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PHARMACOLOGICAL TARGETING OF MUTANT p53 FAMILY MEMBERS

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To my beloved family

ABSTRACT

The tumor suppressor p53 serves as a guardian of the genome and functions mainly as a transcription factor. In response to various stress signals p53 binds to specific DNA sequence motifs and regulates transcription of a large group of target genes involved in cellular processes such as cell cycle arrest, senescence and apoptosis. Inactivation of p53 is critical for the formation of most tumors. Around half of all human cancers carry mutations in the *p53* gene (*TP53*) and mutant p53-harbouring tumors often show increased resistance to conventional chemotherapy. Therefore, pharmacological restoration of wild type function to mutant p53 is a promising strategy for novel cancer therapy. We have identified a low molecular weight compound, STIMA-1, that selectively targets tumor cells in a mutant p53-dependent manner. STIMA-1 contains a reactive double bond that can potentially participate in Michael addition reactions and may restore the tumor suppressive function to mutant p53 by affecting its redox status.

Several other small molecules that reactivate mutant p53 have been identified in our group. PRIMA-1 and its more potent analog PRIMA-1^{MET} (also denoted APR-246) both induce p53 target genes and mutant p53-dependent apoptosis in human tumor cells. PRIMA-1 and PRIMA-1^{MET} are under physiological conditions converted to MQ that binds covalently to the p53 core domain and this modification *per se* is sufficient to endow mutant p53 with pro-apoptotic properties. To further explore the effects of PRIMA-1 and its analogs on tumor cells we analyzed the subcellular distribution pattern of several proteins upon drug treatment. We found that PRIMA-1 and PRIMA-1^{MET}, but not PRIMA-Dead (a PRIMA-1 analog that is unable to induce apoptosis) induced nucleolar accumulation of mutant p53. In addition, PRIMA-1^{MET} induced the levels of heat shock protein (Hsp) 70 and a redistribution of the PML nuclear body-associated proteins CBP, PML, Hsp70, and the Epstein-Barr virus encoded protein EBNA-5 to nucleoli. Our results suggest that relocation of mutant p53 and/or PML nuclear body-associated proteins to nucleoli may play a role in PRIMA-1^{MET}-induced apoptosis.

Since p53 and its family members p63 and p73 share high sequence and structural homology, we examined if PRIMA-1^{MET} also affects mutant p63 and p73. We found that PRIMA-1^{MET} restores wild type activity to some mutant forms of p63 and p73. PRIMA-1^{MET} enhanced mutant p63 DNA binding, and induction of target gene expression and apoptosis in human tumor cells in a mutant p63/p73 dependent manner. PRIMA-1^{MET} also induced a redistribution of mutant p63 to PML nuclear bodies and to nucleoli. Our data indicate that PRIMA-1^{MET} exerts its effects through a common mechanism for all three p53 family members, presumably involving homologous structural domains in the three proteins.

A better understanding of the exact molecular mechanisms of p53-targeting compounds is highly relevant for further drug optimization and the design of novel compounds with improved target selectivity and potency. The effect of PRIMA-1^{MET} on mutant p63 also raises the possibility of pharmacological rescue of p63 mutants in human developmental disorders caused by mutations in p63.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **Rökaeus N.**, Klein G., Wiman K.G., Szekely L. and Mattsson K. PRIMA-1^{MET} induces nucleolar accumulation of mutant p53 and PML nuclear body-associated proteins. Oncogene (2007) 26, 982-992.

II. Zache N. Lambert J.M.R., **Rökaeus N.**, Shen J., Hainaut P., Bergman J., Wiman K.G. and Bykov V.J.N. Mutant p53 targeting by the low molecular weight compound STIMA-1. Molecular Oncology (2008) 2, 70-80.

III. Stuber G., Flaberg E., Petranyi G., Ötvös R., **Rökaeus N.**, Kashuba E., Wiman K.G., Klein G. and Szekely L. PRIMA-1^{MET} induces nucleolar translocation of Epstein–Barr virus-encoded EBNA-5 protein. Molecular Cancer (2009) 8:23.

IV. **Rökaeus N.**, Shen J., Eckhardt I., Bykov V.J.N., Wiman K.G. and Wilhelm M.T. PRIMA-1^{MET}/APR-246 targets mutant forms of p53 family members p63 and p73. Oncogene (2010) 29, 6442-6451.

LIST OF ABBREVIATIONS

APL	acute promyelocytic leukemia
ARF	alternative reading frame
CBP	CREB-binding protein
cDNA	complementary deoxyribonucleic acid
CTD	carboxy-terminal domain
DBD	DNA binding domain
DNA	deoxyribonucleic acid
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EEC	ectrodactyly, ectodermal dysplasia and cleft/lip palate
ER	endoplasmatic reticulum
FLIP	fluorescence loss in photobleaching
FRAP	fluoresence recovery after photobleaching
HAUSP	herpesvirus-associated ubiquitin-specific protease
HIPK2	homeodomain-interacting protein kinase 2
HPLC	high performance liquid chromatography
Hsp	heat shock protein
MDM2	murine double minute 2
MIRA-1	mutant p53-dependent induction of rapid apoptosis 1
MQ	methylene quinuclidinone
mRNA	messenger ribonucleic acid
NB	nuclear body
NCI	National Cancer Institute
OD	oligomerization domain
PCR	polymerase chain reaction
PML	promyelocytic leukaemia
PRD	proline rich domain
PRIMA-1	p53 reactivation and induction of massive apoptosis 1
PUMA	p53 upregulated modulator of apoptosis
RARα	retinoic acid receptor α
RE	response element
RNA	ribonucleic acid
SAM	sterile alpha motif
SCID	severe combined immuno-deficiency
STIMA-1	SH group targeting and induction of massive apoptosis 1
SIM	SUMO interaction motif
SUMO	small ubiquitin-related modifier
TA	transactivation
TAD	transactivation domain
TID	transcription inhibitory domain
ts	temperature sensitive
UV	ultraviolet
wt	wild type

CONTENTS

INTRODUCTION

CANCER

Cancer is a collection of more than hundred diverse diseases characterized by abnormal and uncontrolled cell growth. Malignant tumors invade and destroy adjacent tissues, and tumor cells that have also acquired traits such as motility and adaptation to foreign environments spread and form new colonies, termed metastases, at different sites of the body.

A tumor arises from normal tissue and is a result of changes that have occurred in the DNA sequence of the cancer cells genomes. Genetic changes such as substitutions of one base by another, rearrangements, insertions or deletions of DNA segments, gene amplifications and/or epigenetic changes can result in the activation of oncogenes that promote cell growth, invasion and angiogenesis, or inactivation of tumor suppressor genes whose products operate to constrain cell proliferation or survival.

The somatic mutations present in a cancer cell represent a cumulative record of all the mutational processes the cancer cell has experienced throughout the lifetime of a patient. The mutation rates increase in the presence of substantial exposure to certain exogenous mutagens, such as tobacco smoke carcinogens, by ingestion of food products containing aflatoxins, or various forms of radiation including ultraviolet light. These exposures are associated with distinctive mutation signatures and are associated with increased rates of lung, liver, and skin cancer, respectively. In addition, a cancer cell may have acquired completely new DNA sequences from exogenous sources such as viruses, including human papilloma virus (HPV), Epstein Barr virus (EBV), hepatitis B virus (HBV) and human herpes virus 8 (HHV-8), each of which are known to contribute to the genesis of one or more types of cancer [1].

Individuals can differ in their inherited tendency to develop cancer. Germ-line mutations in certain genes, inherited from parents and transmitted to the offspring, can confer an inborn susceptibility to cancer. Genetic predisposition to cancer often involves mutations in genes involved in cancer progression, many of which have key roles in cell-cycle control, DNA-repair and cell death pathways.

Several acquired capabilities are found in a vast majority of cancer cells and are essential for the formation of a normal cell into a malignant phenotype, including self-sufficiency in growth signals, insensitivity to growth-suppressing signals, evasion of cell death, limitless replicative potential, induction of angiogenesis, and activation of invasion and metastasis [2]. Other emerging hallmarks of cancer are reprogramming of energy metabolism and evasion of immunological destruction [2]. In addition, tumors exhibit another dimension of complexity. They coexist with a variety of extracellular matrix components and cell types, such as fibroblasts, myofibroblasts, endothelial cells, adipocytes, pericytes and immune cells, which collectively form the tumor stroma, often termed its microenvironment [3]. Tumor-

associated stromal cells have been demonstrated to actively promote tumor progression by influencing the growth, survival, invasiveness, and metastatic ability of the tumor cells. Moreover, tumor cells within a single tumor have been shown to exist in multiple states of differentiation with different capabilities to self-renew [4].

ANTI-CANCER THERAPY

Surgery, radiation and chemotherapy are commonly used anti-cancer treatments. However, the lack of specificity of chemotherapeutic agents make them highly toxic to normal tissues, especially those with high proliferation rates, resulting in severe side effects. Approaches aiming to selectively kill cancer cells while protecting normal cells are evolving. Detailed molecular analyses of human cancers have revealed an increasing number of specific genetic mutations that render tumor cells sensitive to a range of targeted therapeutics [5]. Several classes of agents that target different molecular abnormalities in tumor cells are currently being investigated. One class of agents acts specifically on an overabundant or overactive oncogene product, such as the kinase inhibitors erlotinib, imatinib and trastuzumab [6, 7]. Other examples are proteasome inhibitors and poly (ADP-ribose) polymerase (PARP) inhibitors that act on a general target whose partial inhibition seems to be selectively toxic to some tumor types [8]. Angiogenesis inhibitors act on the tumor microenvironment [9]. Targeted therapies represent advances in cancer treatment and targeting of several pathways simultaneously may be important to minimize the risk of resistance. Much effort is made to develop cancer therapies based on the tumor suppressor p53. Nearly all cancers show defects in the p53 pathway and over 50 % of human tumors have mutations in *TP53* [10], the gene encoding the p53 protein.

THE P53 FAMILY

The p53 protein family consists of three transcription factors, namely p53, p63 and p73. The tumor suppressor p53 has been studied for almost three decades and is a central player in protecting the integrity of the genome. Inactivation of the p53 pathway, either by mutations in *TP53* or through interaction with abnormally expressed cellular or viral proteins, is a common denominator to human cancer. Two p53 paralogs, p63 and p73, were identified in 1997. These proteins share high sequence homology to p53, particularly in the DNA-binding domains (approximately 60% similarity), including conservation of essential DNA contact residues [11], allowing them to transactivate p53-responsive genes causing cell cycle arrest and apoptosis. However, despite structural and functional similarities between p53, p63 and p73, mouse knockout studies have revealed that these proteins are not functionally entirely redundant. Each p53-family transgenic knockout mice develop distinct phenotypes, spanning from increased cancer susceptibility to severe developmental defects.

The *p53* gene family has a dual gene structure conserved in drosophila, zebrafish and mammals. Due to multiple splicing, alternative promoter usage and alternative initiation of translation, the *p53, p63* and *p73* genes encode for multiple protein isoforms (Figure 1). Whereas full-length proteins function as transcription factors

(p53, TAp73 and TAp63), the ΔN-isoforms, lacking the transactivation domain or parts of it, have negative effect on p53, TAp73 and TAp63 by blocking their transactivation activity. The ratio and interplay between the p53 family isoforms are likely to be an important cell fate determinant and fundamental to our understanding of tumor formation.

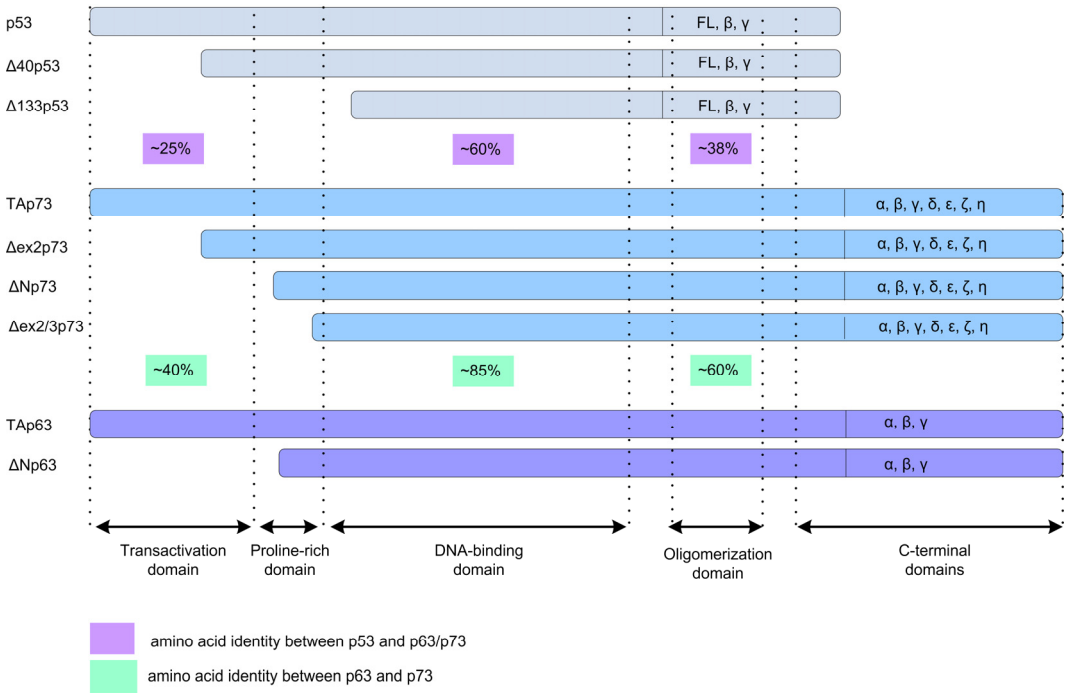

Figure 1. Schematic representation of p53, p63 and p73 isoforms. Alternative promoter usage, alternative initiation of translation and multiple splicing of the C-terminus yield a plethora of different isoforms. An approximate location of the major domains and the percentage of amino acid identity between p53, p63 and p73 in some of these domains are indicated. The α isoforms of p63 and p73 contain a sterile alpha motif (SAM) domain (sharing 50% amino acid identity between p63 and p73), followed by a transcriptional inhibitory domain in the C-terminus. FL - full length.

P53

p53 was originally discovered in 1979 through its interaction with the simian virus 40 (SV40) large-T antigen [12-15]. It was originally regarded as an oncogene, based on the observations that many tumor cell lines expressed high levels of p53 and that p53 could transform cells when co-expressed with the Ras oncogene [16-18]. However, subsequent studies showed that the transforming ability of p53 was a result of mutation and that wild type p53 acts as an inhibitor of transformation whereas certain p53 mutants have transforming activity [19-21]. In addition, p53 point mutations along with loss of chromosome 17p were frequently found in common human tumors, including colorectal carcinomas [22, 23]. All these findings led to a major paradigm shift and established p53 as a key tumor suppressor. p53's role as a tumor suppressor was further strengthened by the identification of germ line p53

mutations as the cause of an inherited human syndrome (Li-Fraumeni) associated with a high risk of developing various tumors [24], and that mice deficient for p53 develop frequent spontaneous tumors [25]. Since the discovery of p53 more than 30 years ago, the complexity of the p53 field has grown enormously. In addition to the role of p53 as a major tumor suppressor, contribution of p53 in numerous other aspects of normal life and in disease is emerging. Some of the aspects of p53-associated biology will be summarized in this chapter.

p53 isoforms

As the *p53* gene, located on the short arm of chromosome 17 (17p13), was discovered several years before PCR and modern molecular biology technologies, understanding of the *p53* gene structure was not as complex then as it is today. Until recently, only one promoter and three mRNA splice variants were described for p53. Revisiting of the *p53* gene expression in normal human tissue using the novel method of Generacer PCR established that the human *p53* gene has a dual gene structure similar to *p63* and *p73* genes [26]. The human *p53* gene can encode at least nine different p53 protein isoforms; p53, p53β, p53γ, Δ133p53, Δ133p53β and Δ133p53γ due to alternative splicing of the intron-9 and usage of an alternative promoter in intron-4, and also Δ40p53, Δ40p53β and Δ40p53γ due to alternative splicing of the intron-9 and alternative initiation of translation or alternative splicing of the intron-2 [27]. The exact function and physiological significance of these isoforms are under intense investigation.

p53 protein domains

The p53 protein consists of 393 amino acids and has a modular protein structure commonly associated with transcriptional regulators (Figure 2). Two transcriptional activation domains (TADs), TAD1 and TAD2, are located at the N-terminus of p53. These domains can independently enhance transcription of p53 target genes by recruiting other factors, such as histone-modifying enzymes, components of the basal transcription machinery and coactivator complexes [28-30]. The TAD1 subdomain can also bind strongly to the negative regulators MDM2 and MDMX that target p53 for proteasomal degradation. Adjacent to the transactivation domains lies the proline-rich domain (PRD), which has been shown to be required for p53-mediated apoptosis [31]. It has also been proposed to participate in protein interactions with SH3-containing proteins [32], as well as having a structural function [33]. The central core of p53 comprises the DNA-binding domain (DBD) that is responsible for sequence-specific binding to p53 response elements in DNA. The key importance of DNA binding for p53-mediated tumor suppression is highlighted by the fact that most cancer-associated *TP53* mutations are missense mutations in this domain and abolish DNA binding. A loop-sheet-helix motif and two large loops make up the DNA binding surface of p53 [34]. The two loops are held together in part by a zinc ion, which is tetrahedrally coordinated by Cys176, His179, Cys238 and Cys242 [34]. p53 binding to DNA has been shown to be dependent on the DBD's ability to coordinate this single zinc ion and p53 that is mutant in these cysteine residues is impaired in DNA binding [35]. p53 binds to its response elements as a

tetramer and the formation of p53 tetramers rely on the oligomerization domain (OD) in the C-terminal region of p53. Finally, the most extreme C-terminus contains a basic, lysine-rich domain (CTD) that binds DNA in a non-sequence-specific manner and plays a role in the ability of p53 to linearly diffuse on naked DNA [36]. It also undergoes extensive post-translational modifications that modulate p53 stabilization and sequence-specific DNA-binding.

Multiple posttranslational modification sites are located throughout the p53 protein and modifications of these are often critical for p53 function by regulating p53's activity, stability and subcellular localization. p53 interacts with a large number of partner proteins. Phosphorylation of serine and threonine residues or acetylation of lysine residues can regulate the affinity of p53 for many of its partners. Ubiquitination of p53 in its C-terminus targets it for proteasomal degradation. Recent data suggest that lysine residues located in the N-terminal region and in the DNA-binding domain may also be ubiquitinated [37]. Multiple monoubiquitination within the C-terminus and DNA binding domain of p53 leads to nuclear export of p53 [38]. In addition, modifications such as sumoylation, methylation and neddylation can in different ways modulate p53 function.

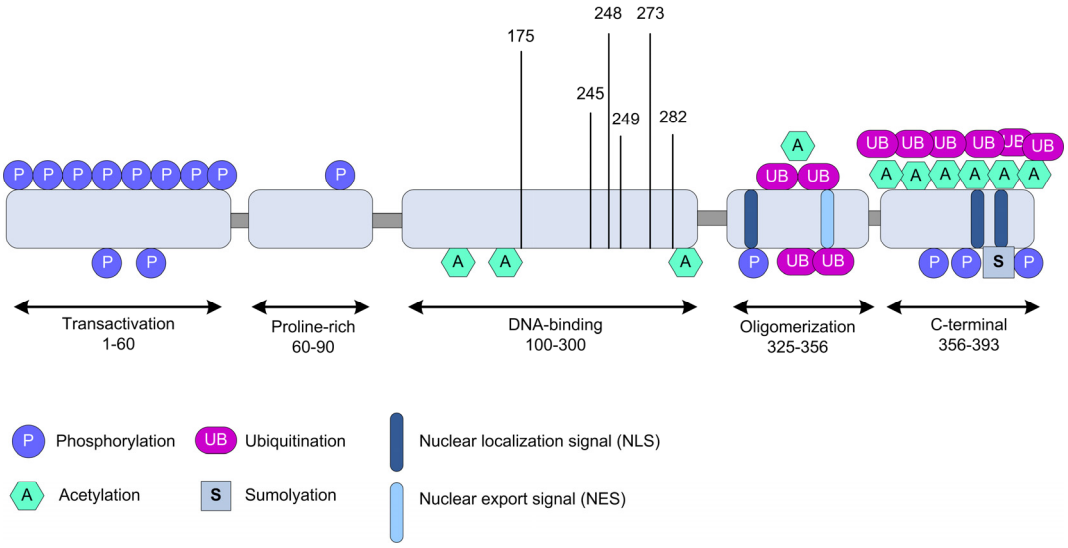

Figure 2. Schematic representation of the p53 protein with its major functional domains. Sites of phosphorylation (P), acetylation (A), ubiquitination (UB) and sumoylation (S) as well as nuclear export and localization signals are indicated. The majority of p53 mutations are located in the DNA-binding core domain, frequently affecting six so-called hotspot residues – Arg175, Ser245, Arg248, Arg249, Arg273 and Arg282.

Regulation of p53

A tight control of cellular p53 levels is of high importance to avoid otherwise lethal activities of p53 in cells under physiological conditions. Even though p53 mRNA is constitutively expressed, p53 is a short-lived protein and remains at a low or undetectable level in most tissues. This is primarily achieved through the ubiquitin-dependent proteasomal degradation of p53 mediated by the E3 ubiquitin ligase mouse double-minute 2 (MDM2). MDM2 (also known as HDM2 in humans) binds to p53 and prevents its transcriptional activity [39, 40], shuttles p53 out of the nucleus and targets p53 for ubiquitin-mediated proteasomal degradation [41, 42]. *MDM2* itself is a p53 target gene and induction of its expression by p53 is part of a crucial negative-feedback loop that maintains low levels of p53 in the absence of stress [43]. MDM2 can also autoubiquitinate itself for proteasome-dependent degradation [44]. In response to stress, p53 is mobilised by inhibition of MDM2 through several different mechanisms. Post-translational modifications of both p53 and MDM2 can disrupt the MDM2-p53 interaction. Lysines within the C-terminus and DBD of p53 can be acetylated by several enzymes, such as CBP and p300 [45, 46]. p53 acetylation is markedly enhanced in response to stress and promotes p53 stabilization and activation [47, 48]. Six C-terminal lysines of p53 are the predominant sites for MDM2-mediated ubiquitination [49]. Acetylation and ubiquitination are mutually exclusive modifications and competition between these modifications is believed to affect p53 stability. Recent work has shown that acetylation of eight different lysine residues on p53 prevents the interaction between p53 and MDM2 [50]. A broad range of kinases, including ATM/ATR/DNA-PK and Chk1/Chk2, can modify p53 by phosphorylation of specific serine residues of p53 leading to p53 accumulation. Phosphorylation of p53 at Ser15 and Ser 20 after DNA damage and other types of stress [51-53] have generally been thought to stabilize p53 by counteracting MDM2-p53 binding. In addition, phosphorylation of certain serine residues of MDM2 can inhibit its activity [54]. Oncogene activation induces expression of the p53 target ARF (p14ARF in humans and p19ARF in mice) that inhibits MDM2 [55]. It does so both by binding MDM2 and sequestering it in the nucleolus [56] and by directly inhibiting the ubiquitin ligase activity of MDM2 [57, 58]. Moreover, nucleolar stress triggers binding of the ribosomal proteins L5, L11 and L23 to MDM2 and inhibit its function [59-61].

Another key regulator of p53 is the MDM2-related protein MDMX (also called MDM4). It inhibits p53 by binding to and blocking the transcriptional activation domain of p53 [62]. Although structurally similar to MDM2, MDMX does not display E3 ligase activity of its own. Instead it forms heterocomplexes with MDM2 and indirectly potentiates the ubiquitination and subsequent degradation of p53 [63, 64]. Rescue of embryonic lethality of MDM2-deficient or MDMX-deficient mice by double knockout of p53 revealed fundamental roles of both MDM2 and MDMX in regulating p53 [65-67].

To add to the complexity of the regulation of p53 stability, p53 and MDM2 can be deubiquitinated and stabilized by enzymes such as HAUSP [68-70], or dephosphorylated by the p53-inducible phosphatase WIP1 [71], which facilitates MDM2- and MDMX-mediated destruction of p53. DAXX, a death domain-

associated protein that regulates HAUSP-mediated deubiquitination was shown to stabilize and enhance the intrinsic E3 activity of MDM2 [72]. RASSF1A stabilizes p53 by disrupting the interaction between MDM2, HAUSP and DAXX and thereby promoting MDM2 ubiquitination [73]. Additional negative regulators of p53 include the E3 ubiquitin ligases Cop1 [74], Pirh2 [75], and CARPs that promote MDM-2-independent degradation of p53 [76].

Low levels of MDM2 can induce monoubiquitination of p53 and promote p53 nuclear export and accumulation in the cytoplasm [38]. This was originally thought to passively block the nuclear function of p53 as a transcription factor. However, accumulating evidence suggest that the cytoplasmic localization of p53 plays an important role in p53-mediated functions such as apoptosis and autophagy [77-79]. MSL2 is another E3-ligase that can promote cytoplasmic localization of p53, independently of MDM2 [80].

p53 is also regulated in a redox-dependent manner. Efficient DNA binding and correct folding of p53 requires a reducing environment, and oxidation of p53 results in loss of wild type conformation and as a consequence loss of DNA binding [81]. The zinc ion that stabilizes the core of p53 is critical for proper conformation and protects p53 from oxidation.

The three members of the ASPP (**a**poptosis **s**timulating **p**rotein of **p**53) family of proteins can affect the activity of p53. ASPP1 and ASPP2 bind the core domain of p53 and specifically induce p53-mediated apoptosis by causing transactivation of *bax* but not *p21* [82]. In contrast, iASPP binds to p53 and inhibits the transactivation of proapoptotic genes [83].

In addition, N-terminal truncated forms of p53 family members can act as dominant negative inhibitors of p53 and interfere with p53's ability to exert its function [84, 85].

Recently, several studies have showed a critical role of both RNA-binding proteins and regulatory RNAs in regulating p53 at the RNA level. The p53 target Wig-1 can bind to AU-rich elements in the 3´untranslated region (UTR) of p53 mRNA and enhance its stability, thus forming a positive feedback loop [86]. Wrap53, a natural antisense transcript of p53, regulates the steady-state levels of p53 mRNA by interacting with its 5´ UTR [87, 88].

Function and biological responses of p53

The discovery that p53 is a sequence-specific DNA-binding protein was an early breakthrough for the p53 field [89, 90]. p53 is activated in response to a variety of cellular stresses, including DNA damage and oncogene activation, and once activated p53 acts as a master transcriptional regulator of several hundreds of genes encoding both proteins and microRNAs [91] (Figure 3). p53 tetramers bind as dimers of dimers to sequence-specific p53 response elements (REs), which are classically defined as two DNA half sites of RRRCWWGYYY (where R is a purine, W is adenine or thymine, and Y is a pyrimidine), separated by a spacer of 0 to 13 base

pairs [92], although many validated REs have mismatches from the consensus [91]. In addition to its role in transcription activation, p53 is also involved in repression of a wide range of targets [93], including the anti-apoptotic *Bcl-2* and *hTERT* [94, 95].

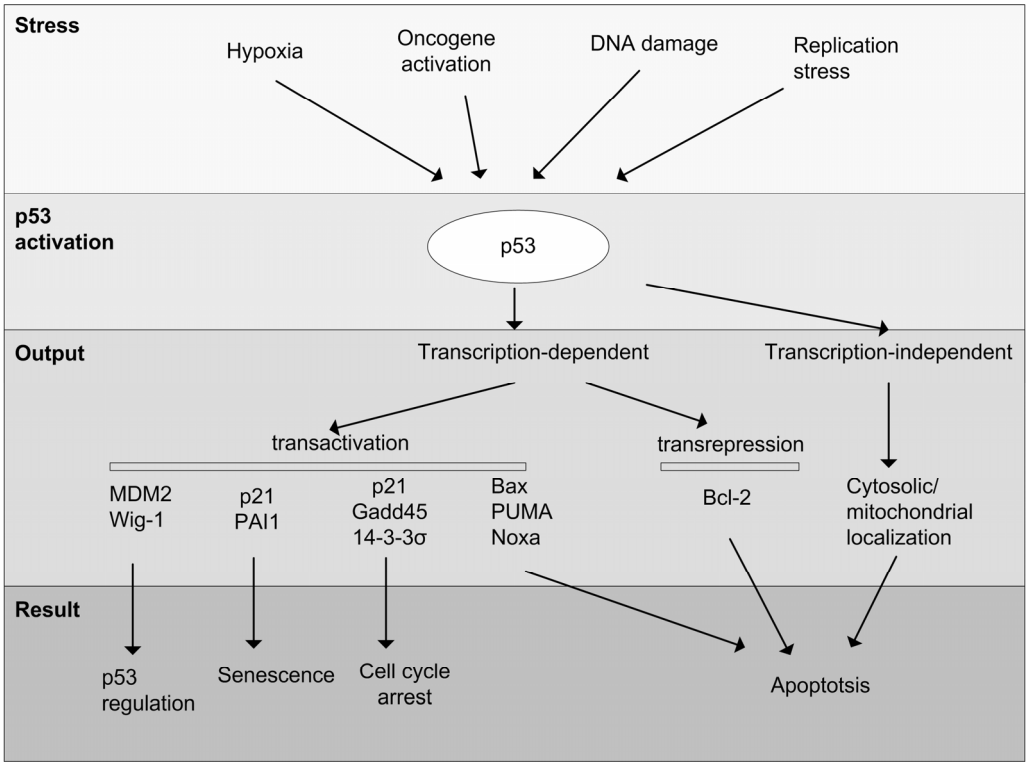

Figure 3. A simplified representation of the complex p53 signaling network. In response to various kinds of stress, the p53 protein becomes stabilized and activated through different mechanisms, such as posttranslational modifications and partner protein-interactions. This results in transcription-dependent and -independent responses.

p53 is implicated in a wide range of cellular processes, such as cell cycle arrest, apoptosis, senescence, DNA repair, cellular metabolism, autophagy, angiogenesis, innate immunity, stem cell renewal, differentiation and meiotic recombination, and the list of possible outcomes of p53 activation is constantly growing [96, 97]. The important role of p53 in tumor suppression is highlighted by the fact that mice functionally deficient for all p53 isoforms are prone to the spontaneaous development of a wide variety of neoplasms by early age [25].

Cell cycle arrest, senescence and apoptosis are processes where the important role of p53 as a guardian of the genome is firmly established. The choice of responses in a cell upon p53 activation depends on several factors, such as cell type, cellular environment and type of stress. Under conditions of lower levels of stress, p53 can limit the propagation of oncogenic mutations by engaging a temporary program of cell cycle arrest and DNA-repair. In response to severe or sustained stress signals, p53 can drive irreversible senescence or apoptosis programs.

Inhibition of cell proliferation and growth is an important ability of p53 that allow the cell to temporary pause and repair any damage that has occurred. p53 can effectively block cell cycle progression by inducing the transcription of the cyclin dependent kinase inhibitor p21. Induction of p21 expression is extremely sensitive to even low levels of p53, and it can induce both G1 and G2 cell cycle arrest [98, 99]. Several other p53 target genes can contribute to this response, such as *14-3-3σ* and *GADD45* [100].

p53 also plays a key role in the induction of senescence, an irreversible cell cycle arrest, that acts as a barrier towards tumorigenesis. p53-induced senescence is triggered by a wide spectrum of stimuli, including telomere shortening (replicative senescence) and non-telomeric signals such as DNA-damaging agents, oxidative stress or activated oncogenes (premature senescence) [101]. Transcriptional activation of target genes such as *p21* [102], the plasminogen activator inhibitor *PAI1* [103], and *PML* [104, 105] have been shown to be involved in p53-induced senescence. The role of senescence as an important p53-activated tumor suppressive response has been shown in mice models, where activation of p53 leads to regression of many different tumor types via cellular senescence [106-108].

p53 can induce the expression of a wide variety of genes involved in apoptosis. These genes can contribute to the induction of apoptosis through multiple pathways. Several proteins involved in the extrinsic (death receptor) pathway are induced by p53, such as the death receptors Fas [109] and KILLER/DR5 [110]. After ligand binding, the cytoplasmic tail of the receptor acts via the FADD protein to assemble the death receptor-inducing signalling complex (DISC) to induce caspase 8, triggering a chain of events resulting in the activation of effector caspases and an apoptotic response. In addition, caspase 8 can mediate cleavage of Bid [111], a proapoptotic member of the Bcl-2 family. Truncated Bid translocates to the mitochondria leading to cytochrome c release, thus establishing a link between the extrinsic and the intrinsic pathways [111]. The intrinsic (mitochondrial) pathway is regulated by the pro- and anti-apoptotic Bcl-2 family proteins, including the pro-apoptotic proteins Bak and Bax, pro-apoptotic BH3-only proteins PUMA and Noxa, and the anti-apoptotic proteins Bcl-2 and Bcl-X_L. This pathway involves mitochondrial depolarization, cytochrome c release, formation of the apoptosome and the subsequent activation of caspases resulting in cell death. p53 can induce the expression of several targets involved in this pathway, such as pro-apoptotic *bax* [112], *puma* [113], and *noxa* [114], and transrepress proteins such as anti-apoptotic *bcl-2* [95]. p53 can also induce expression of *scotin* that can induce apoptosis via an endoplasmatic reticulum (ER) pathway [115]. All these pathways cross-communicate with each other and converge to a common downstream pathway leading to cell death.

In addition, cytoplasmic p53 can localize to the mitochondria where it induces apoptosis by transcription-independent mechanisms. At the outer mitochondrial membrane p53 has been shown to interact with the anti-apoptotic Bcl-X_L and Bcl-2 [116]. This direct action of p53 results in disruption of inhibitory complexes between these proteins and pro-apoptotic Bak and Bax, leading to mitochondrial outer membrane permeabilization, release of cytochrome C and other apoptotic activators

from the mitochondria [116-118]. p53 has also been shown to activate Bak by releasing it from its inhibitory interaction with MCL-1 [119].

Mutations in p53 and cancer

Impairment of p53 function has a crucial role in tumor evolution. Mutation or functional inactivation of p53 is an almost universal feature of human cancer cells. Loss of p53 function can occur either by mutation of *TP53* itself or through partial abrogation of signalling pathways or effector molecules that regulate p53 activity, including overexpression of negative regulators of p53, such as MDM2 and MDMX [39, 120-122], and deletion or epigenetic inactivation of positive regulators of p53, such as ARF [55, 123]. Inactivation of p53 by exogenous factors such as viral infection also contributes to development of human tumors.

Mutations in *TP53* can either be somatic or germline. Germline mutations in *TP53* in humans cause Li-Fraumeni syndrome, which causes predisposition to a variety of early-onset malignancies [24]. Somatic mutation of *TP53* occurs in a high fraction of human tumors, where the highest incidences of p53 mutations are found in small cell lung cancer and ovarian carcinoma. Approximately 95% of somatic p53 mutations lie in the DNA-binding core domain and result in deficient DNA binding and failure to transactivate target genes. Most of these p53 mutations are missense mutations, leading to a tumor-associated full-length form of p53 with a single amino acid change in the core domain [124]. The most frequently mutated amino acid residues in cancer – known as "hotspots" - include Arg175, Ser245, Arg248, Arg249, Arg273 and Arg282 [125] (Figure 2). p53 point mutations can be divided into two main classes. Mutations in the first class affect residues that are involved in DNA binding, such as Arg273. These DNA contact mutants disrupt specific DNA binding but have little or no effect on p53 folding. In contrast, the second class of mutations affects residues important for the structural integrity of the core, for example Arg175. These structural mutations abrogate DNA binding by disrupting the local structure only or by destabilizing the whole protein [126]. Many p53 mutants gain dominant-negative functions and new oncogenic properties (gain-of-function) in addition to loosing their tumor-suppressive function. Dominant-negative activities of mutant p53 include hetero-oligomerization of the mutant protein with the wild type protein from the remaining wild type allele [127-129], but also inhibitory interaction between mutant p53 and p53 family members p63 and p73 [130-132]. In addition, p53 mutants may also have gained other transcriptional regulatory functions, such as illegitimate transactivation of the *c-Myc* oncogene or the *MDR1* (multidrug resistance) gene [129]. Promoting of tumor invasion by affecting integrin and epidermal growth factor receptor trafficking is another recently described feature of mutant p53 [133]. Gain-of-function activities of mutant p53 have been linked to increased invasiveness and metastasis of tumors [133-135].

Targeting p53 for therapeutic gain

The fact that mutation or functional inactivation of p53 is an almost universal feature of human cancer cells makes p53 an attractive target for cancer therapy.

Several different strategies to restore p53 function in human tumors are currently under investigation.

One strategy is the use of gene therapy. Restoration of wild type p53 function in tumors can be achieved by introducing a functional copy of *TP53* using a viral vector, in most cases an adenoviral vector. The vector is injected locally into the tumor and will therefore depend on a bystander effect in order to target all cells in the tumor [136, 137]. Local injection will limit the effect to the tumor site and therefore novel methods that deliver the gene to distant sites would be required in order to target metastases in patients with disseminated disease. The replication-defective p53-producing adenovirus Gendicine (Shenzhen Sibiono Genetech, Shenzhen, China), was clinically approved in China in 2003.

A second strategy is activation of p53 by targeting the p53-MDM2 interaction. MDM2 is frequently overexpressed in tumors that possess wild type p53, leading to inhibition of p53 activity. MDM2 overexpression is often owing to an amplification of a chromosome segment that includes *MDM2* [138]. Similarly, methylation at the *CDKN2A* (*INK4a/ARF*) locus can epigenetically silence the expression of the MDM2 antagonist p14ARF [123]. Disruption of the p53-MDM2 interaction can restore p53 function and sensitize tumors to chemotherapy or radiotherapy in experimental models [139-141]. Several small molecule inhibitors of MDM2 have been identified and are currently being investigated.

A group of compounds called the nutlins were the first reported small-molecule MDM2 antagonists with *in vivo* activity [139]. The nutlins have been shown to displace p53 by binding to the p53 pocket of MDM2, and nutlin-3 potently induces apoptosis in cell lines derived from hematologic malignancies including AML, ALL, myeloma and B-cell CLL [142-147]. Moreover, nutlin-3 induces tumor shrinkage in mouse xenograft models, with few toxic effects reported. Another compound from the nutlin series, RG7112 (F. Hoffmann-La Roche, Basel, Switzerland), is currently being tested in phase I trials in patients with hematologic neoplasms and advanced solid tumors.

The small molecule RITA (**r**eactivation of p53 and **i**nduction of **t**umor cell **a**poptosis) is another activator of the p53 pathway that blocks MDM2 function [148, 149]. RITA binds directly to the N-terminus of p53 and inhibits the interaction of p53 with MDM2. RITA induces apoptosis in wild type p53 expressing tumor cells and inhibits tumor growth *in vivo* [148].

A third strategy is reactivation of mutant p53. Pharmacological restoration of wild type function to mutant p53 should trigger massive cell death and offer the potential for efficient elimination of a wide variety of tumor types. This notion is supported by studies in mice demonstrating that restoration of functional p53 in mouse tumors *in vivo* rapidly eliminates tumors through apoptosis and/or senescence [106-108].

The low molecular weight compound 2,2-bis(hydroxymethyl)-1-azabicyclooctan-3-one, later called PRIMA-1 (**p**53 **r**eactivation and **i**nduction of **m**assive **a**poptosis), was identified in a cell-based screen of the Diversity set (a library of structurally

diverse synthetic compounds) from the US National Cancer Institute (NCI) for its ability to inhibit cell growth in a mutant p53-dependent manner [150]. PRIMA-1 show selective growth-inhibitory and apoptosis-inducing effects on mutant p53-expressing cells, as well as restoration of wild type conformation and sequence specific DNA binding. PRIMA-1^{MET} (also denoted APR-246) is a methylated form of PRIMA-1 that is even more potent in inducing mutant p53-dependent apoptosis than PRIMA-1 itself [151]. PRIMA-1 and PRIMA-1^{MET} induce p53 targets, such as p21, PUMA, Noxa and Bax [150, 152, 153]. Several genes associated with ER stress are also induced by PRIMA-1^{MET} [153]. Induction of activated caspase-2, caspase-3 and caspase-9 by PRIMA-1^{MET} is consistent with induction of apoptosis via the mitochondrial pathway [152]. PRIMA-1 has also been shown to induce apoptosis in a transcription-independent manner [154].

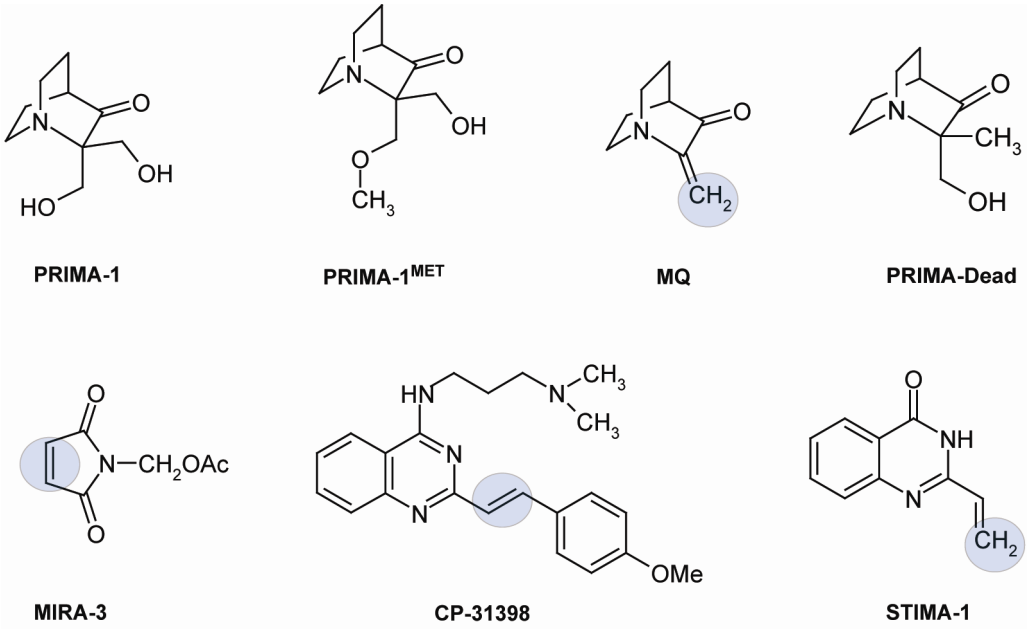

Figure 4. Chemical structures of some mutant p53-reactivating compounds. MQ, MIRA-3, CP-31398 and STIMA-1 share a common chemical activity as Michael acceptors. The reactive groups are encircled.

Systemic administration of PRIMA-1 and PRIMA-1^{MET} can inhibit human xenograft tumor growth in SCID mice [150, 151], suppress growth of mouse tumors in a syngenic host [155], as well as of chemically induced mammary carcinomas in rats [156]. Moreover, PRIMA-1 has been shown to potently induce apoptosis of primary human acute myeloid leukaemia and chronic lymphoid leukaemia cells [157, 158]. Combination with PRIMA-1 and certain chemotherapeutic drugs, including adriamycin, cisplatin and camptothesin, has been shown to act in a synergistic manner to induce apoptosis of tumor cells [151, 159]. PRIMA-1^{MET}/APR-246 has been tested in a phase I clinical trial in patients with hematological malignancies or prostate cancer (Aprea, Solna, Sweden).

Under physiological conditions PRIMA-1 and PRIMA-1^{MET} are relatively rapidly converted to other compounds [160]. One of these decomposition products is methylene quinuclidinone (MQ). MQ has a reactive carbon-carbon double bond which can potentially participate in Michael addition reactions, thus making it a Michael acceptor with a potential ability to react covalently with cysteines in mutant p53. It binds covalently to the p53 core domain and this modification *per se* is sufficient to provide mutant p53 with pro-apoptotic properties [160]. Importantly, PRIMA-Dead (also referred to as PRIMA-D), a PRIMA-1 analog that cannot be converted to MQ, is biologically inactive.

Interestingly, other mutant p53 reactivating molecules, MIRA-1 (**m**utant p53-dependent **i**nduction of **r**apid **a**poptosis), STIMA-1 (**S**H-group **t**argeting compound that **i**nduces **m**assive **a**poptosis, see the Results and discussion chapter) and CP-31398, also share the common chemical activity as Michael acceptors and can potentially modify cysteines in mutant p53 [161, 162]. MIRA-1 is a maleimid that was identified in the same cellular screening of the NCI Diversity set that led to the identification of PRIMA-1. Several MIRA-1 analogs have been tested for biological activity against mutant p53-expressing cells [163]. Importantly, the compounds containing the reactive double bond exhibited mutant p53-reactivating properties, whereas analogs lacking this double bond were inactive. MIRA-3, a more potent analog of MIRA-1, showed inhibition of human xenograft tumor growth in SCID mice but also toxicity at high doses, indicating that the compound has a narrow therapeutic window and is unsuitable for further drug development [163]. The styrylquinazoline compound CP-31398 was identified in a screening of a chemical library for molecules promoting the stability of p53 DNA binding domain using the wild type-specific antibody PAb1620 [164]. CP-31398 was the first compound reported with the ability to induce conformation shift of mutant p53 to a wild type conformation and rescue p53 function in some tumor cell lines and xenografts.

P63

p63 isoforms

p63 is the most recently discovered but most ancient member of the p53 family. The *p63* gene expresses at least six mRNA variants which encode for six different protein isoforms [165] (Figure 1). The transactivating isoforms TAp63α, TAp63β and TAp63γ are generated by the activity of a promoter upstream of exon-1 while an alternative promoter in intron-3 leads to the expression of the N-terminal truncated p63 isoforms ΔNp63α, ΔNp63β and ΔNp63γ.

The transactivation domain is 22% identical between p63 and p53. The core DNA-binding domain of p63 share 60% identity to p53's DBD, and the residues of p53 that directly interact with DNA are identical in p63. As a consequence, p63 is able to bind canonical p53 DNA-binding sites and activate transcription from p53-responsive promoters and thus promoting cell cycle arrest, apoptosis and cellular senescence [165, 166]. The C-terminal oligomerization domain of p63 shares 38% similarity with p53.

Although the ΔNp63 isoforms lack the transactivating domain present in the TAp63 isoforms, they still can transactivate genes through an alternative transactivation domain present in their distinct N-terminal end [167]. The α-isoforms of p63 contain an additional region at the C-terminus that is not found in p53, but also exist in the α-isoforms of p73. This region combines sterile alpha motif (SAM) and transcription inhibitory domains (TID) and has been implicated in lipid-membrane binding and repression of transcription [168-170]. In addition, a helix in the oligomerization domain of p63 has been shown to be crucial for tetramer stabilization by competing with the transactivation domain for the same binding site [171].

Biological activities of p63

Two independent groups generated *p63*-deficient mouse models on different genetic backgrounds and using different targeting strategies [172, 173]. Both *p63*$^{-/-}$ mouse models were characterized by truncated limbs, craniofacial abnormalities, a shiny translucent skin, and an absence of structures such as hair follicles, teeth and mammary glands. *p63*-deficient animals were born viable but died shortly after birth, probably from dehydration.

Heterozygous mutations in the *p63* gene in humans have been shown to cause several autosomal dominantly inherited human syndromes [174-176]. Ectodermal dysplasia, orofacial clefting and limb malformations are key characteristics of these syndromes and various combination of these features are seen in at least five different syndromes: EEC syndrome, AEC syndrome, ADULT syndrome, limb-mammary syndrome (LMS) and Rapp-Hodgkin syndrome (RHS). *p63* mutations can also cause non-syndromic single malformations, such as isolated split hand/foot malformation (SHFM4) and non-syndromic cleft lip (NSCL) [177].

The pattern of heterozygous mutations is distinct for each of these syndromes [178]. In all of these syndromes, the mutation appears to act through both dominant negative and gain of function mechanisms rather than loss of function. Hot spot mutations in the EEC syndrome are clustered in the DNA binding domain, whereas the RHS and AEC syndrome mutations are found in the SAM and TI domains.

The EEC (ectrodactyly, ectodermal dysplasia and cleft/lip palate) syndrome is the most common of the p63 syndromes and was described as early as 1804 by Eckhold and Martens. The main features are split hand/foot or lobster claw deformity (ectrodactyly), ectodermal dysplasia which manifests as the abnormal development or growth of ectodermal tissues and structures such as hair, skin, nails, teeth and sweat glands, and cleft lip with or without cleft palate. Lacrimal tract abnormalities, urogenital defects, conductive hearing loss, mental retardation and chronic respiratory infections are other features frequently found in EEC patients [174]. Most of the *p63* gene mutations found in EEC syndrome patients give rise to amino acid substitutions in the DNA binding domain common to all known p63 isoforms. The most frequently mutated amino acids are the arginine codons 204, 227, 279, 280 and 304, accounting for almost 90% of all EEC syndrome cases [178, 179]. These

mutations appear to impair the p63 protein binding to DNA [174]. Genotype-phenotype analyses for the five hotspot mutations revealed significant differences between the corresponding phenotypes and might reflect that different target genes are affected by each of the hotspot mutations [179]. All of the *p63* missense mutations found in EEC syndrome patients affect amino acids that are strictly conserved among all the p53 family members and they correspond exactly to the somatic p53 hotspot mutations found in human malignancies [178]. However, despite the similarities between p53 and p63 both in amino acid homology and the ability to transactivate many common target genes, there is no indication for an increased susceptibility for cancer development in EEC syndrome patients.

ΔNp63α plays an important role in the development of stratified epithelial tissues by maintaining a stem cell population in the basal layer [180], and inactivation of ΔNp63α seems to be responsible for the severe developmental defects found in p63-deficient mice as well as in human patients with mutations in p63.

TAp63α has been shown to be constitutively expressed in female germ cells during meiotic arrest, and for being responsible for maintaining the genomic stability in mammalian oocytes and elimination of damaged cells [181]. In unstressed oocytes TAp63α is kept in a closed dimeric conformation, while DNA damage triggers phosphorylation that leads to a switch of TAp63α from an inactive dimeric state to active tetramers [171].

p63 and cancer

In contrast to the high incidence of tumors in p53-null mice, the analysis of *p63*-deficient mice has led to often conflicting results in regard to its role in tumorigenesis. The severe developmental abnormalities of the two independently generated $p63^{-/-}$ mouse models, made it difficult to study whether germ line *p63*-deficiency causes a tumor-prone phenotype [172, 173]. Instead, several studies on $p63^{+/-}$ mice have been performed. One study showed that a number of $p63^{+/-}$ mice are cancer-prone, that p63 functions as a tumor suppressor, and that a combined loss of p53 and p63 cooperates in malignancy and enhances metastasis [182]. However, other independent studies concluded that $p63^{+/-}$ mice on a different inbred strain show premature aging by inducing cellular senescence [166], but are not prone to either chemically or spontaneously induced tumors [183], γ-irradiation induced lymphomas [184], and that p63 heterozygosity does not accelerate tumorigenesis when p53 is compromised [183]. These conflicting data might result from different influences on tumorigenesis by the different genetic backgrounds. Also the fact that the p63 mouse models used in these tumor studies are deficient for all p63 isoforms, masks the contribution of individual p63 proteins in cancer. A recent study using a TAp63-specific conditional mouse model showed that TAp63 isoforms are strong mediators of senescence that inhibit tumorigenesis *in vivo* [185].

Unlike p53, p63 is rarely mutated in human cancers [186]. Although p63 expression is reduced in a small subset of advanced tumors [187-189], the majority of squamous cell

carcinomas express high levels of p63. However, many of these tumors actually overexpress the ΔNp63 isoforms.

ΔNp63 acts as an oncogene. Imbalance between the TA and ΔN isoforms of all the p53 family members may lead to tumor development by preventing the transcriptionally active TA isoforms from exerting their tumor-suppressive functions [188, 190-192]. Overexpression of ΔN-isoforms can counteract the cell-cycle arrest and proapoptotic transcription activities of p53 and TA proteins and this may lead to a proliferative advantage and tumorigenic potential to cancer cells [193, 194]. ΔNp63 plays a fundamental role in the development of epithelia, and alteration in ΔNp63 is often seen in epithelial tumors. Amplification of the p63 locus as well as overexpression of the ΔNp63α isoform have been found in squamous cell carcinomas of the lung, head and neck [195-197]. Breast, bladder and aggressive cases of prostate cancers have also been shown to overexpress ΔNp63. Moreover, ΔNp63α expression is directly correlated with the clinical response to cisplatin and the expression level of ΔNp63α can be used to predict the response of head and neck squamous cell carcinoma patients to anti-cancer therapy [198].

Loss of p63 in human squamous cell carcinoma cell lines has been shown to lead to increased cell migration and up-regulation of genes associated with invasion and metastasis [199]. Moreover, TAp63 was previously demonstrated to play a primary role as an antagonist of TGFβ-driven tumor invasiveness and metastasis [200]. Loss of TAp63 promotes invasion and migration, and the invasive activity of mutant p53 correlates with inhibition of TAp63 function [133].

P73

p73 isoforms

The *p73* gene expresses at least thirty-five mRNA variants; seven alternatively spliced C-terminal isoforms (α, β, γ, δ, ε, ζ, and η) and at least four alternatively spliced N-terminal isoforms (Figure 1). The N-terminal protein isoforms encoded by alternatively spliced exon-2 and/or exon-3 mRNA variants are initiated at different ATG and contain different parts of the transactivation domain.

p73 and p53 share 30% amino acid identity in the transactivation domain and 38% in the C-terminal oligomerization domain. In the DNA-binding domain, 63% identity is found between p73 and p53 and the residues in p53 responsible for direct interaction with DNA are completely identical in p73 as well. As a consequence, p73 can bind to and activate transcription from p53-responsive promoters and as a result induce cellular responses such as cell cycle arrest, apoptosis and senescence [201-203].

In addition to the TID and SAM domains present in the C-terminus of the α-isoforms of p73 [168-170], a C-terminal helix has recently been found in p73 that is not present in p53 and this helix is necessary to keep the oligomerization domain of p73 in a tetrameric and conformational stable state [204].

Biological activities of p73

Expression of *p73* is essential for neurogenesis of specific neural structures, for normal dynamics of cerebrospinal fluid, and for pheromonal signalling [205]. Mice functionally deficient for all p73 isoforms exhibit profound defects, including hippocampal dysgenesis due to massive apoptosis of sympathetic neurons in superior cervical ganglion [206], hydrocephalus due to hypersecretion of cerebrospinal fluid, immunological problems characterized by chronic infections and inflammation, runting, as well as abnormalities in pheromone sensory pathways leading to abnormal reproductive and social behaviour [205]. The majority of *p73*-deficient mice die at approximately 4 to 5 weeks of age due to chronic infections [205].

In mice lacking all p73 isoforms, the defects in hormonal and sensory pathways contribute to both male and female infertility through abnormal reproductive behaviour. TAp73 has been shown to play an important role in maintaining the fidelity of the genome [207]. Mice selectively deficient for the TAp73 isoforms mate normally, instead they are infertile due to genomic instability of the oocyte which leads to impaired embryonic development [207]. TAp73 regulates the formation of proper mitotic and meiotic spindles required for chromosome alignment and genomic stability [208]. Interestingly, loss of TAp73 may be responsible for the compromised developmental ability of aged normal oocytes since natural aging leads to a loss of TAp73 expression in oocytes [207].

Mice that are selectively deficient in ΔNp73 show signs of neurodegeneration but are viable and fertile [209]. Importantly, this mice model revealed a novel role of ΔNp73 as a negative regulator of the DNA damage response by inhibiting signal transduction from sites of DNA damage [209]. This function may explain the increased resistance to chemotherapy seen in tumors which express high levels of ΔNp73 [209].

No genetic disorder has yet been associated with germ line mutation of the *p73* gene in humans.

p73 and cancer

$p73^{-/-}$ mice show no increased susceptibility to cancer [205]. However, $p73^{+/-}$ mice develop malignant lesions, demonstrating that p73 can act as a tumor suppressor. Moreover, $p63^{+/-}$;$p73^{+/-}$ mice develop malignant tumors at high frequency and $p53^{+/-}$;$p73^{+/-}$ mice displayed a higher tumor burden and metastasis compared to $p53^{+/-}$ mice [182]. Importantly, phenotypical characterization of mice selectively deficient for TAp73 isoforms revealed that loss of TAp73 *in vivo* predisposes for an increase in spontaneous as well as carcinogen-induced tumorigenesis [207].

The *p73* gene is rarely mutated in human cancers [186]. However, elevated levels of ΔNp73 isoforms and loss of TAp73 expression have been linked to poor prognosis,

reduced disease-free survival and chemotherapeutic response in a number of human cancers [190-192].

Accumulating evidence show that p73 plays a significant role in curative anti-cancer treatment. TAp73 is induced by a large variety of chemotherapeutic agents, such as doxorubicin, cisplatin, camptothecin, etoposide and taxol [210]. Inhibition of p73 with siRNA or a dominant-negative mutant has been shown to suppress apoptosis and lead to resistance of tumor cells to chemotherapy, irrespective of the p53 status [210-212].

Interactions between different isoforms of the p53 family may lead to chemoresistance when dominant-negative ΔN isoforms suppress the transcriptionally active counterparts. In addition, ΔNp73 acts as a negative regulator of the DNA damage response [209]. A majority of the tumors in a study of 35 different human cancers, including cancers of the breast, ovary, endometrium, cervix, vagina, vulva, kidney and colon, exhibited up-regulation of ΔNp73 [213]. In rhabdomyosarcoma ΔNp73 is frequently overexpressed and has been shown to play an essential role for tumor progression in *vivo* [214]. The importance of assessment of p73 isoform expression in a tumor when it comes to cancer prognosis and prediction of tumor chemosensitivity have been confirmed in several studies and in different tumor types [215]. Increased expression of dominant-negative p73 isoforms have been linked to resistance to conventional chemotherapy in ovary carcinomas and in childhood acute lymphoblastic leukaemia [216, 217]. High levels of ΔNp63 in head and neck squamous cell carcinomas have been shown to suppress TAp73-dependent apoptosis both by physical interaction between the proteins and by direct promoter binding, and inhibition of endogenous p63 expression correlates with increased tumor sensitivity to chemotherapy and/or radiation [194].

Mutant p53 can induce chemoresistance by neutralizing TAp73 and downregulation of mutant p53 has been shown to enhance chemosensitivity [210]. Moreover, a p53 polymorphism in patients with head and neck cancers influences the response to cancer therapy by inhibiting p73-dependent apoptosis, underlining the potential effect of these factors in the prediction of clinical responses [218].

p73 as a target for antitumor therapy

Therapeutic strategies to directly and selectively activate TAp73 are generating interest because of the ability of TAp73 to induce apoptosis independently of p53.

A small peptide (named 37AA) containing 37 amino acids from p53 has been shown to induce cell death in multiple cell types irrespective of p53 status [219]. This molecule binds iASPP, a common negative regulator of p53 family members, resulting in derepression of TAp73, p73-mediated gene activation and cell death of p53-null cells [219]. Systemic nanoparticle delivery of a transgene expressing 37AA has been shown to induce p73-dependent tumor regression *in vivo* [219].

Screening of chemical libraries for small molecules that activate a p53 response in tumor cells in the absence of p53 have led to the discovery of compounds able to induce the expression of p53 target genes and apoptosis [220, 221]. The complex between mutant p53 and p73 is a promising and highly specific potential target for cancer therapy. A small molecule called RETRA (**re**activation of **t**ranscriptional **r**eporter **a**ctivity), is able to release p73 from the blocking complex with mutant p53 [220]. Treatment of mutant p53-expressing tumor cells with RETRA results in a substantial activation of a set of p53-regulated genes and specific suppression of mutant p53-expressing tumor cells *in vitro* and in mouse xenografts. Targeting of mutant p53-p73 protein complexes by small interfering peptides have been shown to enhance the response of mutant p53 tumor cells to commonly used anticancer drugs [222].

Several studies have supported a role for p73 in mediating nutlin-3-induced apoptosis in the absence of *TP53*. Nutlin-3 can stabilise p73 in p53-deficient cells by disrupting p73-HDM2 binding, leading to increased p73 transcriptional activity, up-regulation of p73 targets p21, PUMA and Noxa and enhanced apoptosis [223]. Moreover, Nutlin-3 increases the sensitivity of p53-null neuroblastoma cells to doxorubicin via up-regulation of TAp73 and activation of E2F1 [224].

Another strategy could be to identify molecules or siRNA-based therapeutic agents that can interfere with overexpressed ΔNp73.

THE PML NUCLEAR BODY AND ITS RESIDENTS

Electron microscopy and auto-antibodies made it possible to explore the field of nuclear structures. In the early 1960s, the work from several pioneers revealed the presence of dense spherical objects in the nucleus by electron microscopy. 30 years later, by using autoimmune sera from primary billiary cirrhosis patients allowed the identification of the first PML-NB-associated protein, SP100 [225], and an initial characterization of these structures [226].

Characteristics, structure and spatial distribution

The promyelocytic leukaemia nuclear bodies (PML-NBs, also known as Kremer bodies, nuclear domains-10, ND10, PML oncogenic domains, PODs, NBs or nuclear dots, NDs) are discrete doughnut-shaped subnuclear macromolecular structures with a diameter of 0.2-1.0 μm and localized within the interchromatin space [227]. They are present in most mammalian cells, typically 1-30 PML-NBs per nucleus, depending on cell type, cell cycle phase and differentiation state [227]. Studies using electron microscopy showed that PML-NBs are composed of a ring-like protein structure and do not in general contain RNA or DNA in the centre [227, 228]. At the periphery of the ring, however, PML-NBs have been shown to make extensive contacts with chromatin fibres and thereby maintaining the integrity and positional stability of PML-NBs in the nucleus [229]. However, the position of PML-NBs in the nucleus with respect to chromatin is not random. Immunofluoresence *in situ* hybridization experiments have shown that PML-NBs associate non-randomly

with transcriptionally active genomic regions [230]. The major histocompatibility complex (MHC) class I gene cluster region and the p53 gene locus are two chromosomal loci that PML-NBs have been shown to associate to [231-233].

PML protein

The promyelocytic leukaemia (PML) protein (also called MYL, RNF71, PP8675 and TRIM19) is a tumor suppressor and the key organizer of PML-NBs. The *PML* gene was originally identified because of its involvement in acute promyelocytic leukemia (APL), where a reciprocal chromosomal translocation event t(15;17) of the *PML* gene results in a fusion to the retinoic acid receptor α gene (*RARα*) [234, 235]. In cells from APL patients, the PML-RARα fusion protein physically interacts with wild-type PML expressed from the non-translocated locus. This leads to disruption of PML-NBs in a dominant negative manner and delocalization of PML-NB-components, including PML, into an uncountable number of tiny speckles [236]. Re-formation of PML-NBs occurs upon treatment with arsenic trioxide (As_2O_3) or retinoic acid, and results in apoptosis or terminal differentiation in APL cells [237-242]. In cells from PML-deficient mice [243], the PML-NB-associated proteins Sp100, DAXX and CBP show aberrant localization, and this is reversible upon exogenous expression of PML [244, 245].

The PML protein exists as seven groups of isoforms (PML-I to VII) due to alternative splicing of C-terminal exons, and some recently described cytoplasmic cPMLs [246]. All PML isoforms contain the RBCC/TRIM motif, which is a tripartite structure containing a zinc-finger called the RING motif, two additional zinc-finger motifs (*B*-boxes) and a coiled-coil domain mediating homodimerisation [246]. Many TRIM/RBCC containing proteins are ubiquitin ligases that generate subcellular structures through autoassembly [247, 248]. Distinct PML-NBs are observed when expressing single PML isoforms in *pml* -/- cells, implying that isoform-specific sequences contact different nuclear constituents that influence morphogenesis [249-251]. However, in the physiological situation in the cell multiple isoforms appear to be expressed in combination and contribute to PML-NB structure [252]. PML-IV, even though not the most abundant isoform [250], is probably the best investigated one. It has been shown to regulate the recruitment and activation of p53 to PML-NBs, facilitating apoptosis or cellular senescence upon cytotoxic stress [253]. Transcription of the *PML* gene is tightly controlled by interferons [254], and also by p53 [255].

PML contains three covalent SUMO (small ubiquitin-related modifier) modification sites and one SUMO interaction motif (SIM), which non-covalently binds SUMO [256]. SUMO seems to play a fundamental role in PML-NB formation and recruitment of other proteins to PML-NBs. Most PML-NB components are also sumoylated or bind to SUMO [257]. Sumoylation of PML seem to be necessary for the formation of PML-NBs, since a PML mutant that cannot be modified by SUMO fails to recruit classical PML-NB components such as SP100 and DAXX [244, 245].

Function of PML-NBs

PML-NBs are diverse and proteinaceous structures. An ever-increasing number of partner proteins (now in the range of 100) have been shown to reside, constitutively or more often transiently, in the PML-NBs [258]. As a consequence, PML-NBs have been implicated in the regulation of a variety of cellular functions, such as maintenance of genomic stability, induction of apoptosis and cellular senescence, inhibition of proliferation, antiviral responses and angiogenesis.

The diverse nature of the partner proteins that accumulate in PML-NBs has made it difficult to attribute a specific biochemical function to these structures. Three main models have been proposed to explain how PML-NBs exert their biological functions [227, 259, 260]. In a first model, PML-NBs operate as nuclear storage compartments for accumulation of proteins both under normal conditions (accumulation and release of proteins when necessary) and under pathological conditions (sequestration of foreign or misfolded proteins). In a second scenario, PML-NBs form sites for specific protein-protein interactions where proteins accumulate to be post-translationally modified. The third of these models proposes that PML-NBs are active sites for distinct nuclear functions such as transcriptional and chromatin regulation.

PML-NBs and cellular stress

PML-NBs are dynamic structures that undergo significant changes in size, number and position particularly in response to cellular stress [227, 261]. For example, heat shock and heavy metals cause the redistribution of PML-NBs into numerous small nuclear dots that are devoid of SUMO and most partners [262-264]. Transcriptional inhibition by actinomycin D can either induce formation of dispersed micro-bodies or larger peri-nucleolar bodies depending on cellular context [229, 265, 266]. DNA-damaging agents such as UV-irradiation and alkylating agents cause the dispersal of PML-NBs into smaller bodies or a diffuse pattern [267-269]. However, different responses in PML-NB organization were seen for other DNA-damaging agents. Doxorubicin causes PML to accumulate around the nucleolus [270], and γ-irradiation results in an increase of the number and size of PML-NBs [265, 271, 272]. Inhibition of proteasomes can result in PML-NBs to increase in number and size [273, 274], and redistribution of PML-NBs to nucleoli [265, 275]. PML is induced at the transcriptional level by interferons, which cause an increase in both the number and size of PML NBs [254]

PML-NBs and p53 family members

The role of PML in the control of cell growth, senescence and apoptosis prompted researchers to investigate a link with p53. This led to the demonstration that p53 binds to PML and is consequently recruited to PML-NBs [104, 105, 253, 276]. PML overexpression led to relocalization of endogenous p53 to PML-NBs, so did also expression of activated Ras [104, 105], UV-irradiation combined with As_2O_3 treatment [276], and γ-irradiation [271]. Lack of PML dramatically impairs p53-dependent Ras-

induced senescence as well as p53-dependent apoptosis following γ-irradiation, and the requirement of PML for these processes is intimately related to the formation of PML-NBs and the recruitment of p53 to them [104, 105, 253, 276]. PML itself is a p53 target gene, suggesting a positive feedback loop for p53-dependent cell fate regulation [255].

Strikingly, several proteins that play key roles in post-translational modifications of p53 have been found within or in association with PML-NBs. PML can bind to both p53 and MDM2 [277-279], and regulates p53 stability by sequestering MDM2 in the nucleolus [270]. CREB-binding protein (CBP), an acetyltransferase that activates p53 by acetylating it at Lys383, has been found to colocalize with PML-NB as a consequence of either ras-induced senescence, PML overexperssion or γ-irradiation of human cells [104, 105, 253, 276]. Homeodomain-interacting protein kinase 2 (HIPK2) is a protein known to phosphorylate p53 at Ser46 [280, 281], an event that has been linked to induction of apoptosis following high doses of UV-irradiation [282]. Upon cellular stress, degradation of HIPK2 is inhibited and a fraction of HIPK2 is recruited to PML-NBs where it colocalizes with p53 and presumably phosphorylates p53 on Ser46 [280, 281]. In addition, HIPK2 binds to the acetyltransferase CBP and enhance CBP-dependent acetylation of p53 at Lys382 [281]. Efficient HIPK2-mediated phosphorylation of p53 is dependent on the presence of PML, and also the PML-NB component Sp100 seems to be important for HIPK2-mediated p53-phosphorylation, indicating that PML-NBs could be the site of Ser46 phosphorylation of p53 [283, 284]. Another enzyme affecting p53 activity and localization to PML-NBs is herpesvirus-associated ubiquitin-specific protease (HAUSP) [285]. HAUSP de-ubiquitinates p53, resulting in its stabilization and induction of apoptosis [68]. Induced accumulation of the acetyltransferase TIP60 at PML-NBs has been shown upon UV-irradiation [286]. TIP60 is able to acetylate p53 at Lys120, and this acetylation was also shown to facilitate p53-dependent apoptosis [287, 288]. NAD-dependent deacetylase SIRT1, a negative regulator of p53 function [289, 290], can localize to PML-NB and reduce the acetylation of p53 at Lys382 and thereby counteract DNA damage-induced apoptosis [291].

Both p63 and p73 have been found to localize to PML-NBs. PML recruits p73 to the PML-NBs where it inhibits the degradation of p73 and potentiates its transcriptional and proapoptotic activities [292], and similarly protein stability and transcriptional activity of p63 increases through interaction with PML [293].

PML-NBs and EBNA-5

Epstein-Barr virus (EBV) is a human gamma herpesvirus that immortalizes B-cells. The EBV-encoded nuclear antigen EBNA-5 (also known as EBNA-LP) is a latency associated antigen and one of the proteins essential for human B-cell transformation [294]. EBNA-5 can bind to p14ARF [295]. It has also been shown to form trimolecular complexes with MDM2 and p53, leading to inhibition of the transactivating function of p53 [296]. In EBV-transformed lymphoblastoid cell lines EBNA-5 locates to the inner core of the PML-NBs [297]. Upon heat shock or high cell density, EBNA-5 and Hsp70 have been shown to translocate to nucleoli [298], and inhibition of

proteasomes with MG132 resulted in nucleolar translocation of EBNA-5 and mutant p53 [299]. Proteasomal inhibition was also followed by an induction of Hsp70 protein levels as well as redistribution of Hsp70 to nucleoli and cytoplasm [299]. In addition, proteasomes and the PML-NB-associated proteins PML, Sp100 and SUMO-1 accumulated in nucleoli upon treatment with MG132, suggesting that nucleoli may be involved in the regulation of proteasome-dependent protein degradation and that a natural turnover of these proteins involves trafficking through the nucleoli [275].

THE NUCLEOLUS

The nucleolus is a plurifunctional, nuclear organelle organized around the repeated ribosomal gene clusters. Because the nucleolus is easily visualized by light microscopy, it was one of the first subcellular organelles discovered by early microscopists in the late 1890's. It occupies a considerable portion of the nucleus, but its size varies greatly depending on the species, cell type and metabolic conditions. Its major functions are ribosomal RNA (rRNA) synthesis, processing and assembling into ribosomal subunits. However, in the late 1990's, several other functions began to appear, some of which will be mentioned below.

At least three morphologically distinct regions of the mammalian nucleolus can be distinguished – the fibrillar centres (FCs), which are surrounded by the dense fibrillar component (DFC), and the granular component (GC) which constitutes the remainder of the nucleolus. Each of these subcompartments has distinct protein compositions and functions [300]. For example, the FCs contain the transcription factor UBF and are rich in RNA polymerase I. Nucleolin and the RNA methyltransferase fibrillarin are found in the DFCs, and the GC contain the nucleolar phosphoprotein B23 (also known as nucleophosmin or NPM) and is enriched with ribosomal proteins and assembly factors. During ribosome subunit biogenesis, pre-rRNA transcripts are transcribed by RNA polymerase I from the repeated clusters of rDNA genes. Transcriptionally active ribosomal genes are located in the FCs and the intimately associated DFCs. The initiation of transcription is believed to occur either within the FCs or at the border between FC and DFC. The resulting pre-rRNA transcripts then emerge into the DFC, where they are cleaved and modified by the small nucleolar RNPs (snoRNPs) and other processing enzymes. In the DFC, the rRNAs also begin the pathway of assembly with ribosomal proteins and continue this as they pass through the GC and are exported to the cytoplasm. Therefore, FCs and DFCs are considered to represent the structural-functional units of the nucleolus producing rRNA molecules that migrate to the GC where they undergo maturation for ribosome subunit composition. The GC might also contain regions that include protein complexes that are devoid of RNA [301].

The nucleolus is not enclosed by a membrane, but its unique density in combination with its robust structure makes it one of the most convenient structures to purify. This ability has made it possible to analyse the nucleolar protein composition in great detail. Examination of the nucleolar proteome provides a glimpse into its

functional complexity. More than one third of the nucleolar proteome are factors involved in different steps in rRNA transcription, processing and modification, as well as ribosome subunits proteins, confirming its central role in ribosome subunit biogenesis [302]. However, factors not obviously connected to ribosome biogenesis were also found in nucleoli, including viral proteins, polyadenylated mRNAs, cell cycle regulatory and tumor related proteins, suggesting its involvement in a wide range of cellular processes, such as viral replication, RNA processing, cell cycle regulation, tumor suppression, control of aging and modulation of telomerase function.

A novel role for the nucleolus is its participation of the regulation of p53. It has been proposed that the nucleolus also functions as a major cellular stress sensor and transmits signals for regulation of p53 activity, for example by releasing ARF to the nucleoplasm. Nucleolar sequestration has been reported to regulate protein activity by preventing proteins from reaching their targets in other cellular compartments. MDM2 is sequestered in the nucleolus by ARF in response to replicative senescence or activation of the oncoprotein Myc [56, 303]. This prevents MDM2 from exporting p53 into the cytoplasm for degradation, allowing p53 to exert its function.

The nucleolus is frequently altered in tumor cells. Nucleolar size is typically assumed to reflect the rate of ribosome production and more prominent nucleoli are often observed in many types of cancer cells.

HEAT SHOCK RESPONSE

The stress or heat shock protein response is a highly conserved cellular defense mechanism. It is activated in response to a wide variety of physiological and environmental stresses, such as hyperthermia or pharmacological agents, allowing cells to withstand a subsequent metabolic stress that would otherwise be lethal. Heat shock response is characterized by increased expression of stress proteins that provide cellular protection by acting as molecular chaperones, catalyzing the proper folding of misfolded proteins and preventing their aggregation [304]. Hsps are also powerful anti-apoptotic proteins able to associate with key effectors of the apoptotic machinery, resulting in interference with the cell death process at different stages [305-307]. Moreover, Hsps contribute to cell survival also by providing either stability or the proteasomal degradation of selected proteins under stress conditions [308, 309].

An increased surface exposure of hydrophobic amino acids discriminates a nonnative, partially or globally unfolded protein from its native counterpart. This feature is recognized by molecular chaperones that subsequently bind to hydrophobic patches, specific peptide sequences or to structural elements of the nonnative protein. This interaction is controlled by ATP as well as by several different co-chaperones that modulate the ATPase cycle.

The molecular chaperones comprise of five major and broadly conserved families that are classified according to their molecular weight: Hsp100s, Hsp90s, Hsp70s,

Hsp60s and small Hsps. All molecular chaperons interact promiscuously with a broad range of unfolded proteins.

Hsp70 is one of the most abundant heat shock proteins. It is under physiological conditions involved in *de novo* folding of proteins, the assembly and disassembly of multiprotein complexes and the transport of proteins across cellular membranes [310, 311]. Moreover, they regulate signal transduction pathways by controlling the stability and activities of transcription factors and protein kinases. During stress conditions they prevent aggregation of unfolded proteins and can even refold aggregated proteins. Hsp70 can bind to mutant p53 [312] through the hydrophobic core of the DNA binding domain accessible in mutant but not wild type p53 [313]. Overexpression of Hsp70 increases transformation of several cell types [314-316], and consistent with these observations inhibition of Hsp70 causes inhibition of tumor cell proliferation. Constitutively high expression of Hsp70 is essential for the survival of most cancer cells [317], and has been correlated with metastasis and poor prognosis [318, 319]. Hsp-targeting drugs have therefore emerged as potential anti-cancer agents [320].

AIMS OF THE THESIS

The general aims of this thesis were to investigate the mutant p53-rescuing properties of the molecule STIMA-1 and to further characterize the effects of the mutant p53-reactivating compound PRIMA-1. Identifying molecules that can selectively target mutant p53-harbouring tumor cells and understanding of the effects of such compounds on different cellular targets are important for further development of potent and selective mutant p53-targeting anti-cancer drugs.

The specific aims were:

Paper I: To investigate the effect of PRIMA-1 analogs on the subcellular distribution of mutant p53 and some p53-interacting or modifying proteins.

Paper II: To characterize the mutant p53-targeting compound STIMA-1.

Paper III: To study how the subcellular localization of the Epstein-Barr virus-encoded protein EBNA-5 is affected by PRIMA-1^{MET}.

Paper IV: To analyze the effect of PRIMA-1^{MET}/APR-246 on the p53 family members p63 and p73.

RESULTS AND DISCUSSION

PAPER I - PRIMA-1^{MET} induces nucleolar accumulation of mutant p53 and PML nuclear body-associated proteins

When we started this project in 2003, little was known about the molecular mechanism behind PRIMA-1-induced reactivation of mutant p53. In order to obtain clues about what happens in the cell upon treatment with PRIMA-1 we aimed to investigate the effect of different PRIMA analogs on the subcellular distribution of mutant p53 and some proteins known to modify or interact with p53 and/or mutant p53.

In this paper we investigated how PRIMA-1, PRIMA-1^{MET} and PRIMA-Dead affect the subcellular localization of mutant p53 and several other PML-NB-associated proteins. PRIMA-1 is the original PRIMA compound, PRIMA-1^{MET} is a methylated form of PRIMA-1 with higher potency in inducing mutant p53-dependent apoptosis than PRIMA-1 itself, and PRIMA-Dead is a PRIMA-1 stuctural analog lacking the apoptosis-inducing properties. We treated human tumor cells with different p53 status with the PRIMA compounds separately and analyzed the distribution of mutant p53, PML, CBP and Hsp70 by immunofluoresence staining.

We started by examining mutant p53 localization in H1299-His175 cells that express exogenous mutant p53, and SW480 cells containing endogenous His273 mutant p53, cultured in the presence or absence of PRIMA-1^{MET}. We found that PRIMA-1^{MET} induced nucleolar accumulation of mutant p53 in both cell lines, as verified by double immunofluorescence staining for p53 and the nucleolar protein B23. The H1299-His175 cells showed a higher percentage of cells with mutant p53 relocated to nucleoli, 79% as compared to 33% of SW480 cells. At a later time point the fraction of p53-positive cells that showed nucleolar accumulation of mutant p53 was even higher, 99% of the H1299-His175 cells and 68% of the SW480 cells, respectively.

Next we treated H1299-His175 cells with three different PRIMA structural analogs; PRIMA-1, PRIMA-1^{MET}, and PRIMA-Dead, and analyzed the mutant p53 localization pattern. PRIMA-1^{MET}, and to a lesser extent PRIMA-1, induced nucleolar accumulation of mutant p53. In contrast, PRIMA-Dead did not change the localization pattern of p53. The amount of caspase-positive cells after drug treatment were assessed by incubation with FAM-VAD-FMK and analysis by flow cytometry, and confirmed that PRIMA-1^{MET} was more potent in inducing apoptosis compared to PRIMA-1, and that PRIMA-Dead was inactive in this regard. Induction of cell death by cisplatin or the mutant p53-reactivating compound MIRA-1 did not result in nucleolar accumulation of mutant p53 in H1299-His175 cells, suggesting that nucleolar accumulation of mutant p53 is not a general response to apoptosis-inducing and/or mutant p53-reactivating drugs, but a specific effect of PRIMA-1^{MET} on these cells. The cellular distribution pattern of wild type

p53 in the MCF-7 and U2OS cell lines did not change upon treatment with PRIMA-1^{MET} under similar conditions.

Several proteins found in the PML-NB are known to interact with p53. The PML-NBs might be involved in regulating apoptosis by serving as activation or modification platforms for p53, and by controlling the subcellular availability of p53 and other apoptotic proteins. In order to investigate the effect of PRIMA-1^{MET} on some of these PML-NB-associated proteins, we treated H1299-His175 and SW480 cells with PRIMA-1^{MET} and analyzed the subcellular distribution pattern of PML, CBP and Hsp70. We found that PML, CBP and Hsp70 all accumulated in the nucleoli upon treatment with PRIMA-1^{MET} and that redistribution of these proteins to nucleoli was enhanced by the presence of mutant p53. Moreover, no nucleolar accumulation of mutant p53, Hsp70 or PML was observed upon treatment with PRIMA-Dead, not even at higher concentrations.

The acetyltransferase CBP can acetylate p53 at Lys383, leading to increased cellular susceptibility to p53-mediated apoptosis. Therefore we tested if treatment with PRIMA-1^{MET} induced the levels of acetylated p53. However, at the conditions tested we did not observe any increased p53 acetylation at Lys383 in H1299-His175.

Finally, we observed a stronger intensity of the Hsp70 staining in PRIMA-1^{MET}-treated H1299-His175 and SW480 cells, as well as the p53 null cell line H1299. Western blot analysis confirmed that Hsp70 protein levels are increased by PRIMA-1^{MET}-treatment in all three cell lines. Treatment with PRIMA-Dead did not induce the level of Hsp70 protein.

Our findings presented in this paper suggest that redistribution of mutant p53 to nucleoli, and/or redistribution of other PML-NB-associated proteins, may play a role in PRIMA-1^{MET}-induced apoptosis. Our data also indicate that PRIMA-1^{MET} induces a heat shock response in cells independently of mutant p53. Elucidation of the molecular mechanism underlying the effect of PRIMA-1^{MET} is important for further drug optimization.

PAPER II - Mutant p53 targeting by the low molecular weight compound STIMA-1

The high frequency of p53 mutations in human tumors, together with the often high levels of mutant p53 in those tumors and the fact that increased resistance to conventional chemotherapy is frequently seen in mutant p53-harbouring tumors, makes p53 an attractive target for cancer therapy. Restoration of wild type function to mutant p53 in human tumor cells should evoke a massive apoptotic response and result in elimination of the tumor.

Some derivatives of 2-styrylquinazolin-4(3H)-one have been found to possess biological activity against cancer cells [321], and these compounds show some resemblance to CP-31398 – a newly characterized mutant p53-reactivating

compound [164]. Therefore, a series of 2-styrylquinazolin-4(3H)-one-related derivatives were synthesized in order to investigate their anti-tumor activity and possible effect on mutant p53.

In this paper, we used a cell-based assay to screen for molecules among these 2-styrylquinazolin-4(3H)-one-related derivatives, that could inhibit cell growth in a mutant p53-dependent manner. Out of 26 compounds, one was found to suppress growth of mutant p53-expressing cells but not their corresponding p53 null counterparts. This low molecular weight compound, called STIMA-1 (**S**H-group **t**argeting and **i**nduction of **m**assive **a**poptosis), was shown to target p53 mutants belonging to the two main classes, conformational mutants (His175) and DNA contact mutants (His273). Flow cytometry analysis of sub-G1 DNA content and active caspase-positive cells showed a mutant p53-dependent increase of both upon treatment with STIMA-1, indicating cell death by apoptosis. By using an ELISA assay with an immobilized DNA oligonucleotide containing a p53 binding site we observed that STIMA-1 stimulated DNA binding of mutant p53 His175. We also found that STIMA-1 induced protein levels of p21, Bax, and PUMA in mutant p53-expressing tumor cells, while no induction of these p53 targets was seen in the corresponding p53 null cells.

Cisplatin is a widely used compound in clinical cancer therapy and is known to form adducts with DNA. To further investigate the properties of STIMA-1, we compared its effects on tumor cells with those of cisplatin and found opposite patterns of activity. In agreement with previously published results [151, 322, 323] cisplatin inhibited growth of tumor cells in a wild type p53-dependent manner, whereas STIMA-1 preferentially killed mutant p53-harbouring tumor cells. This suggests that the mechanism of action of STIMA-1 differs from that of cisplatin.

Next we studied the effect of STIMA-1 on tumor cells with different p53 status and on normal human diploid fibroblast. We analyzed STIMA-1-induced growth suppression in these cells and calculated IC_{50} values. These data showed that human tumor cells expressing mutant p53 were more sensitive to STIMA-1 as compared to p53 null or wild type-p53-carrying human tumor cells. Importantly, human diploid fibroblasts showed a significantly higher resistance to STIMA-1-induced growth suppression compared to mutant or wild type p53-carrying tumor cells.

It is known that the redox status of p53 is important for its tumor suppressor function and that oxidation of cysteines in p53 results in the formation of inter- and intramolecular disulfide bridges resulting in loss of wild type conformation [81]. STIMA-1 and CP-31398 share a common chemical activity as Michael acceptors. They both contain reactive carbon-carbon double bonds that enable them to participate in reactions of nucleophilic addition. Therefore, we investigated whether STIMA-1 and CP-31398 could react with thiol groups and whether this interaction is required for biological activity of these compounds. Our HPLC data showed that both compounds could form adducts with N-acetylcysteine, a cysteine derivative containing an acetyl group instead of an aminogroup, leaving one SH group as the only target for alkylation. The reactivity of STIMA-1 towards N-acetylcysteine was higher (90%), as compared to CP-31398 (20%). To investigate whether covalent

modification of thiol groups plays a role in the biological effects of STIMA-1 and CP31398, we pre-treated mutant p53-expressing cells with the thiol group donor N-acetylcysteine and found that STIMA-1-induced growth suppression in H1299-His175 cells was completely blocked by N-acetylcysteine, while the effect on CP-31398 was only partially blocked. Moreover, STIMA-1 and CP-31398 were found to react with thiol groups in recombinant GST-His175 mutant p53.

In conclusion, we have identified a low molecular weight compound with the ability to modify thiol groups. It selectively targets tumor cells in a mutant p53-dependent manner and may restore mutant p53's tumor suppressive function by affecting its redox status. This finding raises the possibility that modification of thiol groups plays a role in mutant p53 reactivation in tumor cells. Understanding of the molecular mechanisms of mutant p53 rescue is important in the development of novel anticancer drugs that target mutant p53-carrying tumors.

PAPER III - PRIMA-1^{MET} induces nucleolar translocation of Epstein–Barr virus-encoded EBNA-5 protein

The Epstein-Barr virus-encoded, latency associated antigen EBNA-5 is a nuclear protein that upon heat shock or inhibition of proteasomes translocates to the nucleolus along with mutant p53, PML, Hsp70, MDM2 and proteasome subunits. It has been shown to locate to PML-NBs in EBV-transformed lymphoblastoid cell lines and to associate with ARF-MDM2-p53 complexes. Considering the similarities between the proteasome inhibitor- and PRIMA-1^{MET}-induced nucleolar translocation of mutant p53, PML and Hsp70 and the intimate relation between EBNA-5 and the p53 pathway, we aimed to investigate the effect of PRIMA-1^{MET} on the subcellular distribution pattern of EBNA-5.

In this paper we investigated how the subcellular localization of EBNA-5 was affected by PRIMA-1^{MET}-treatment. We started by treating EBV transformed lymphoblastoid cell lines and tumor cells transfected with either native EBNA-5 or EBNA-5 conjugated with the fluorescence proteins EGFP or DSRed. We found that PRIMA-1^{MET}-treatment induced nucleolar translocation of EBNA-5 in all cell lines tested. EBV transformed human blastoid cells carries virus encoded EBNA-5 and harbours wild type p53. In these cells EBNA-5 was found in the PML-NBs and upon treatment with PRIMA-1^{MET} it relocated to the nucleolus. The EBNA-5 transfected tumor cell lines tested have different p53 status: SW480 cells contain endogenous His273 mutant p53, MCF-7 cells carry wild type p53, H1299 cells are p53 null and H1299-His175 express exogenous His175 mutant p53. EBNA-5 localized to the low DNA density areas in the nucleus in these cell lines, and upon treatment with PRIMA-1^{MET} the localization pattern of EBNA-5 changed with an overall increase in the nucleolus and also numerous distinct foci scattered throughout the nucleoplasm, independently of p53 status. Even though the cells showed extensive nucleolar EBNA-5 accumulation, the presence of EBNA-5 did not seem to influence on the survival rate of PRIMA-1^{MET}-treated cells as compared to empty vector control cells.

We used an automated confocal microscopy method for live cell imaging to study the dynamics of the movements of EBNA-5 induced by PRIMA-1^{MET}. EBNA-5 was evenly distributed throughout the nucleoplasm in untreated MCF-7 cells stably transfected with DSRed-EBNA-5, and between 6 and 10 hours after adding PRIMA-1^{MET} EBNA-5 successively accumulated in the nucleoli. The accumulated EBNA-5 was distributed in 15-20 round or ovoid particles that showed a limited movement inside the nucleolus. The process of nucleolar accumulation of EBNA-5 started from a single focus in a given nucleolus, and at different time points with up to four hours delay between different nucleoli in the same nucleus. At the point when the nucleoli were saturated with EBNA-5 some of the DSRed-EBNA-5 particles were released from the nucleoli and moved around in the nucleoplasm by rapid Brownian movement.

To study the effect of PRIMA-1^{MET} on the mobility of DSRed-EBNA-5 in different subnuclear compartments of MCF-7 cells we performed fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) experiments. In untreated cells, a homogenously distribution of DSRed-EBNA-5 throughout the nucleoplasm was seen. Single bleaching FRAP showed a very high mobility rate of nuclear DSRed-EBNA-5, with the average half recovery time of 1.5 second. FLIP demonstrated a quick depletion of the homogenously DSRed-EBNA-5 from the entire nucleoplasm. In the nucleolus of untreated MCF-7 cells, two differently equilibrating compartments were seen, one with intermediate level of recovery and one with a very slow recovery. PRIMA-1^{MET}-treatment resulted in accumulation of DSRed-EBNA-5 in the very low molecular mobility compartments of the nucleolus as shown by FLIP experiments. In addition, rigid bodies of DSRed-EBNA-5 were seen in the nucleoplasm of the cells after prolonged PRIMA-1^{MET}-treatment, and these showed random Brownian movements restricted to the dimensions of the average distance between the nucleoplasmic chromatin fibres.

Next, we tested if the PRIMA-1^{MET}-induced accumulation of DSRed-EBNA-5 is reversible or if this is a feature of an advanced stage of cellular agony. PRIMA-1^{MET}-treatment induced nucleolar accumulation of DSRed-EBNA-5 in H1299 cells and a complete disappearance of nucleolar DSRed-EBNA-5 aggregates was seen upon removal of PRIMA-1^{MET} with repeated washing with drug-free medium. Phase contrast images in parallel with the fluorescence imaging showed intact cellular morphology of the cells during the entire length of the experiment.

We suggest that EBNA-5 moves around in the nucleoplasm with high mobility and more slowly when it passes the nucleolus probably because of the more densely arranged chromatin fibres in the fibrillar area of the nucleolus. In this scenario, treatment with PRIMA-1^{MET} would lead to gradual precipitation of EBNA-5 and these aggregates then start to clog the chromatin fibre meshwork of the nucleolus. Our results in this study strengthen the belief that mutant p53 is not the only target of PRIMA-1^{MET}.

PAPER IV - PRIMA-1^{MET}/APR-246 targets mutant forms of p53 family members p63 and p73

The p53 family members p63 and p73 share high sequence and structural homology with p53. We therefore hypothesized that PRIMA-1^{MET} might also rescue wild type function to mutant forms of p63 and p73.

In this paper we investigated the effect of PRIMA-1^{MET} on several different mutants of p63 and p73. We started by analyzing the effect on temperature-sensitive (ts) mutants of TAp73α, TAp73β and TAp63γ, stably expressed in H1299 cells. These mutants are transcriptionally inactive at 39°C and adopt a transcriptionally active conformation upon temperature shift to 32°C. We found that PRIMA-1^{MET} treatment of ts mutant TAp73β or TAp63γ expressing cells at 39°C resulted in significant growth suppression, shown by using the WST-1 cell proliferation reagent, as compared with parental H1299 cells. Ts mutant TAp73α expression did not confer any significant increase of sensitivity to PRIMA-^{MET}-induced growth arrest compared with control cells. These results were in concordance with initial experiments using the same ts p63/p73 mutants expressed in Saos-2 cells. To check whether PRIMA-1^{MET} could act at the transcription level as well, we treated H1299 cells expressing ts mutant TAp73α, TAp73β or TAp63γ with PRIMA-1^{MET} at 39°C and analyzed the effect on p21 mRNA and protein levels by real-time reverse transcriptase PCR and western blot analysis respectively. p21 was induced both at the mRNA and protein level by PRIMA-1^{MET} in the presence of ts mutant TAp73β or TAp63γ, but not in TAp73α-expressing cells.

Next we tested whether PRIMA-1^{MET} could restore the apoptosis-inducing function to ts p63/p73 mutants. We started by analyzing the DNA content profile by propidium iodide staining and flow cytometry analysis of H1299 cells expressing the ts mutant TAp73α, TAp73β or TAp63γ treated with PRIMA-1^{MET} at 39°C. We found that PRIMA-1^{MET} increased the sub-G_1 cell population in the presence of the ts p63 and p73 mutants, indicating DNA fragmentation and cell death. Also here ts mutant TAp73β or TAp63γ expressing cells conferred a greater sensitivity towards PRIMA-1^{MET} compared with ts mutant TAp73α expressing cells. Active caspase-positive cells were then assessed using FAM-VAD-FMK substrate and flow cytometry. A dramatic increase in number of active caspase-positive cells were seen upon PRIMA-1^{MET} treatment in the presence of ts mutant TAp73β or ts mutant TAp63γ, indicating cell death by apoptosis. Cells expressing TAp73α were fairly resistant to PRIMA-1^{MET}-induced apoptosis.

In order to test if PRIMA-1^{MET} could also restore wild type function to naturally occurring p63 mutants, we choose two p63 point mutants, R204W and R304W, which are both known to cause the human EEC syndrome. Interestingly, these point mutants correspond exactly to p53 hot spot mutations at residues 175 and 273 that are commonly found in human tumors. We generated expression vectors for the mutants TAp63γ-R204W and TAp63γ-R304W, as well as for TAp73α-R193H and TAp73β-R193H (corresponding to p53 hot spot mutation at residue 175). To test the effect of PRIMA-1^{MET} on these mutants, we transiently transfected HCT116 p53-/- cells and analyzed the fraction of annexin V-positive cells after treatment

with PRIMA-1^{MET}. Cells expressing mutant p63 or p73 showed a significant increase in the number of annexin V-positive cells as compared with empty vector control cells.

In order to further investigate the effect of PRIMA-1^{MET} on the EEC syndrome-associated p63 mutants, we generated Saos-2 cells stably expressing Tet-inducible (Tet-On) TAp63γ-R204W or TAp63γ-R304W. We assessed cell proliferation in several individual TAp63γ-R204W or TAp63γ-R304W clones after PRIMA-1^{MET}-treatment and found that PRIMA-1^{MET} suppressed cell growth in all TAp63γ clones in a mutant p63-dependent manner. We also found that treatment with PRIMA-1^{MET} increased the fraction of mutant TAp63γ-expressing cells with sub-G_1 DNA content and active caspases, which indicates cell death by apoptosis. The increased sensitivity to PRIMA-1^{MET}-induced apoptosis was more pronounced in the presence than in the absence of doxycycline in the cells expressing the TAp63γ-R304W mutant, confirming that the presence of mutant p63 conferred the increased sensitivity. In the case for TAp63γ-R204W, increased expression did not enhance DNA fragmentation in response to PRIMA-1^{MET}, but instead we observed a significant increase in cells in the G1 phase and a decrease in the S-phase fraction, indicating that TAp63γ-R204W promotes cell cycle arrest in response to PRIMA-1^{MET}-treatment.

Next, we asked the question whether PRIMA-1^{MET} could stimulate mutant p63 DNA binding and up-regulation of p63 target genes to TAp63γ-R204W and TAp63γ-R304W mutants. Increased binding of mutant TAp63γ-R204W and TAp63γ-R304W to a p53/p63 consensus DNA-binding motif was observed in the presence of PRIMA-1^{MET} by using a modified TransAM enzyme-linked immunosorbent assay. We also found that PRIMA-1^{MET} induced p63 downstream targets related to cell cycle control, apoptosis and differentiation. p21 protein level was increased, and induction of pro-apoptotic Noxa was observed both at the mRNA and protein levels after PRIMA-1^{MET}-treatment in the presence of TAp63γ-R204W and TAp63γ-R304W mutants. Moreover, at low concentrations of PRIMA-1^{MET} we found induction of the differentiation marker and p63 target keratin 14 in cells expressing TAp63γ-R204W.

Since we previously found that PRIMA-1^{MET} causes a redistribution of mutant p53, along with PML, CBP and Hsp70 to the nucleolus (Paper I), we tested if mutant p63 also relocates in the presence of PRIMA-1^{MET}. We found that PRIMA-1^{MET}-treatment induced a redistribution of both TAp63γ-R204W and TAp63γ-R304W to PML-NBs and nucleoli in up to ~40% of the cells. No change in mutant p63 distribution was observed after treatment with the inactive PRIMA-1 analog PRIMA-Dead.

Taken together, our results show that PRIMA-1^{MET} in addition to p53 also target mutant forms of p63 and p73, indicating that PRIMA-1^{MET} exerts its effects through a common mechanism for all three p53 family members, presumably involving homologous structural domains common to all three proteins. All new clues underlying the molecular mechanism of PRIMA-1^{MET}-mediated effects are indeed important for further development of PRIMA-1^{MET} toward efficient anti-cancer

drugs. Our findings here may also raise the possibility of pharmacological rescue of p63 mutants in human developmental disorders with mutations in p63.

CONCLUSIONS

Paper I. In this paper we show that the mutant p53-reactivating compounds PRIMA-1 and PRIMA-1^{MET} induce a redistribution of mutant p53 and the PML nuclear body-associated proteins PML, CBP and Hsp70 to nucleoli. We also show that PRIMA-1^{MET} induces the levels of Hsp70 in cells independently of mutant p53. PRIMA-Dead, a compound structurally related to PRIMA-1 but unable to induce apoptosis, does not change the subcellular localization of mutant p53. This suggests that redistribution of mutant p53 and/or PML-nuclear body associated proteins may play a role for mutant p53 reactivation and induction of tumor cell apoptosis by PRIMA-1 and PRIMA-1^{MET}.

Paper II. In this study we identified STIMA-1 as a low molecular weight compound that inhibits cell growth and induces apoptosis in a mutant p53-dependent manner in human tumor cells. It stimulates p53 DNA binding *in vitro* and induces the level of several p53 target proteins in mutant p53-carrying tumor cells. Importantly, human diploid fibroblasts are significantly more resistant to STIMA-1-induced growth suppression than mutant p53-carrying tumor cells. We also show that STIMA-1 and CP-31398 have similar chemical activity as Michael acceptors and that this activity is related to the mutant p53-dependent growth suppression. This raises the possibility that modification of thiol groups plays a role in mutant p53 reactivation in tumor cells.

Paper III: In this paper we show that the Epstein-barr virus encoded protein EBNA-5 translocates to nucleoli in EBV-transformed B lymphoblasts and in transfected human tumor cells upon treatment with PRIMA-1^{MET}. Our results from live cell imaging studies show that in addition to nucleolar accumulation, PRIMA-1^{MET} reduces the mobility of DSRed-EBNA-5. Our results suggest that PRIMA-1^{MET} induces aggregation of EBNA-5 and that mutant p53 is not the only target of PRIMA-1^{MET}.

Paper IV. In this paper we show that PRIMA-1^{MET} restores the growth-suppressive and pro-apoptotic function to mutant forms of the p53 family members p63 and p73. Moreover, PRIMA-1^{MET} improves the DNA-binding capacity of p63 mutants, causes upregulation of p53/p63/p73-target genes and a redistribution of mutant p63 to PML nuclear bodies and nucleoli. Our results indicate that PRIMA-1^{MET} exerts its effect through a common mechanism for p53, p63 and p73, presumably involving homologous structural domains present in all three family members. Our findings also raise the possibility of pharmacological targeting of mutant p63 in human syndromes.

CLOSING REMARKS

Mutant p53 serves as a unique target for cancer therapy, given the high frequency of p53 mutations in human tumors, together with the often high levels of mutant p53 in those tumors and the fact that increased resistance to conventional chemotherapy is frequently seen in mutant p53-harbouring tumors. Restoration of wild type function to mutant p53 in human tumor cells should evoke a massive apoptotic response and result in elimination of the tumor.

The intense effort to target the p53 pathway have led to discoveries showing that drugs exploiting the loss of function of the p53 pathway could in the future have an impact on anti-cancer therapy. Design and/or identification of compounds that rescue p53 function and induce massive apoptosis in tumors have moved from a purely theoretical concept to a realistic proposition in recent years. Further understanding of the exact molecular mechanisms of such compounds will be the basis for novel approaches and further development into effective and selective p53-targeting drugs, with an improved cancer therapy with fewer side effects as the final goal.

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