Lee C et al., 2005). *In vitro* interaction analyses using GST-pull down experiments showed that interaction is dependent on the N- and

C-terminal domains of Mdm2, containing its p53 binding site and the RING domain (II, Figure 2C). Although p53 requires the same binding domain for its interaction with Mdm2, the *in vitro* competition assays suggested that p53 is able to promote the interaction between NPM and Mdm2. Higher amounts of p53, however, competed for the interaction and decreased NPM-Mdm2 complex formation. As p53-NPM interaction has recently been shown to require the N-terminal site of p53 (Maiguel et al., 2004), also essential for its Mdm2 binding, the higher nucleoplasmic NPM levels upon cellular stress might be able to disrupt the interaction between p53 and Mdm2, leading to inhibition of the p53 degradation by the proteasome and its transcriptional activation.

As we had detected transient interactions between PML, Mdm2 and p53 as well as NPM, Mdm2 and p53 following UV stress, we further studied the association of PML and NPM proteins in these early complexes. These proteins were found to interact within similar kinetics like the other UV-induced transient complexes (III, Figure 1A). Furthermore, the interaction between PML and NPM was independent of the presence of either p53 or ARF (data not shown). *In vitro* interaction analyses of the interacting domains between NPM and PML showed that several PML isoforms associate with NPM, which was dependent on the intact N-terminus of NPM and its oligomerization domain (III, Figure 2A-C). Interestingly, Mdm2 also required this oligomerization domain for binding to NPM (unpublished results). As all of these transient interactions took place with similar kinetics (I, II, III), the results strongly implicate the existence of a UV-induced multiprotein complex (Figure 13). The presence of NPM and PML in p53-Mdm2 complex may thus potentiate the early events in p53 functional activation following UV damage of the cells.

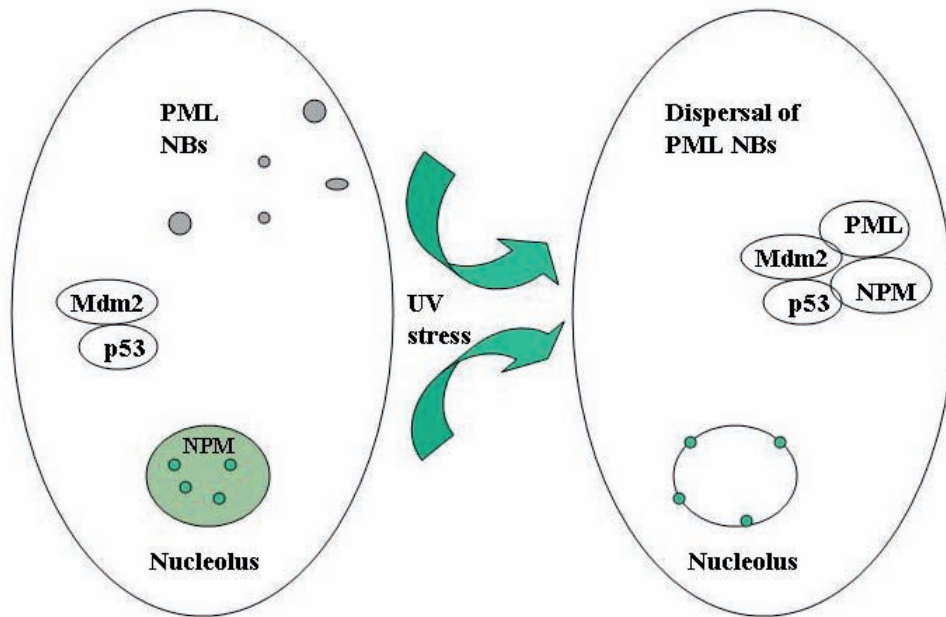


Figure 13. Model for the early events in p53 activation following UV stress. Exposure of cells to UV induces site-specific translocations of the p53 pathway proteins, associated with transient interactions, possibly causing formation of a multiprotein complex involved in p53 stress response.

The impact of PML and NPM on p53 stability and activity (I, II)

p53 stabilization has been shown to take place upon numerous cellular stress situations, like exposure to UV radiation and transcriptional inhibition (Maltzman and Czyzyk, 1984; Ljungman and Zhang, 1996; Ljungman et al., 1999). The stabilization of p53 could involve disruption of the interactions with its negative regulators or direct inhibition of their E3-ligase activities. Our results showed that Mdm2 and PML interact rapidly and kinetically in a transient manner in UV-stressed cells prior to p53 stabilization (I, Figure 5A). This could reflect a role for PML in p53 activation or stabilization. In addition, the *in vitro* data showed binding of PML to Mdm2 RING finger, possibly affecting its E3-ligase activity towards p53 (I, Figure 3B). An increase in the levels of endogenous p53, associated with enhanced PML-Mdm2 complex, was also detected following ectopic PML expression in U2OS cells (unpublished observations). Moreover, p53 in Pml^{-/-} MEFs is present in a multimodified form compatible with ubiquitinated p53, suggesting that PML is necessary for the inhibition of p53 degradation (Louria-Hayon et al. 2003). Recent data from several laboratories has suggested that PML could influence p53 stability by inhibiting the abil-

ity of Mdm2 to degrade p53, either by directly blocking the E3-ligase activity (Louria-Hayon et al., 2003), through direct interactions (Zhu et al., 2003) or by PML-mediated Mdm2 translocation to nucleoli in stressed cells (Bernardi et al., 2004). One study in a breast carcinoma cell line, MCF-7, also showed that stable suppression of PML expression results in enhanced p53-Mdm2 complex formation and a decrease in p53 levels due to enhanced degradation (Bao-Lei et al., 2006). Several reports have thus shown the importance of PML in the control of basal and stress-induced p53 levels.

PML was shown to be required for p53 stabilization upon γ radiation and certain cytotoxic drugs (Louria-Hayon et al., 2003; Bernardi et al., 2004). To test whether it could also be essential for UV radiation-induced p53 stabilization, we performed both immunofluorescence and western analysis from UV-treated wt and Pml^{-/-} MEFs. Although p53 was present in the PML null cells in more ubiquitinated forms, it was stabilized in a similar manner as in the wt MEFs upon UV exposure (unpublished results, Figure 14). Similar findings were presented in a study of Salomoni et al. (2005), showing equal increase in p53 levels in both wt and Pml^{-/-} MEFs in response to UV. Thus, PML seems essential for p53 stability in unstressed cells and in some, but not all stress-induced pathways. Even though PML does not seem to play a role in UV-promoted stabilization of p53, it could still influence the p53 posttranslational modifications or protein-protein interactions, finetuning p53 target gene activation or repair functions. This is supported by several studies suggesting a role for PML in modifying p53 activity. Furthermore, PML is itself a p53 target, forming in this way a positive-feedback loop in the p53 activation (de Stanchina et al., 2004).

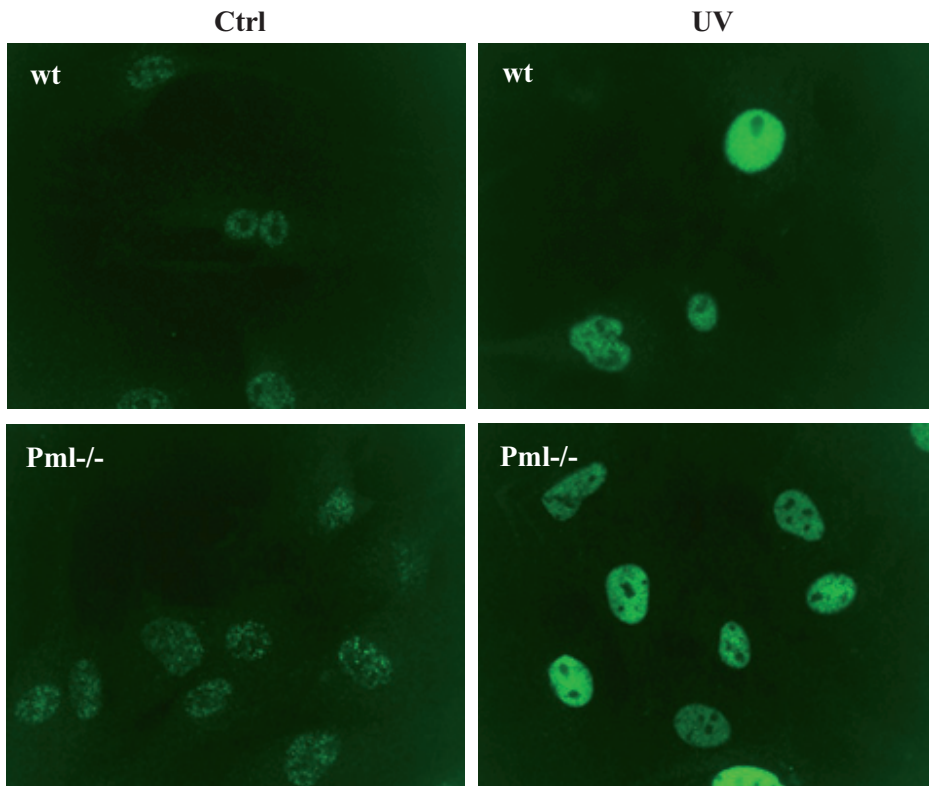


Figure 14. PML is dispensable for p53 stabilization following UV radiation. Immunofluorescence staining of p53 in control and UV-treated (35 J/m², 6 h) wt and Pml^{-/-} MEFs.

NPM has been implicated in the regulation of p53 activity in several studies with contrasting results, some suggesting that NPM acts as p53 activator (Colombo et al., 2002; Zou et al., 2005) and others as repressor (Maiguel et al., 2004; Li et al., 2004; Li et al., 2005). We studied the effect of NPM overexpression on p53 levels in U2OS cells and found that ectopic expression of NPM stabilized both p53 and Mdm2 (II, Figure 4A & B). Similar effect of NPM on p53 stability has been shown by Colombo et al. (2002). Additionally, we found that the increase in Mdm2 levels was clearly independent on the p53 transactivation, as the same phenomenon was evident in SaOS-2 cells (II, Figure 4C). The mechanism of stabilization of p53 by NPM could involve inhibition of the Mdm2 E3-ligase activity, as NPM binds Mdm2 RING finger domain (II, Figure 2C). Alternatively, NPM could disrupt the interaction between p53 and Mdm2 due to competing binding domains (II, Figure 2D and Maiguel et al., 2004). To verify that NPM increases p53 stabilization by inhibiting Mdm2, we silenced NPM using siRNA in U2OS cells (II, Figure 5A & B). Depletion of NPM from these cells reduced the basal levels of p53 as well as the UV-induced stabilization of p53 (II, Figure 5C). Moreover, the decrease in the levels of p53 was associated with enhanced

p53 complex-formation with Mdm2, suggesting that NPM can control p53 levels through blocking its interaction with Mdm2 (II, Figure 5C). Further, depletion of Mdm2 together with NPM was able to rescue the negative regulation of p53 by NPM siRNA (II, Figure 5D), confirming the ability of NPM to block Mdm2-mediated degradation of p53 through controlling their interaction.

Several nucleolar proteins have lately been associated with p53 stabilization in stressed cells. Ribosomal proteins L5, L11 and L23 have been shown to inhibit the Mdm2-mediated degradation of p53 (Lohrum et al., 2003; Dai and Lu, 2004; Jin et al., 2004; Zhang et al., 2004). The general mechanism controlling these proteins and their downstream effects on p53 could be the reorganization and disruption of the nucleolar structure upon cellular stress. Cells can tolerate relatively high amounts of DNA damage without stabilizing p53. On the other hand, nucleolar disruption alone, even in the absence of DNA damage, is able to stabilize p53 (Rubbi and Milner 2003b). The structural and functional integrity of the nucleoli was though proposed to be the main signal for p53 stress response (Rubbi and Milner, 2003b). Interference of rRNA processing has also been shown to lead to p53-dependent cell cycle arrest (Pestov et al., 2001). The possibility that nucleoli function in maintaining low p53 levels also fits to the regulation of p53 stability and nucleolar disassembly during the cell cycle (David-Pfeuty et al., 1996). Inhibition of the CDKs also leads to disruption of the nucleolar structure and accumulation of p53 (David-Pfeuty, 1999; David-Pfeuty et al., 2001). In accordance, we also found that NPM is translocated to nucleoplasm upon CDK2 inhibition by roscovitine treatment, with concomitant p53 stabilization (unpublished results). p53 can also be localized to nucleoli in unstressed cells. It is colocalized with the sites of rRNA transcription, suggesting that it can sense inhibition of transcription immediately even before disruption of the nucleolar structure (Rubbi and Milner, 2000). Alternatively, the nucleolar compartment could play a role in p53 degradation, as the ubiquitinated p53-Mdm2 complex has been proposed to travel through the nucleoli on its way to the proteasome machinery. The inhibition of this pathway upon various stress situations could thus affect the nucleolar structure. In stressed cells, NPM may be one of the main factors affecting p53 stability upon nucleolar reorganization. However, p53 is stabilized and activated in *Npm*^{-/-} cells, suggesting that NPM may not be essential in the maintenance of p53 levels (Colombo et al., 2005; Grisendi et al., 2005). The deletion of *NPM* from mice resulted in p53 activation probably indirectly due to checkpoint activation in cells with mitotic aberrations and DNA damage (Colombo et al., 2005). Some of the opposite results on the effect of NPM on p53 activity may result from different experimental settings and cell lines.

Modification of p53 by SUMO has been proposed to affect p53 transcriptional activity (Gostissa et al., 1999; Rodriguez et al., 1999). Conjugation of several proteins by SUMO has been linked to regulation of the cellular localizations, interactions with other proteins and stability of the proteins. We detected a slower migrating form of p53 in the insoluble fraction of the cells early after UV exposure, correlating kinetically with p53 protein complexes (I, Figure 5B).

The slower migrating p53 form corresponded to a SUMO-modified p53, migrating around 65 kDa. Although PML NBs have been linked to sumoylation of certain proteins and the 65 kDa form was detected at the same time frame with p53-PML interaction, we did not find any evidence for p53 translocation to NBs (I, data not shown). Alternatively the 65 kDa p53 form could be bound to the chromatin fraction. Due to the kinetics of the early interactions, NPM was tested for its ability to induce p53 sumoylation. Ectopic expression of NPM led to an increase in a slower migrating, sumoylated p53 form (II, Figure 4F). Furthermore, NPM preferred binding to the sumoylated p53 (II, Figure 4F) and associated with this form in UV-treated cells (II, Figure 4D & E). Although we could not find any evidence for p53 sumoylation taking place in NBs, PML could still potentiate the sumoylation event of p53 by acting as a platform for protein interactions, in other cellular compartment than PML NB.

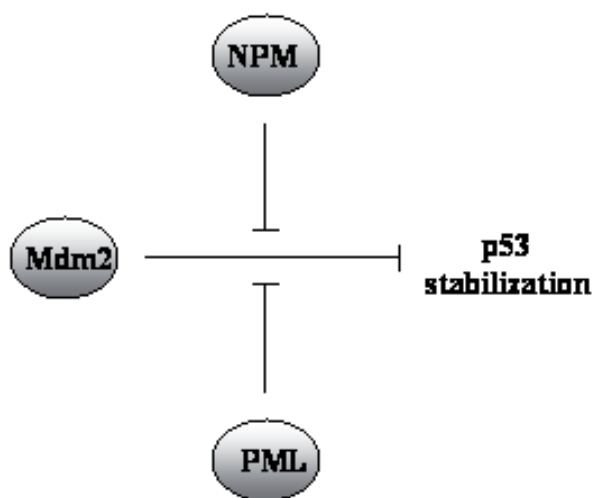


Figure 15. NPM and PML inhibit Mdm2-mediated degradation of p53. Both NPM and PML are regulators of p53 stability and may block the Mdm2-mediated degradation of p53 (Kurki et al., 2003; Louria-Hayon et al., 2003; Zhu et al., 2003; Bernardi et al., 2004; Kurki et al., 2004). NPM seems to be essential for p53 stabilization upon UV radiation (Colombo et al., 2002; Kurki et al., 2004), γ radiation (Colombo et al., 2002), polyamine depletion (Zou et al., 2005) as well as upon viral stress (Kurki et al., 2004). PML plays a role in p53 stabilization upon γ radiation (Louria-Hayon et al., 2003) and after exposure to cytotoxic drugs (Bernardi et al., 2004).

NPM is associated with p53 stabilization in viral insult (II)

NPM interacts with several viral proteins like Rev, HIV and Tat (Fankhauser et al., 1991; Miyazaki et al., 1995; Li, 1997). p53 function is also altered by several viral proteins, including viral cyclin (K-cyclin) (Verschuren et al., 2002). This viral protein is a cyclin-D homologue, encoded by the Kaposi's sarcoma-associated herpesvirus (KSHV) and is known to induce p53 stabilization, concomitant

with its activation leading to either growth arrest or apoptosis (Verschuren et al., 2002). As NPM was required for p53 stabilization upon DNA damage, we wanted to assess its possible role in p53 stress response.

We transiently expressed K-cyclin in U2OS cells. Immunofluorescence analysis showed a major translocation of NPM to the nucleoplasmic fraction following expression of this protein (II, Figure 6A). This was also evident from the soluble fraction of the cells in western analysis (II, Figure 6B), while the total levels remained unaltered. NPM may be attracted to the nucleoplasmic fraction through its interaction with this viral protein (II, Figure 6C). Alternatively, the expression of this protein could affect the function of the nucleoli as nucleolus is targeted by several viral proteins (Hiscox, 2002). Thus, viral stress by K-cyclin may lead to release of the nucleolar proteins and promote NPM-K-cyclin interaction in the nucleoplasmic compartment.

The effect of K-cyclin expression on p53 levels was similar in U2OS cells as described before in MEFs (Verschuren et al., 2002), leading to stabilization of the protein (II, Figure 6D). Mdm2 protein was stabilized as well (II, Figure 6E). These inductions in the levels of p53 and Mdm2 were associated with increased interactions with NPM as well as decreased interaction between p53 and Mdm2, suggesting that also following this kind of cellular stress NPM is able to affect the negative pressure of Mdm2 on p53 (II, Figure 6D & E). As several viruses are able to target nucleoli (Hiscox, 2002) and adenovirus infection for instance blocks the rRNA synthesis (Castiglia and Flint, 1983), causing nucleoplasmic distribution of NPM (Matthews, 2001), the general pathway affecting p53 in the viral infections could take place through the interference of nucleolar functions.

PML controls the localization of p53 pathway proteins (I, III)

The plurifunctional PML NBs can be divided into subgroups according to their protein composition, size and movement. PML is able target several proteins with variable functions to PML NBs in a cell cycle phase and stress-dependent manner (Dellaire and Bazett-Jones, 2004). Nowadays over forty proteins are found in the database for PML NB proteins, many of these being RING finger proteins (Dellaire et al., 2003). As we had detected colocalization of Mdm2 with PML following DNA damage, inhibition of the proteasome and treatment with ATO, we addressed whether Mdm2 localization was altered by PML itself. Ectopically expressed PML III or IV was able to relocalize Mdm2 in a dose-dependent manner to large PML NB structures in a p53-null background (I, Figure 2). As we did not detect a strong interaction between Mdm2 and PML III *in vitro*, this relocalization could involve the endogenous PML IV isoform or other associated proteins. Interestingly, in studies of the capacity of PML to relocalize different Mdm2 deletion mutants, we found that Mdm2 lacking its nucleolar localization signal was found in the nucleoli in cells treated with a proteasome inhibitor only when PML was colocalizing with it, pointing towards a role of PML in Mdm2 nucleolar entry (I, data not shown). The nucleolar localization of Mdm2 is usually affected by its nucleolar localization signal (NoLS) in its C-terminal site (Lohrum et al., 2000), but

the results suggest that either PML or some PML associated protein is able to direct Mdm2 to this subnuclear compartment independently of its NoLS sequence. This observations was corroborated by a study of Bernardi et al. (2004), in which they showed the absence of Mdm2 nucleolar localization in stressed Pml^{-/-} cells.

Additionally, PML was able to control the localization of NPM, relocalizing it from the nucleoli to either perinucleolar area, mature PML bodies or to nucleoplasm in a dose dependent manner (III, Figure 2D). NPM translocation was detected with PML III, PML IV, PML IV-3 (sumoylation defective mutant) and PML-RAR α fusion protein (III, Figure 2D and data not shown). The immunofluorescence data further confirmed the *in vitro* data on association of NPM with several PML isoforms and suggested that NPM binding occurs through a common domain of different PML isoforms through its N-terminus. To further test whether PML could have a role in the UV-promoted translocation of NPM, we performed immunofluorescence stainings of the wt and Pml^{-/-} MEFs. The data showed a striking difference already in unstressed Pml null and wt MEFs, NPM being prominently nucleoplasmic in the absence of PML (III, Figure 4A). Further, translocation following UV stress to the nucleoplasmic fraction was also not as evident as in wt MEFs, proposing a defect in the UV response of NPM. Additionally, NPM perinucleolar staining pattern, usually detected at the border of nucleoli within one hour in UV-damaged cells, was delayed in the Pml null cells. Whether this reflects a defect in the reorganization of the nucleolar structure upon transcriptional inhibition remains to be studied.

NPM has an essential role in the maintenance of genomic integrity and Npm^{-/-} cells show more increased staining for γ -H2AX repair foci (Colombo et al., 2005). NPM is also linked to DNA repair and binds chromatin following IR-induced DSBs (Wu et al., 2002; Lee et al., 2005). To verify that the different subcellular localization of NPM in Pml^{-/-} cells was not due to increased DNA- damage of the PML null cells, we performed immunofluorescence stainings with γ -H2AX (III, results not shown). The staining was comparable in wt and Pml null MEFs, suggesting that NPM translocation probably does not occur through a mechanism involving damaged DNA. However, PML may affect NPM localization directly, or alternatively, it could act as a platform protein, mediating some essential modifications or protein-protein interactions involved in the control of NPM localization and its stress response.

PML dictates NPM-chromatin association and NPM-p300 complex formation in DNA-damaged cells (III)

NPM has been shown to associate with histones (Okuwaki et al., 2001) and bind chromatin in γ radiated cells (Lee SY et al., 2005) A recent paper also suggested a role for NPM as a general transcriptional regulator through control of histone acetylation and nucleosomal disassembly (Swaminathan et al., 2005). We studied the possible association of NPM with chromatin in UV-treated U2OS cells (III, Figure 3A). The results showed that NPM is associated with chromatin, without a major change in this property after UV treatment of the cells. As

DNase treatment of the cells only partially released NPM from chromatin, this finding suggested that NPM may actually be more tightly bound to chromatin associated proteins than to DNA itself. In contrast, p53 was increasingly associated with chromatin upon UV radiation, and this association was clearly diminished in DNase-treated cells, in similar manner than the association of acetylated histones (AcH3, lysine9, respectively) with DNA (III, Figure 3A). Furthermore, AcH3 was found to be released from chromatin fractions even without DNase-treatment, in response to UV radiation, indicating a conversion of acetylated histones to a more soluble form upon DNA relaxation.

To address whether NPM association with histones was regulated upon UV treatment, we studied their interaction using coimmunoprecipitation analyses of UV-treated MEFs (III, Figure 3B). The results showed transient complex formation between AcH3 and NPM shortly after radiation. As NPM acetylation by p300 has been linked to its association with histones (Swaminathan et al., 2005) and p300 is also known to regulate chromatin structure through histone modifications in damaged cells (Chan and La Thangue, 2001), we tested a possible involvement of NPM in p300-complexes following UV treatment. p300-NPM interaction was also transiently increased in UV-treated cells, although it was clearly detectable already in control cells (III, Figure 3C). p53, known to bind p300 (Grossman, 2001), was also tested for its p300 interaction upon UV. Their complex formation was also enhanced early after radiation (unpublished results). The association of NPM and p300 was further increased by the presence of trichostatin A (TSA), a known histone deacetylase inhibitor or by over-expression of PML, suggesting that histone acetylation and PML could play a role in regulating this interaction (III, Figure D).

To verify whether PML could control the association of NPM with p300 and chromatin, we isolated chromatin from wt and Pml null MEFs. Regulation of NPM in UV-treated wt MEFs was similar to U2OS, and its nucleoplasmic levels increased upon UV radiation and there was no change in its association with the chromatin (III, Figure 4B). The increase in the nucleoplasmic NPM levels could be due to its translocation from the nucleoli upon rearrangement of the nucleolar structure (II, Figure 1A). Interestingly, this increase was not as evident in the samples of Pml^{-/-} MEFs, suggesting a role for PML in the proper UV response of NPM. The results correlated well with the immunofluorescence data, showing that in Pml^{-/-} MEFs NPM exists in a more soluble fraction, without any major changes in its localization pattern upon UV radiation (III; Figure 4A).

We further asked whether PML influences p300-NPM interaction (III, Figure 3D). Wt and Pml^{-/-} MEFs behaved completely differently with respect of p300 binding upon radiation, as the NPM in wt MEFs transiently interacted with p300 within one hour timepoint and this same interaction was negligible in cells lacking PML (III, Figure 4C). It is therefore possible that PML controls the acetylation of NPM through its association with p300 and influences the histone and chromatin binding properties of NPM.

The dynamic movement of NPM is affected by UV radiation (III)

NPM is translocated to nucleoplasm in response to UV (II) and this translocation is associated with transient complex formation with p53, Mdm2, PML, p300 and AcH3 early after radiation, prior to p53 stabilization (II, III). To address whether these events affect the mobility and movement of NPM, we utilized optical manipulation of the cells using fluorescence recovery after photobleaching (FRAP). In previous studies NPM mobility in unstressed cells has been shown to be high (Phair and Misteli, 2000; Chen and Huang, 2001). We observed that the high mobility of this protein was transiently retained one hour after radiation (III, Figure 3E). The observed kinetics correlate well with the transient NPM protein complexes, suggesting that NPM interactions with its nucleoplasmic partners may affect its dynamic movements in response to UV radiation. Alternatively, the mobility of NPM could be retained by its association with chromatin (III, Figure 3B, C & D). The mobility of NPM was increased with one hour and further six hours after UV damage NPM was almost completely mobile in the nucleoplasm (III, Figure 3E and data not shown). This mobility correlated with the staining pattern, observed in immunofluorescence analysis that could be lost by pretreatment of the cells with NP-40 lysis buffer prior to staining with NPM antibodies (III, Figure 4A).

Hypothetical models for the function of early multiprotein complexes following UV (I, II, III)

The detected stress-induced interactions between p53, Mdm2, PML, NPM, p300 and AcH3 took place at similar kinetics after radiation, and could possibly indicate the formation of a multiprotein complex required for p53 stress response. Several possibilities for the function of these complexes exist. As the interactions occurred prior to p53 stabilization, they could be required for the stability of p53 by inhibiting Mdm2 E3 ligase activity or its interaction with p53. NPM is essential for the elevation in p53 levels in UV-stressed cells (II, Figure 5C). Interestingly, NPM localization is not affected by γ radiation (Syrjäkari et al., unpublished results) and the ubiquitination of p53 is also not affected following this treatment (Maki and Howley, 1997), indicating separate mechanisms leading to p53 stabilization following different kinds of DNA damage. We could not find any evidence for the role of PML in the UV-induced stabilization of p53. Still, PML could provide a platform for the protein-protein interactions and specific modifications, required for p53 stability or transcriptional activity. These interactions could also play a role in the regulation of p53 target gene selectivity. As p300 and AcH3 were also involved in the complexes, they could as well participate in the promoter-specific histone acetylation, required for the activation of specific p53 targets. p53-dependent histone acetylation of certain promoters, including p21 and PUMA, has been shown to occur upon p53 activation (Kaeser and Iggo 2004). In addition, it has been suggested that PML and PML NBs are involved in chromatin remodel-

ling and could also mediate the access to certain promoter regions (Seeler et al., 1998).

To effectively repair the UV-induced DNA damage, the lesions have to be recognized and repaired in the highly condensed chromatin fibers. Histone modifications like acetylation affect structure of the nucleosomes in a modification-dependent manner. This may lead to relaxation between the tight histone-DNA interface and facilitate binding of NER machinery to these sites. Histones are known to be acetylated in response to UV radiation (Ramanathan and Smerdon, 1986), and the repair of UV-induced lesions by NER is also associated with increased histone acetylation (Brand et al., 2001). Specific chromatin accessibility factors are required for this task. p53 was shown to act in this manner and to mediate histone acetylation in a p300-dependent way upon UV damage (Rubbi and Milner, 2003a). Tumor cells lacking functional p53 also have lower levels of acetylated histone H3. The basal acetylation of K9 residue of H3 and increase in acetylation of K9 and K14 after UV damage have been shown to be affected by p53 (Rubbi and Milner, 2003; Allison and Milner, 2003). A recent paper also showed the p53-dependent increase in K9 H3 acetylation upon UV damage in *Drosophila* (Rebollar et al., 2006), suggesting that this function of p53 is conserved. Further, p53 can tether Mdm2 to chromatin, where it is able to bind histones and promote monoubiquitination H2A and H2B through its RING-domain (Minsky and Oren, 2004). PML and NPM have also been proposed to play a role in chromatin modifications due to their protein associations. NPM binds histones and controls their acetylation (Okuwaki et al., 2001; Swaminathan et al., 2005). PML on the other hand interacts with HDACs and p300 and has been linked to both condensation and decondensation of the chromatin structure (LaMorte et al., 1998; Doucas et al., 1999; Von Mikecz et al., 2000; Bandobashi et al., 2001; Boisvert et al., 2001; Wu et al., 2001). PML could as well control chromatin structure and act as a chromatin accessibility factor in UV-radiated cells. Given that the association of NPM with p300 is PML dependent (III, Figure 4C) and that PML controls NPM localization in UV-treated cells (III, Figure 4A) as well as forms transient complexes with it and p53 (I, Figure 5A; III, Figure 1A), the apparent multiprotein complex early after radiation could possibly have a role in modifications of the chromatin structure.

p53 is involved in the repair of UV-induced DNA lesions through affecting NER functions by several ways. p53 also controls the localization of PML to the sites of DNA damage and nucleotide excision repair in UV-treated cells (Seker et al., 2003). PML itself and PML NBs have been proposed to act as damage sensors and control the release and localizations of many different repair proteins (Dellaire and Bazett-Jones, 2004), in this way linking it either directly or indirectly to the repair processes. NPM also has the ability to promote repair of lesions by upregulating PCNA protein (Wu et al., 2002) and through regulating the localization of GADD45 (Gao et al., 2005), although evidence about a direct association to the lesion sites is missing. The possibility that the detected early NPM protein complexes (I, II, III) could affect repair functions, exists, although a direct involvement in the repair process seems quite unlikely.

PML-RAR α fusion protein in acute promyelocytic leukemia cells affects the normal function and localization of NPM (III)

Acute promyelocytic leukemia is most often caused by a translocation between *PML* and *RAR α* genes (de The et al., 1990; Goddard et al., 1991; Kakizuka et al., 1991; Pandolfi et al., 1991). In some cases, the partner with *RAR α* in the fusion protein can also be *PLZF*, *NPM*, *NuMA* or *Stat5b* (Redner, 2002). However, these different subtypes of leukemia display different cytomorphological features as well as penetrance of the disease in transgenic mouse models (Rego et al., 2006). The *NPM-RAR α* fusion protein in APL does not interact with *PML* and localizes to nucleoli, probably affecting the normal *NPM* functions and the development of APL (Rego et al., 2006; Rush et al., 2006). During this study we observed the association of *PML-RAR α* with *NPM* (III) and became interested in their relationship in the development of APL. Even though both *NPM* and *PML* have been linked to hematological malignancies and are associated with APL, this connection has not been studied before.

We used a APL cell line, NB4, which has a wt *PML* allele in addition to the fusion protein *PML-RAR α* (Lanotte et al., 1991). Even though *p53* mutations are extremely rare in this type of cancer, *p53* status in NB4 cells is mutant (Fleckenstein et al., 2002). Correlating with this status, the levels of *p53* were also very high in this cell line, as observed by Western blot experiments (III, Figure 5A). Interestingly, the *NPM* levels were comparable to other tumor cell lines, A375 and U2OS, but it was prominently localized to the nucleoplasm (III, Figure 5B). This could be due to its sequestration by nucleoplasmic *PML-RAR α* (III, Figure 5B), which clearly formed complexes with *NPM* (III, Figure 5C). The findings are concordant with the *in vitro* interaction data and immunofluorescence data showing that the effect of *PML-RAR α* expression on *NPM* localization pattern (III, Figure 2A, B & D). Nucleolar *NPM* localization may be crucial for its proper function, as mutant *NPMc+* expression disrupts the *ARF* pathway (Falini et al., 2005), suggesting that the capacity of *PML-RAR α* to sequester *NPM* may alter cellular functions and subject the neoplastic transformation.

The APL phenotype can be reversed by *ATO* and *RA* treatments, which induce degradation of the fusion protein and clinical remission in most APL patients (Zhu J et al., 2001). To verify whether the abnormal localization of *NPM* in the NB4 APL-cells is due to *PML-RAR α* expression, we treated the cells with these drugs and analyzed *NPM* localization. The nucleolar staining of *NPM* became more intense in the *ATO*- and *RA*-treated cells without any change in its protein levels, suggesting that a relocalization event occurs in response to *PML-RAR α* degradation (III, Figure 6 A& B). *ATO* treatment induced an almost complete degradation of the cellular *PML* and *PML-RAR α* , while *RA* treatment promoted the formation of normal *PML* NB structures (III, Figure 6A&B). Immunoprecipitation experiments from cells with similar treatments revealed that *RA* promoted complex formation between *p53*, *NPM*, *Ach3* and *p300* (III, Figure 6C) and that this was associated with *p53* activation as determined by luciferase reporter assays in the presence of exogenous *p53* (III, Figure 6D) (Figure 14.). Based on

these results we propose a role for this multiprotein complex in the activation of p53 pathway following RA treatment of APL. Whether PML acts as a crucial factor in the activation of p53 upon this treatment, remains to be studied.

APL cells, expressing PML-RAR α , are blocked at the promyelocyte phase. Enhanced co-repressor binding of PML-RAR α and inhibition of the RA-pathway have been proposed to result in this differentiation block and APL development (Grignani et al., 1993; Grignani et al., 1998; He et al., 1998; Lin & Evans, 2000; Lin et al., 1998; Minucci et al., 2000). PML-RAR α maintains a more condensed chromatin structure due to its association with co-repressor complexes together with HDACs and inactivates target genes by these means (Wu et al., 2001; Segalla et al., 2003). Additionally, the fusion protein maintains the silenced chromatin state by recruiting DNA methyltransferase activities (Villa et al., 2006). Expression of PML-RAR α also promotes relocalization of PML from PML NBs, affecting the normal functions of these structures (Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994) and the apoptotic pathways that PML and PML NB proteins are involved in (Takahashi et al., 2004). Furthermore, tumor suppressor protein p53 has been suggested to be inactivated in APL by the complex of PML-RAR α and HDACs, blocking the proper p53 response in these cells (Insinga et al., 2004). Given that HDACs would be the only player in p53 inactivation in these types of cancer, one could expect to see p53-mediated apoptosis in response to histone deacetylase treatment. However, TSA promotes p53-independent apoptosis and we could not detect any p53 activation upon this treatment (III, results not shown). On the other hand, another histone deacetylase inhibitor, RA, was able to induce formation of a multiprotein complex, associated with the activation of p53 pathway (III, Figure 6C & D). Although NPM overexpression has been shown to decrease the sensitivity of human leukemia cells (HL-60) to retinoic-acid-induced differentiation and apoptosis (Hsu and Yung, 1998; Hsu and Yung, 2000; Yung, 2004), we find here that it could possibly act as an activating component of the p53 pathway. Histone modifications and alterations in the chromatin structure seem to be one of the outcomes in p53 binding to its target sequences upon stress. Our findings indicate that PML-RAR α , in addition to its various effects on chromatin structure, also disrupts the normal localization and function of nucleolar protein NPM, possibly having an effect in the association of p53 with histone modifying factors and blocking the activation of p53 pathway in APL.

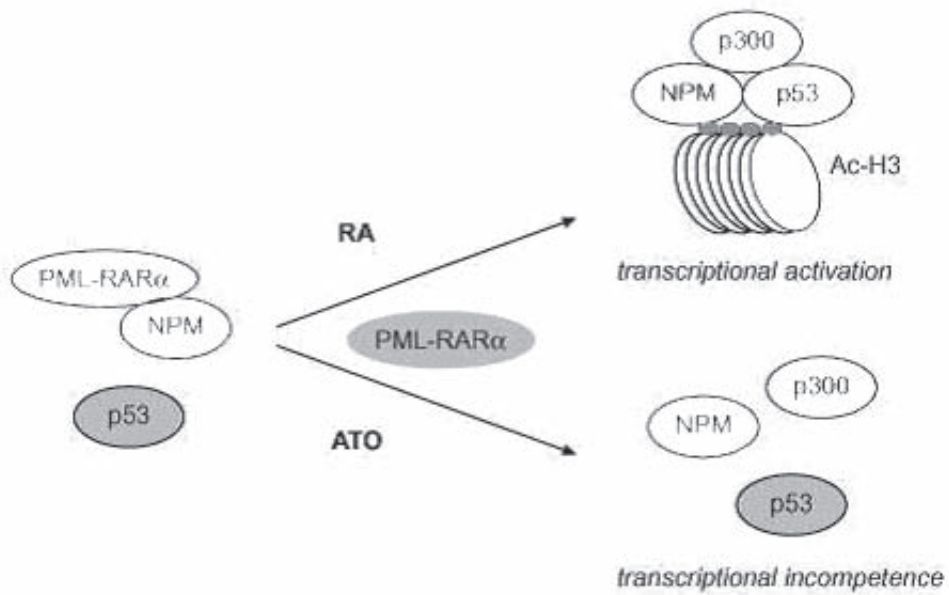


Figure 16. Model for p53 activation in APL cells. NPM associates constitutively with PML-RAR α , and is dissociated from this inhibitory interaction by ATO and RA induced degradation of the fusion protein. RA treatment leads to formation of normal PML NBs, and binding and activation of p53 through its interactions with NPM, AcH3 and p300. ATO does not support PML NB formation or interactions between p53, NPM and p300.

CONCLUSIONS

Cancerous cells can be potentially destroyed by activation of the p53 pathway. This thesis work has aimed in finding new contributors to p53 stabilization and activation as well as unravelling the molecular mechanisms behind them. Although many p53 inducing agents have been shown to decrease the levels of p53 negative regulator Mdm2 and in this way lead to p53 accumulation (Wu and Levine, 1997; Arriola et al., 1999; Ashcroft et al., 2000; Inoue et al., 2001; Wang et al., 2002), the stabilization of p53 following transcriptional inhibition does not occur due to diminished Mdm2 protein levels (Ashcroft et al., 2000; O'Hagan and Ljungman, 2004). The blockage of the p53-Mdm2 interface by modifications and newly formed interactions, followed by UV-induced transcriptional inhibition, plays a major role in the regulation of p53 response in this type of damage .

Transcriptional inhibition by UV exposure of the cells promoted subcellular translocations of the p53 pathway proteins Mdm2, NPM and PML, but not p53 itself. The subsequent rapid and transient interactions of NPM and PML with each other as well as with p53 and Mdm2 could be prerequisite for the induction of a proper p53 cellular response. Although, the transcriptional inhibition leading to nucleolar stress response and concomitant release of NPM was found to be essential for p53 stabilization, the exact function of the potential multiprotein complex between p53, Mdm2, NPM and PML is currently not clear and needs to be verified in future studies. The fact that Mdm2 and PML are associated with the nucleolar compartment upon cellular stress, underlines the importance of this subnuclear organelle in the regulation of p53 pathway and suggests that cellular compartmentalization is important in this type of damage response.

NPM and PML are often altered in hematological malignancies and could thus contribute to the oncogenesis through alterations in the p53 pathway. Moreover, PML exerts control over the cellular localization of NPM, its UV response and association with chromatin binding factor p300, events which are disrupted in cells lacking functional PML. The pathogenesis of APL could so be affected through NPM inactivation. The relevance of the UV-induced interactions between these particular p53 pathway proteins is underscored by the finding that therapeutically relevant RA, reversing the APL phenotype, induced similar complexes with p53 and its partners leading to transcriptional activation of p53. The transcriptional changes by UV radiation or oncogenic PML-RAR α could thus be overcome by p53 association with NPM and PML, emphasizing their importance in the regulation of the p53 pathway. To address whether NPM and PML dysregulation influences p53 function in other types of cancers and whether they could have potential as therapeutic targets, will have to be determined by future work.

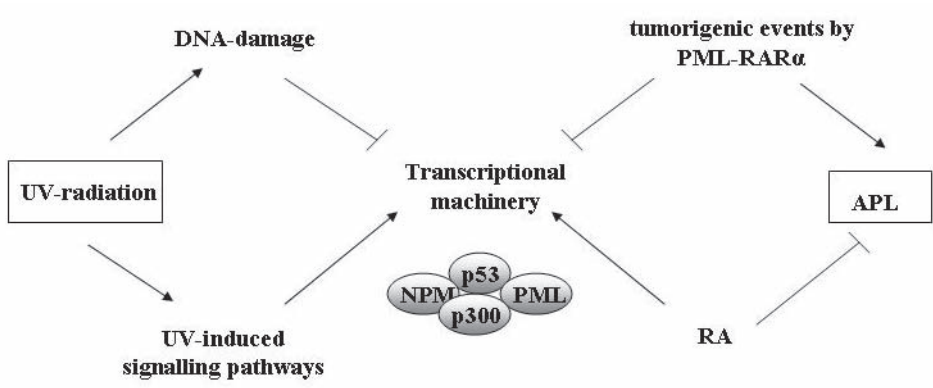


Figure 17. Hypothetical model of the RA- and UV-induced p53 complex. Transcriptional inhibition by UV-induced damage as well as PML-RAR α promoted transcriptional repression in APL is overcome by the formation of a hypothetical p53-NPM-PML-p300 multi-protein complex, associated with the recovery of transcriptional competence. APL, acute promyelocytic leukemia; RA, retinoic acid.

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