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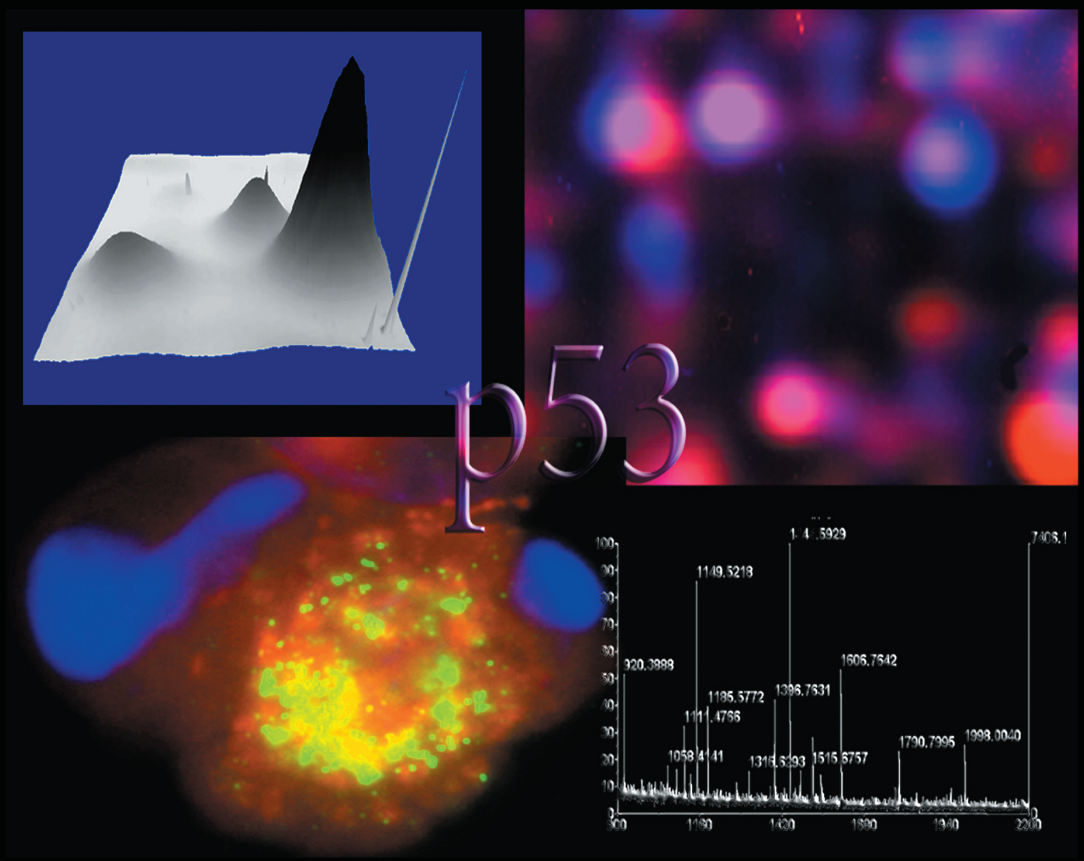
The p53 Pathway: Role of Telomerase and Identification of Novel Targets

Acts of a Master Regulator of Tumor Suppression



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THE P53 PATHWAY: ROLE OF TELOMERASE AND IDENTIFICATION OF NOVEL TARGETS

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av

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To Mom and Dad

If we begin with certainties, we shall end in doubts, but if we begin with doubts, and are patient in them, we shall end in certainties.

Francis Bacon

ABSTRACT

A key role of the p53 protein in tumor suppression is reflected by its frequent mutations in human tumors. p53 is activated by various types of stress conditions such as DNA damage, hypoxia and oncogene activation that results in p53-mediated cellular responses including DNA repair, cell cycle arrest, and apoptosis. p53 is a transcription factor that upon stabilization regulates expression of multiple genes involved in the above cellular responses. Thus, depiction of novel p53 targets is important for a better understanding of its tumor suppressive function. In addition, p53 also induces transcription-independent apoptotic activity.

The catalytic subunit of human telomerase, hTERT involved in telomere length maintenance is repressed in normal cells, but is highly expressed in most human tumors. We have previously shown that hTERT is downregulated upon activation of wtp53 suggesting that p53-mediated repression of hTERT is important for p53-induced apoptosis. We investigated the effects of constitutive expression of hTERT on p53-dependent apoptosis using two different cell systems, the BL41-tsp53 and HCT116 wtp53$^{+/+}$ and wtp53$^{-/-}$. We showed that hTERT expression antagonizes p53-induced apoptosis in both cells. This inhibitory effect was independent of hTERT telomerase activity as expression of catalytically inactive hTERT efficiently blocked apoptosis. To further explore the mechanism of hTERT anti-apoptotic function we studied the mitochondrial pathway and found that hTERT acts upstream of the mitochondria. hTERT expression specifically inhibits activation of Bax. Thus, hTERT exerts its anti-apoptotic function by inhibition of Bax. Our data suggests that p53-dependent downregulation of hTERT is important for p53-induced Bax activation and apoptosis.

Identification of novel p53 targets is important for elucidation of p53 function. Several studies have addressed p53-dependent gene expression by microarray analysis. However, analysis of p53-dependent expression at the protein level can help to identify targets that are regulated by transcription-independent mechanisms. We examined the effects of p53 activation on the proteome using 2D gel electrophoresis analysis of mitomycin C-treated HCT116 colon carcinoma cells and identified 55 novel proteins, differentially expressed in a p53-dependent manner by mass spectrometry. The proteins identified are involved in different cellular processes. Several of them lack putative p53-binding sites and thus are likely to be regulated independently of p53-mediated transcription. This could be due to posttranslational modifications such as phosphorylation of these targets. To further investigate this possibility, we analyzed p53-dependent phosphorylation of proteins using a fluorescent phosphoprotein dye and 2DE and mass spectrometry. Forty-four proteins showed changes in phosphorylation in a p53-dependent manner. This suggests that active p53 not only regulates gene expression but also triggers posttranslational modification of proteins.

This thesis provides two new aspects of p53 function, namely the significance of p53-mediated downregulation of hTERT for induction of apoptosis and regulation of protein expression by different mechanisms that further demonstrate the multiple dimensions of p53's role as tumor suppressor.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their roman numerals (I-IV) throughout the text.

I. **Rahman R**, Latonen L and Wiman KG.
hTERT antagonizes p53-induced apoptosis independently of telomerase activity
Oncogene., (2005), 24, 1320–1327.

II. **Rahman-Roblick R**, Wiman KG and Stridh H.
hTERT antagonizes p53-induced apoptosis through inhibition of Bax activation
Submitted

III. **Rahman-Roblick R**, Roblick UJ, Hellman U, Conrotto P, Liu T, Becker S, Hirschberg D, Jörnvall H, Auer G, and Wiman KG
p53 targets identified by protein expression profiling
PNAS., (2007), 104, 13, 5401–5406.

IV. **Rahman-Roblick R**, Hellman U, Roblick UJ, Becker S, Auer G and Wiman KG
Proteomic identification of p53-dependent protein phosphorylation
Submitted

LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli
APAF-1	Apoptosis protease-activating factor 1
ASPP	Apoptosis stimulating proteins of p53
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia related kinase
Bcl-2	B cell lymphoma 2
bp	Base pair
BRCA-1	Breast cancer antigen gene
CBP	CREB-binding protein
Chk 1 or 2	Checkpoint kinase 1 or 2
CK	Casein kinase
COP1	Constitutively photomorphogenic 1
DNA-PK	DNA-dependent protein kinase
DYRK2	Dual-specificity tyrosine-phosphorylation-regulated kinase 2
E2F1	E2 promoter binding factor 1
FADD	Fas-associated death domain
GADD45	Growth arrest and DNA damage-inducible 45
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HIPK2	Homeodomain-interacting protein kinase 2
HNF-3	Hepatic nuclear factor 3
HSP70	Heat shock protein 70
IGF-BP3	Insuline-like growth factor-binding protein 3
IR	Ionizing radiation
MDM2	Mouse double minute2
NF-Y	Nuclear factor Y
PERP	p53 apoptosis effector related to PMP-22
PKC	Protein kinase C
p53AIP	p53-regulated Apoptosis-Inducing Protein 1
PML	Pro myelocytic leukaemia
PRIMA-1^{MET}	p53-reactivation and inducer of massive apoptosis methylated
Puma	p53 upregulated modulator of apoptosis
RITA	Reactivation of p53 and induction of tumor cell apoptosis
TAF	TBP associated factors
TFIID	Transcription factor IID
TNF	Tumor necrosis factor
Tsp-1	Thromobspondin-1
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TSA	Trichostatin A
USPa	Ubiquitin-specific protease-2a
VEGF	Vascular endothelial growth factor
WT-1	Wilms' tumor suppressor gene
YY1	Yin Yan1
53BP 1 or 2	p53-binding protein 1 or 2

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INTRODUCTION

CANCER

Ever since complex organisms evolved, they have been susceptible to cancer. It has been around for thousands of years, in fact, the oldest description of cancer was documented in an Egyptian papyrus written in between 3000 to 1500 BC and referred to tumors of breast. Cancer is the common term for numerous distinct diseases characterized by uncontrolled growth of abnormal cells as a result of mutations in specific genes.

There are at least 200 different types of cancer, as many as there are cell types in the human body. Cancer or tumor growth kills when it spreads throughout the body preventing the normal function of vital organs. The worldwide burden of cancer is a major health problem, with more than 10 million new cases and 6 million deaths per year, making cancer the second largest cause of death next to heart diseases [1]. However, progress toward better cancer treatments are being made every day much thanks to the past and present years of intense cancer research. Thus with improved knowledge about the underlying mechanisms of tumor progression a cure for cancer is in our grasp within the near future.

TUMORIGENESIS – A MULTI-STEP PROCESS

Tumor progression is a complex process governed by a series of arbitrarily occurring genetic and epigenetic alterations of DNA. Whereas genetic changes include point mutations, deletions, chromosomal translocations and amplifications, the epigenetic alterations include three different types of changes i.e. DNA methylation, histone modifications and changes in genomic imprinting [2]. The genes affected are the ones in charge of maintenance of proper cellular homeostasis. Hundreds of distinct types of cancer and tumor subtypes can be found in specific organs. The vast majority of cancer cells harbor six common properties that are essential for the transformation of normal cell into malignant phenotype [3]. The acquired capabilities: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis mediate the successful breakage of the pre-existing anticancer defense mechanism of the organism. These changes involve activation of oncogenes, inactivation of tumor suppressor genes and DNA repair genes conferring an imbalance between pro- and anti-growth signaling. For example, in human colon cancer - the best studied human cancer - the function of the tumor suppressors APC and p53 is lost whereas the K-ras oncogene is activated by mutations [4]. Characterization of the functional properties of these proteins and their multiple downstream effectors is of utter importance for unveiling the mechanisms of tumor progression and for the discovery of new anti-cancer drugs.

P53

The History

During the 1960s and 70s when studies on tumor viruses and oncogenes was the focus of many cancer researchers, several groups reported about the existence of a cellular protein that was overexpressed in many tumors. This protein, with an approximate molecular weight of 53 kDa was termed p53 (protein 53). In one of the first studies, p53 was identified in complex with the simian virus 40 (SV40) large-T oncoprotein in SV40-transformed cells [5]. Subsequently, p53 was shown to bind several other oncoproteins produced by different tumor viruses, including the human papillomavirus (HPV) E6 protein and the adenovirus E1B 55K protein [6,7]. The ability of p53 in cooperation with ras, a well known oncogene to transform rat embryo fibroblasts further confirmed the "oncogenic" property of p53 [8]. Together these findings led to the conclusion that p53 may be a novel cellular oncogene whose overexpression associated with cellular transformation when coexpressed with viral oncoproteins and mutant Ras.

This story was far from the whole truth. Overexpressed p53 in tumors was later shown to be a result of accumulation of mutant p53 and missense mutations in the p53 gene indeed conferred strong transforming potential [9,10]. Also the originally cloned p53 cDNAs used in the early studies contained dominant negative missense mutations within a region important for the biological activity of wild type (wt) p53 protein. The idea of p53 as an oncogene was in transition and the wt p53 was instead considered a key tumor suppressor. Furthermore, binding of p53 to the viral oncoproteins was shown to cause inactivation or degradation of p53 [11,12]. These oncoproteins through complex formation with p53 abrogated p53 transactivation function, an activity that was later found to be essential for its role in tumor suppression. Other observation that further strengthened this new view of p53 was that certain cellular stresses such as DNA-damage caused by UV-irradiation or chemicals increased the cellular level of wt p53 in non-transformed mouse cells [13]. Such genotoxic agents are known to act in a cytostatic manner, forcing cells to either arrest their progression through cell cycle or trigger activation of cell suicide program.

p53 the Gatekeeper

The most important clue on p53 function as a tumor suppressor emerged from the findings that p53 deficient mice are susceptible to spontaneous tumorigenesis [14]. p53 null mice, although develop normally, show high incidence of sarcomas and lymphomas at an early age. Another crucial piece of evidence came from the observation that patients with the cancer-prone Li-Fraumeni syndrome, an inherited susceptibility disorder carry a germline mutation in the p53 allele [15]. These patients have an increased risk of developing a variety of cancer including soft tissue sarcoma, tumors of breast, bone, brain and bladder [16]. Subsequently, mutations in the p53 locus have been identified in nearly half of all human tumors.

It is well established that the tumor suppressor p53 is a loyal cellular watchman in charge of ensuring the maintenance of the cellular household in order. The level of p53 in normal cells is tightly controlled by its major inhibitor MDM2 that binds and targets p53 for cytoplasmic shuttling and degradation [17]. When the genomic integrity of a cell is threatened by metabolic or genetic disorder such as oncogene activation, DNA-damage, nutrient deprivation and hypoxia, latent p53 is stabilized and transformed into an active form. Upon activation it triggers a wide range of cellular responses depending on the type of cell and stress. These processes include cell cycle arrest, DNA-repair, programmed cell death (apoptosis), differentiation, senescence, inhibition of angiogenesis and metastasis and possibly other unknown cellular responses [18,19] (Fig. 1). Cell cycle arrest and apoptosis are the two most studied p53-mediated cellular responses that reflect the choice of p53 leading to a cell's ultimate fate, life or death.

How does p53 function? The molecular functions of p53 have been a subject of intensive research over the past 25 years. The first activity to be depicted was p53's ability to bind DNA in a sequence specific manner through its central core domain [20]. When fused to a DNA-binding polypeptide GAL4, p53 mediates transcriptional activation function [21]. These groundbreaking findings laid the foundation for the future characterization of the major p53 function, the transcriptional regulation of target genes. Numerous targets of p53 that participate in various cellular processes have been identified and actions of these targets ultimately decide the final destiny of a cell in stress. Recent data suggests that p53 can induce apoptosis directly at the mitochondria and indeed, the tumor suppressive function of p53 also comprises transcriptional independent mechanism which is a growing field of research today.

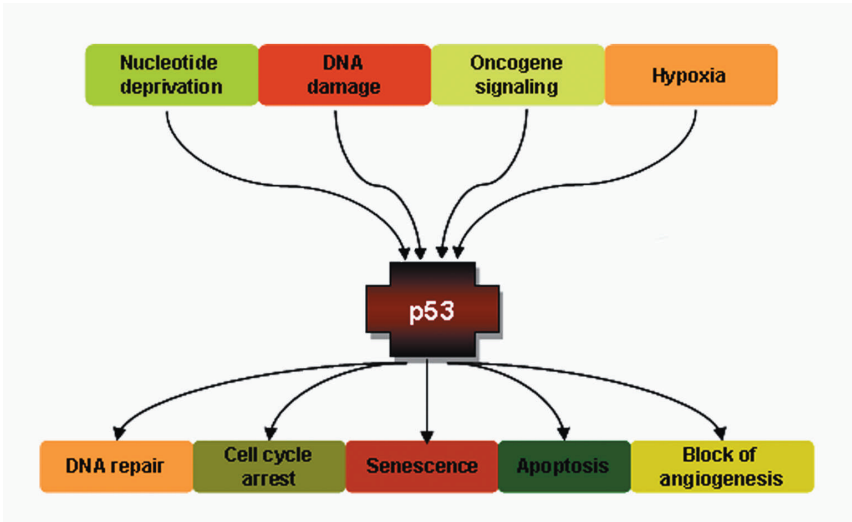

Figure 1. p53 activating signals and its downstream cellular responses.

The protein structure

Localized on the short arm of chromosome 17 (17p13), the p53 gene encodes a protein of 393 amino acids (aa) that is conserved during evolution. The gene consists of 11 exons, where the first exon is non-coding. Amino acid analysis has revealed existence of five highly conserved regions that extends from exon 2 to 8 and that are crucial for p53 function [19,22]. The protein is composed of four functional domains, an N-terminal transactivation domain (TAD), a central DNA-binding core domain (DBD), C-terminal oligomerization domain (OD) and a regulatory domain (RD) as shown in Fig. 2.

Molecular characterization of the p53 functional domains has revealed numerous interaction partners as well as posttranslational regulatory sites. The N-terminal TAD mediates recruitment of the basal transcriptional machinery such as TBP, TAFs and TFIID [23]. Several viral proteins and MDM2 bind to the amino-terminal region to inhibit its transactivation function [24,25]. The N-terminal region also harbors a proline rich domain (PRD) which is required for p53-mediated apoptosis [26]. Further, this domain is implicated in p53-mediated repression of target genes [27].

The central region comprising the sequence-specific DBD includes the four conserved regions II-IV, and it is where the most cancer associated mutations are detected. This part readily recognizes the 10 bp consensus sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' present in two copies separated by 0-13 bp in the regulatory region of the target gene promoters [28]. The central region, according to some studies also functions as a protein binding domain that interacts with 53BP1 and 53BP2 and SV40 large T antigen [29,30]

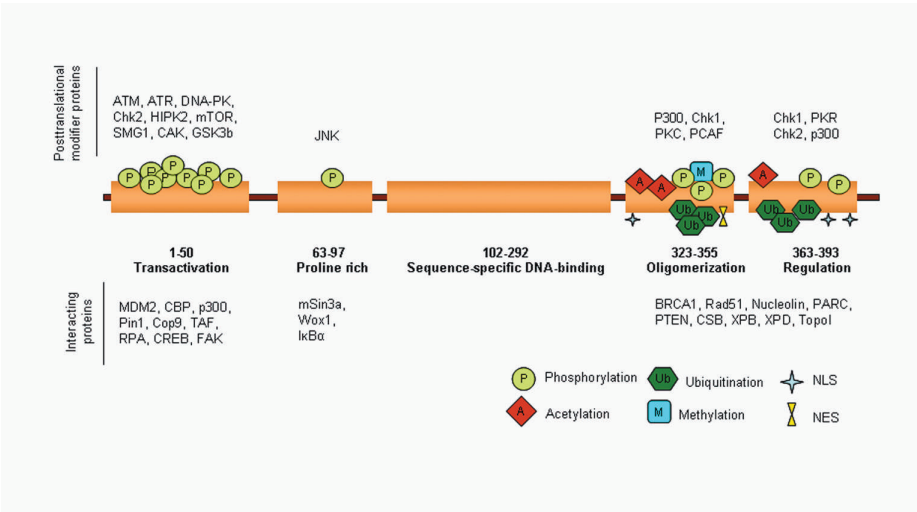

Figure 2. Schematic representation of the p53 protein. Major functional domains and the positions of posttranslational modifications are indicated. In the upper part, proteins that phosphorylate, acetylate, methylate are outlined. Interacting proteins are indicated in the lower part.

Upon stabilization p53 forms highly symmetrical tetramers, dimers of two homodimers through the C-terminal oligomerization domain. Tetramerization of p53 is required for efficient p53-dependent transactivation and p53-mediated growth suppression [31]. Finally, the most extreme C-terminal regulatory domain is a basic region that has been implicated in apoptosis, transcriptional regulation as well as DNA damage recognition [32,33]. In addition, the three nuclear localization signals (NLS) of p53 are located in the C-terminal region and the most N-terminal NLS is required for nuclear localization of p53 [34]. Of note, multiple posttranslational modification sites are located throughout the p53 protein and many of these modifications have been shown to be critical for p53 function (Fig. 2).

p53 Regulation

When activated p53 has the ability to determine between life and death of a cell by inducing cell cycle arrest or apoptosis. Therefore, in normal, undamaged cells it is extremely important to keep it under strict control to avoid unnecessary killing by unleashed p53. Regulation of p53 occurs at both mRNA and protein levels, although most published data deals with protein regulation [35]. Upon cellular stresses p53 undergoes a series of posttranslational modifications depending on the cellular context. These modifications including phosphorylation, acetylation, ubiquitination, glycosylation and sumoylation conduct p53 stabilization as well as its transcriptional activity (Fig. 2).

Stabilization

MDM2, in humans also referred to as HDM2, plays the most central role in p53 regulation. Originally identified as overexpressed in a large proportion of sarcomas, MDM2 was demonstrated to co-purify and interact with p53 *in vitro*. Subsequent analysis showed that MDM2 binds to the N-terminal (aa residues 17-27) part a region containing several phosphorylation sites of p53 and inhibits p53–dependent transcription [36,37]. MDM2 functions as an E3 ligase, the final component of the enzyme cascade that leads to the conjugation of ubiquitin to their substrate proteins and the following degradation by the proteosome. It binds and ubiquitinates p53 already in the nucleus and shuttles it to the cytoplasm where protesome-mediated degradation takes place [38]. MDM2 also contributes to its own degradation as it can auto ubiquitinate itself. The promoter of MDM2 gene carries a p53-binding motif and is transcribed in a p53-dependent manner [39,40]. Thus, high levels of MDM2 by increased p53 activity generate an autoregulatory loop that leads to rapid turnover of the p53 protein, allowing induced p53 to act during a short time window before it is withdrawn to its normal cellular levels [41,42]. The critical role of MDM2 for p53 regulation is further supported by the rescue of the embryonic lethal phenotype of MDM2 null mice through elimination of p53 [43]. Furthermore, two other ubiquitin ligases, COP1 and Pirh-1 independently of each other form autoregulatory loop by promoting p53 degradation [44,45].

Deubiquitination of p53 by the originally identified as p53-associated factor HAUSP has been shown to cause p53 stabilization. A recently published paper showed that USP2a, also a deubiquitinating enzyme unlike HAUSP stabilizes only MDM2 by deubiquitination and consequently promotes p53 degradation [46]. Several proteins cooperate with MDM2 in the regulation of p53. The transcription factor YY1 with a

key role in development has recently been shown to enhance MDM2-mediated p53 degradation by facilitating the interaction between p53 and MDM2 [47,48].

Clearly, inhibition of MDM2 is a prerequisite for p53 stabilization which is accomplished by various independent pathways. Upon DNA damage one of the key p53 stabilization processes involve phosphorylation of p53 by its upstream DNA-damage-induced kinases. The checkpoint kinases Chk1 and Chk2 phosphorylate p53 at several DNA-damage-inducible sites [49]. More specifically phosphorylation of p53 on Ser20 by Chk2 counteracts MDM2-p53 binding to stabilize p53 after DNA damage [50,51]. It was later shown that Chk2 is dispensable for p53-dependent G1 arrest but is required for p53-mediated apoptosis [52]. ATM, a further upstream kinase and a sensor of double-stranded DNA breaks causes phosphorylation of p53 on Ser15 after IR-induced DNA-damage and this phosphorylation although not essential contributes to efficient p53 stabilization and activation [53].

Interruption of MDM2-p53 interaction is also achieved by phosphorylation of MDM2. The non receptor tyrosine kinase, c-Abl positively regulates p53 by targeting MDM2. It phosphorylates MDM2 on Tyr394 *in vivo* and inhibits MDM2-mediated degradation of p53 and thereby promotes p53 accumulation and p53-induced cell death upon DNA damage [54].

Activation

After stabilization of p53, its function as a DNA-binding transcription factor is also regulated by protein interaction and in particular, by its posttranslational modifications including phosphorylation, acetylation and sumoylation.

MDM2 binds in the vicinity of p53 TAD concealing this region from the cellular transcription machinery and inhibits p53-mediated transcription. Similarly the structural relative of MDM2, MDMX, which is not an E3 ligase has been reported to inhibit p53-dependent transctivation through interaction with p53 TAD [57]. Notably, unlike MDM2 null mice that die by p53-dependent apoptosis, the MDMX null mice die by inhibition of cell proliferation also in a p53-dependent manner implicating differential purposes of these two regulators for determining p53-mediated cell fate [58].

The cellular activators of p53, the ASPP family proteins ASPP1 and ASPP2 interact with p53 through its DBD and specifically induce p53-induced apoptosis [59]. In contrast, iASPP, an inhibitor of ASPPs and an oncoprotein upregulated in human breast carcinomas, preferentially binds Pro72 polymorphic p53 and inhibits its proapoptotic function [60].

A recent study showed that the RNA-binding protein, hnRNP K engaged in multiple cellular processes is a target of MDM2 and serves as coactivator of p53. hnRNP K together with p53 is recruited to the promoters of p53-responsive genes in mutually dependent manner and plays a key role in p53-dependent transcription [55,56].
Multiple posttranslational modifications have been shown to enhance p53-mediated DNA-binding and transactivation [61,62]. Phosphorylation of p53 on N-terminal and C-terminal regulatory domains by several kinases has been demonstrated. Besides the pivotal regulators of DNA damage ATM and ATR,

DNA-PK has also been reported to induce phosphorylation on N-terminal residues including on Ser37 which is necessary but not sufficient for p53 DNA-binding and its transcriptional activity [63]. C-terminal phosphorylation of p53 by CDKs, PKC and CKII on Ser315, Ser378, and Ser392 respectively also mediates p53 sequence-specific binding in vitro [64-66]. Phosphorylation of p53 on Ser46 by the autophosphorylating kinase, DYRK2 following severe DNA damage results in apoptosis by p53-mediated transactivation of the pro-apoptotic gene p53AIP [67,68]. Another kinase, HIPK2 also phosphorylates p53 on Ser46 dissociating p53-MDM2 complex and induces p53-mediated transcriptional activity and apoptosis [69]. Inactivation of p53 by inhibition of its phosphorylation has also been demonstrated. Upon expression of the intracellular domain of Notch-1 (Notch1-IC), a transmembrane receptor involved in Notch signaling, it binds to p53 and inhibits its phosphorylation on Ser15, 37 and 46 causing downregulation of p53-targets including p21, MDM2 and Bax [70].

Histone acetyltranferases such as p300/CBP and PCAF mediate acetylation of the C-terminal lysine residues of p53 through interaction with its N-terminal region. DNA damage induced phosphorylation of p53 in the N-terminal region increases its association with p300/CBP that augments p53 acetylation and leads to enhanced p53 activity [71-73]. Of course, MDM2 prevents this interaction between p53 and p300 in the absence of stress [74]. The DNA-damage inducible p33ING2, a candidate tumor suppressor increases p53 acetylation on Lys382 and thus facilitates p53-induced G1/S checkpoint and apoptosis [75,76]. Additionally, PML a protein induced by various stimuli localizes to nuclear bodies together with p53 and CBP where it triggers N-terminal phosphorylation and C-terminal acetylation of p53 to facilitate transcriptional activation of p53 and apoptosis and senescence [77,78].

Other modifications that regulate p53 activity include methylation mediated by methyltransferases such as Set9 that when overexpressed causes hyperstabilization and activation of p53 through lysine methylation on Lys372 and enhances apoptosis [79]. Increased translation of p53 mRNA has also an impact on p53 activity. Recent data suggests that overexpressed ribosomal protein RPL26 interacts of with 5'UTR of p53 and subsequently results in increased G1 arrest [80].

Cellular Localization

Nuclear import and export of p53 is a tightly controlled process. Nuclear localization is required for p53-mediated transcriptional regulation. p53 contains three nuclear localization signals (NLS) that upon stimuli enable its nuclear import whereas nuclear export of p53 is mediated by two nuclear export signals (NES) [81]. However, efficient nuclear export of p53 to the cytoplasm requires the ubiquitin ligase function of MDM2 [82,83]. Mutations of the lysine residues in the C-terminus, where MDM2-mediated ubiquitination of p53 occurs, abrogate MDM2-directed nuclear export [84-86]. This is possibly due to the exposure or activation of the nuclear export sequence of p53 caused by MDM2-mediated ubiquitination which affects p53 oligomerization state [87]. Nuclear export of p53 is necessary for efficient p53 degradation [88].

Loss of p53 function in some tumor types such as in neuroblastomas carriyng wt p53 is coupled with its failure to accumulate in the nucleus. The observed nuclear exclusion may be an effect of hyperactive MDM2 or the activity of glucocorticoid receptors (GR) [89,90]. The latter involves complex formation

between p53 and GR, resulting in cytoplasmic sequestration of both p53 and GR. Dissociation of this complex by GR antagonists, results in accumulation of p53 in the nucleus, activation of p53-responsive genes, growth arrest and apoptosis. Other proteins that directly or indirectly effect p53 nuclear import/export are importin-α, PI3/Akt, p14ARF, Pacr, actin, vimentin and mot2 [81].

p53 in cancer – loss and consequences

The p53 function is disrupted by mutations, in about 50% of all sporadic human tumors and in the other half the p53 protein is inactivated by various cellular and viral antagonists, including MDM2 [91,92]. In fact, p53 mutation is the most frequent genetic event found in a broad range of human tumors to date including cancers of the ovary (48%), stomach (45%), colon (43%), esophagus (43%), lung (38%), and breast (25%). More information on p53 mutation database can be found at http://www-p53.iarc.fr/P53main.html or http://p53.free.fr/.

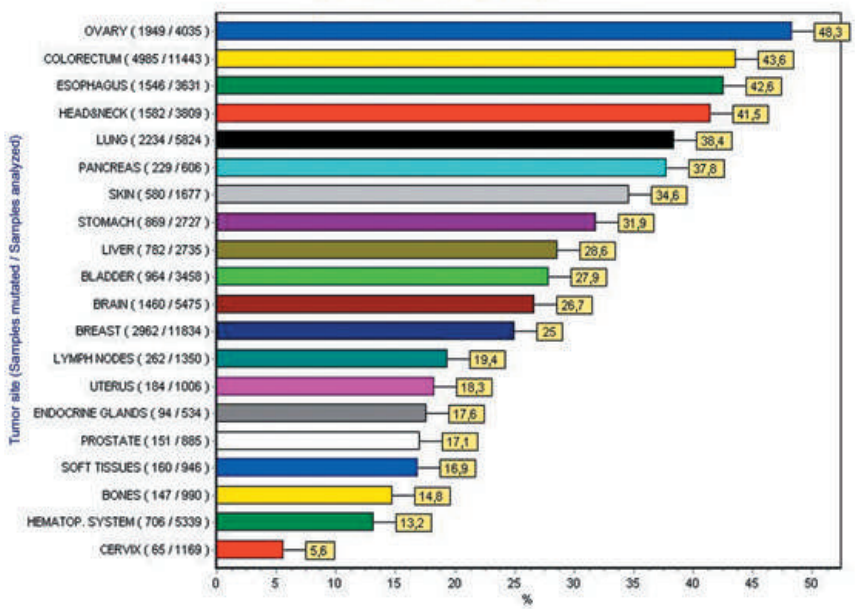

Figure 3. Frequency of mutant p53 allelels in human tumor cell genomes as recorded in the IARC database, 2006. (http://www-p53.iarc.fr/P53main.html).

Unlike other tumor suppressor genes that are inactivated by truncation or deletion of their gene more than 80% of p53 alterations are caused by missense point mutations that result in full length stable p53 levels [93]. Therefore, high expression of mutant p53 is often detected in human tumors. In tumors, p53

mutations are distributed in all coding exons, however with a strong predominance in exons 5-8, encoding the DNA-binding domain. Mutation of p53 typically occurs in one allele whereas the other one is often lost by loss of heterozygosity (LOH). Some mutants exhibit dominant-negative activity that disrupts the p53 function produced by the wt allele through hetero-oligomerization of mutant p53 with the wt p53. Other evidence suggests that some mutants possess oncogenic activity by gain-of-function mechanism [94-96]. Mutant p53 is capable of activating alternate subset of promoters such as of c-myc oncogene and MDR1induction of these genes facilitates cell proliferation and multiple drug resistance respectively [97,98]. Mutant p53 also interacts with transcriptional co-activators including p300 and CBP to promote deregulated gene expression. Thus, human tumors favor selection for accumulation of p53 mutations bestowing growth advantage properties by mutant p53 to endorse tumor progression.

Most of the point mutations (90%) occur in the hot spot region (aa residues 110-290), targeting the sequence-specific DNA-binding core domain and thereby rendering p53 transcriptionally inactive [99]. About 30% of them strike 6 hotspot codons, R175, R245, R248, R249, R273 and R282. The p53 mutations can be divided into two groups. Group I mutations e.g. codon R248, the most frequently occurring mutation in tumors, affect DNA-binding of p53 but generate a protein with wt conformation [100]. The second group of mutations e.g. codon R175 produces a protein with altered conformation conferring more severe phenotype in vitro [91]. Of note, a fraction of the mutations is located outside the DNA-binding domain, including in the transacivation and oligomerization domain.

What are the clinical consequences of these different mutations? According to several reports, specific p53 mutations are associated with poor prognosis or weak response to treatment. For instance, colon cancer patients with p53 mutations in codon 175 have shorter survival compared to patients with other mutations [101]. Likewise breast cancer patients with DNA-contact mutation also have poor prognosis as compared with patients with structural mutations [102]. Polymorphism in codon 72 resulting in the expression of two different p53 proteins, either Arginine or Proline in this codon has been identified [103]. The Arg72 form of p53 found to be more efficient than the Pro72 form at inducing apoptosis is more sensitive to degradation meditated by HPV E6 protein that favors the progression of HPV-associated tumors e.g. cervical cancer [104,105]. In addition, mice with missense inherited p53 mutation exhibit high metastatic potential as compared to the p53 null mice [106].

As mentioned earlier in half of the tumors p53 inactivation occurs via non-mutational mechanism (i.e. in the p53 gene). Instead, the p53 pathway is disrupted either through inactivation of p53 protein by its antagonizers or via disruption of its upstream regulators. Overexpression of the p53 negative regulator MDM2, through gene amplification is a common strategy that appears in a variety of tumors. Deregulated expression of MDM2 leads to continuous degradation of p53 rendering the cell with p53 null condition. Disruption of the p53-MDM2 complex is the critical step for activation of p53 during stress. The upstream modulators executing this process are often inactivated in many tumors. Mutation in the p53 upstream kinase such as the DNA-damage-induced protein kinase, ATM in the human disease ataxia-telangiectasia (AT), renders p53 unphosphorylated at Ser15, Ser20 and MDM2 at Ser395 and thus abrogating IR-induced p53 activation [107]. The AT-patients show multiple abnormalities including increased risk for lymphomas. Chk2, another DNA-damage-checkpoint kinase and a major effector of ATM also phosphorylates p53 at Ser20 upon γ-irradiation and enhances apoptotic response

[50]. Chk2 heterozygous germline mutation has been identified in Li-Fraumeni-like syndrome patients that lack p53 mutations [108]. In addition, the tumor suppressor, ARF can bind directly to MDM2 and free p53 from MDM2-mediated degradation upon mitogenic signals such as oncogene activation [109]. Inactivation of ARF frequently occurs in human tumors and as a consequence leads to inhibition of p53 induction upon mitogenic insult [110].

The Family

Nearly after two decades of undivided attention p53 finally had to step down from the spotlight. It appears that the one-of-a-kind tumor suppressor has a family of two additional members, named p63 and p73. Although identified almost 20 years after the discovery of p53, evidence suggests that the two members are in fact ancestors of p53 with original function in development, and that p53 evolved later by gene duplication and mutation at a time when tissue renewal and thus risk of cancer was deployed in higher organisms [111]. Furthermore, mutations in *p63* and *p73* are rarely found in cancer with the exception of *p73* LOH in some tumors [112]. The *p63* gene is in fact amplified in squamous cells of lung and cervical carcinomas [113]. The gene structure of *p53* and its cousins is highly conserved from fly to man and all three exhibit strong homology in their overall domain structure and conformation. However, despite their striking similarity the older relatives seem to possess functions in developmental processes including stem cell biology and neurogenesis in addition to their role in tumorigenesis [114]. Unlike p53 that has a single promoter, both *p63* and *p73* contain two promoters and as a consequence two classes of proteins, one containing the N-terminal transactivation domain (TAp63, TAp73) and the other lacking it (ΔNp63, ΔNp73), are encoded. This scenario is further complicated by alternative exon splicing and C-terminal exon splicing of both transcripts.

To date, at least six transcripts of both *p63* and *p73* have been depicted and the role of the multiple transcripts is a subject of intensive research. Cellular assays have shown that the isoforms of p63 and p73 containing the TAD are capable of transactivation of certain p53 target genes and induction of cell death [115,116]. Among the classical p53 targets *p21*, *Bax*, *GADD45* and *MDM2* have been shown to be regulated by TAp63/p73 [117,118]. In addition, an essential cooperative function of the p53 family members for induction of apoptosis-related genes e.g. *Bax*, *Noxa* and *PERP* has been demonstrated in double knockout mouse embryonic fibroblasts. In the absence of p63 or p73, p53 cannot bind to these promoters, and is thereby unable to induce apoptosis upon DNA-damage [119]. In contrast, induction of the cell cycle mediator, p21 occurs normally by p53 alone. The ΔNp63/p73 proteins however not only lack p53-like functions, but can also act as dominant negative regulators of p53. Several observations suggest that the ΔNp63/p73 proteins compete with p53 and TAp63/p73 for target gene DNA-binding through interaction with their DBD and thus disrupt apoptotic response [116]. In addition, p53 targets ΔNp63 for protein degradation and both the ΔN promoters contain potential p53-binding motifs [120].

p53 family and diseases

It is well known p63 and p73 are critical for proper development whereas p53 is necessary for genomic integrity. Although the p53 null mice show high rates of specific tumors, they develop normally suggesting that p53 is dispensable for development. In contrast, p63 plays a fundamental role in the development of the epidermis. The p63 null mice exhibit severe defects including defects in formation of limb, absence of skin and craniofacial malformations, and die within five days after birth [121,122]. Heterozygous germ line mutations in humans cause six rare autosomal dominant developmental disorders reminiscent of the mouse knockout phenotype [123]. On the other hand, p73 expression is necessary for neurogenesis. The p73 null mice show hippocampal dysgenesis and other malformations

but no increased susceptibility to tumorigenesis [124]. Nevertheless, mice heterozygous for p63 or p73 together with one p53-deficient allele show a shift in tumor spectra with an increased tumor burden and risk for metastases [125]. One possible reason for this difference in tumor spectrum is the tissue specific expression of p63 and p73. Notably, several tumor-derived p53 mutants interact with wt p63 and p73 and prevent their transcriptional activity, a possible explanation for the rare events of p63 and p73 mutations in cancer [126]. These data clearly suggest that the p53 siblings, although do not fall in the category of classical tumor suppressor, do have tumor suppressive properties.

Choice of Response – Life or Death

When a cell faces cancer threats p53 gets activated to elicit a response that either repairs the damage and keeps the cell halted but alive or eliminates the damaged cell from the system. An obvious question is how p53 makes the decision between life and death. This choice is primarily dependent on the balance between the availability of cellular factors as well as the extracellular signaling events. Firstly, the type of stress versus the extent of damage that triggers p53 is an important factor since the damage caused by it dictates whether or not it is worth repairing the injury. If the damage is too extensive apoptotic response might be the preferred choice. In addition, the survival signals such as high levels of secreted growth factors, cell-matrix interactions conferring anti-apoptotic features render cells resistant to apoptotsis. In these cells p53 activation may facilitate transcient or irreversible termination of cell cycle. Secondly, the intracellular molecular events, that in various ways affect p53 and thus also determine the outcome of its effects. This includes the availability and activity of p53 regulators, positive and negative. The genetic alterations, characteristics of cancer cells are potential modulators of p53-mediated responses. Hence, the specific genotype of a cancer cell governs the biological consequence of p53 activation [127].

p53 is primarily a sequence-specific transcription factor and the proteins encoded by its target genes contribute to the biological outcome of p53 induction. However, transcription independent function of p53 also has substantial impact on its effects. The choice between life and death will ultimately depend on its ability to preferentially switch on or off particular subsets of genes, i.e. cell cycle inhibitory genes or pro-apoptotic genes. The first studies separating the two functions of p53 i.e. activation of cell cycle arrest genes and pro-apoptotic genes came from the analysis of tumor-derived p53 mutants capable of activation of p21 promoter and G1 arrest but not of Bax or IGF-BP3 [128,129]. These apoptosis deficient mutants with slight conformational changes can interact with high affinity binding sites but are unable to bind lower affinity motifs present in pro-apoptosis genes. This model has been supported by others confirming the presence of high or low affinity binding sites in different p53 target gene promoters [130,131]. Furthermore, earlier work demonstrated differential binding affinity of p53 by the observation that low levels of p53 induces cell cycle arrest but higher levels or DNA-damage-induced p53 triggers apoptosis [132].

Many other molecular mechanisms participate in p53 target gene selectivity. Posttranslational modification of p53 has been shown to affect promoter selectivity by p53. The selective DNA binding caused by covalent modifications is possibly a result of changes in p53 conformation altering its DNA binding specificity. Recent data suggests that the E2F1 transcription factor, in the absence of mouse p19ARF, can induce phosphorylation of p53 at multiple sites with striking similarity as observed upon DNA damage. These p53 modifications were shown to be crucial for E2F1-mediated apoptosis [133]. An example of a modifier that favors p53-mediated apoptosis without inducing covalent modifications of p53 is the p300 coactivator JMY, which during stress interacts with p300 and is recruited to activated p53 to conduct transactivation of Bax and induction of apoptosis [134].

Although most work has identified pro-apoptotic modulators of p53, factors that direct p53 selectivity to growth inhibitory, DNA repair or anti-apoptotic genes also have been identified. The WT-1 tumor suppressor has been shown to stabilize and lead p53 towards cell cycle arrest but inhibit p53-mediated apoptosis [135]. Likewise, the BRCA1 tumor suppressor can shift p53-mediated transcriptional response en route for cell cycle arrest and DNA repair. In contrast upon DNA-damage p53 can switch to apoptotic response by downregulating BRCA1 [136,137].

The corepressor mSin3a and histone deacetylases play an important role in repression of several p53-downregulated targets and p53-mSin3a-mediated transrepression has been shown to trigger apoptosis [138,139]. Presence of mSin3a in cells overexpressing p53-repressed proteins can facilitate the apoptotic response by assisting p53 to downregulate these targets. In addition, binding of MDM2 to the N-terminal region of p53 also inhibits p53-regulated repression possibly through masking the interacting region in p53 and making it inaccessible for the repression cofactors. The RB tumor suppressor can enter the MDM2-p53 complex and prevent p53 degradation. Formation of this trimeric complex contributes to regained repression function by p53 and increased apoptosis but no effect on its transcriptional activation function [140] (Fig. 4).

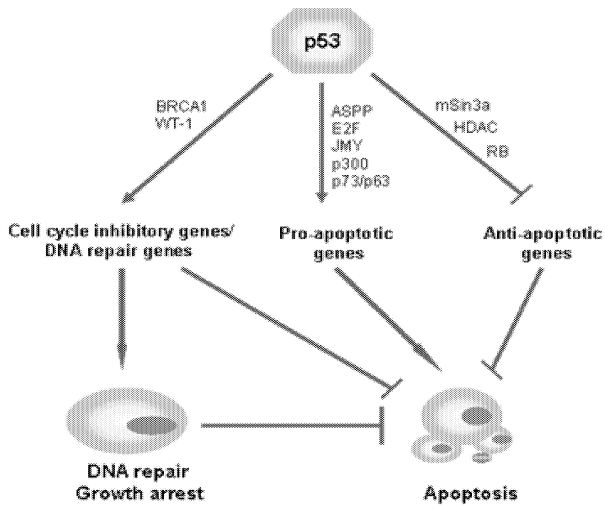

Figure 4. Decision making by p53. The coactivator and corepressor proteins as well as posttranslational modifications of p53 dictate the choice between life and death decision by p53.

Evidence suggests that ER (endoplasmic reticulum) stress caused by protein misfolding can induce p53-mediated cell cycle arrest involving MDM2. ER stress enhances interaction between MDM2 and ribosomal proteins inhibiting MDM2-mediated ubiquitination and degradation of p53 [141]. The role of p21 seems to be important for p53-induced cell cycle arrest by ER.

The p53 family members, p63 and p73 are also contributors to the choice of apoptotic response. p53 needs at least one of its relatives for activation of apoptotic genes upon DNA damage [119]. In the p63/p73 double knockouts, p53 fails to bind the promoters of specific apoptotic genes. The significance of this finding is currently unknown as wt p53 does not interact with p63 or p73 suggesting an indirect mechanism of this cooperative action.

p53 functions

p53 exerts its various cellular functions by transcriptional-dependent and independent pathways. The transactivating function was established by the discovery that p53 interacts with specific double stranded DNA sequences. Subsequent analysis revealed the consensus sequence and its presence in many p53 activated gene promoters [28]. However, transrepression by p53 relies on interactions with other DNA-binding activators, corepressors or with the basal transcriptional machinery since many of the repressed genes are devoid of recognizable p53-binding sites. Function of multiple p53 targets has been analyzed and linked to diverse cellular processes including cell cycle arrest, apoptosis, DNA repair, senescence, angiogenesis and metastasis. Information on cell cycle arrest and in particular, apoptosis by p53 nonetheless beat the other events by many publications which is not surprising since p53-induced apoptosis is crucial for tumor suppression. However, knowing the complexity of cellular mechanisms it is logical to assume that some of the other events may ultimately diverge by one or the other way into the apoptotic response.

Cell Cycle Arrest

Cell cycle is a complex process and is tightly regulated by multiple actors with cyclins and their corresponding cyclin dependent kinases (CDKs) and the CDK inhibitors being the core components of this process. Successful completion of cell cycle is monitored and controlled by cell cycle checkpoints that block progression when flaw in terms of damaged DNA is detected. p53 transactivates components of both G1/S and G2/M checkpoints upon physiological stress.

p21 is the most famous p53-induced cell cycle inhibitory gene that can induce both G1 and G2 arrest by inhibiting cyclinE/CDK2 and cyclinB/Cdc2 respectively [142-144]. Cdc2 (also known as CDK1) required for entrance to mitosis is downregulated at RNA and protein level in a p53-dependent manner after IR and involves p21 for this negative regulation [145]. Furthermore, p21 is also involved in activation of the RB tumor suppressor pathway. RB is inactivated through hyperphosphorylation mediated by cyclin/CDKs during active cell cycle process. Upon induction, p21 inhibits cyclinE/CDK2 causing hypophosphorylation of RB that binds E2F to inhibit E2F-mediated transcription of cell cycle genes. Hence, p21 integrates the two tumor suppressor pathways following cellular stress [146]. Others have demonstrated that activation of p21 protects cells from Fas-mediated apoptosis by direct binding and inhibition of pro-caspase-3 (one of the downstream effectors of apoptosis signaling). Protein kinaseA-mediated phosphorylation of p21 is essential for this complex formation that takes place at the mitochondria [147]. This could be an additional strategy for cells to avoid cell death.

Another p53-induced target 14-3-3σ (sigma) belongs to a family of proteins that regulate cellular activity by binding and sequestering phosphorylated proteins. Induced 14-3-3σ inactivates Cdc25 and Cdc2 by sequestering them to the cytoplasm to trigger pre-mitotic G2/M block upon DNA damage [148,149]. Furthermore, 14-3-3σ has been shown to promote translocation of pro-apoptotic Bax out of the cytoplasm and thereby delay apoptotic signal and enforce cells into G2 arrest [150].

Apoptosis

The first proof of p53 function in apoptosis comes from studies of temperature sensitive mutant p53 that acquires the conformation of wt p53 when the cells are grown at a permissive temperature of 32°C. The observed rapid cell death upon temperature shift to 32°C implicated a role of p53 in cell death [151]. p53-mediated tumor suppression is primarily dependent on its apoptotic function. This notion is based on several mouse model studies. In one such model where p53-dependent growth arrest is defective due to expression of SV40 large T-antigen whereas the apoptotic pathway is intact, the mice develop slow growing tumors. In contrast crossbreeding of these mice on p53 null background generates mice with aggressive tumor growth [152]. Another study showed that in myc-driven lymphoma, block of p53-mediated apoptosis does not confer any selection for inactivating p53 mutations unlike their apoptosis proficient counterparts and that no cell cycle defects could be detected in the apoptosis defective cells [153]. Hence, the function of p53 as an inducer of apoptotic cell death is the most significant one. The relevance of p53-dependent transactivation has been debated. According to some analyses, p53 DNA binding is crucial for tumor suppression, whereas other studies have shown that transactivation mutant p53 can still or even more potently induce apoptosis and therefore p53-mediated transactivation is dispensable for this function [154,155]. Many transcriptional dependent and independent p53 targets have been implicated in p53-mediated apoptosis. However, only a few seem to be essential for induction cell death. Members of both the extrinsic and intrinsic apoptotic club have been identified. Although initiated by different actors both lead to caspase-mediated apoptosis.

The extrinsic pathway

In this pathway the initiators are specific transmembrane death receptors and their respective ligands. Formation of so called death-receptor-inducing-signaling-complex (DISC) including receptor, ligand, the adapter molecule FADD and caspase 8, triggers a chain of events resulting in activation of effector caspases. The cell surface receptor, Fas or CD95 is a key inducer of this pathway that is activated through binding to its ligand, FasL. *Fas* is a p53 target that is upregulated transcriptionally upon chemically induced DNA damage [156]. Induction by γ-irradiation is however tissue-specific and occurs in lung, thymus, spleen and lung but not in heart or liver [157]. The death-domain-containing receptor DR5/KILLER of the TRAIL family is also a target of p53 that upon DNA damage can induce apoptosis specifically in some tissues [158,159]. A third transmembrane protein that is induced by p53 in response to DNA damage is PERP that probably in collaboration with E2F1 induces apoptosis [160]. Furthermore, caspase-8, an initiator caspase of the death receptor pathway is activated by p53 independent of transcription in nucleus-depleted cell-free system in response to γ-irradiation [161].

The intrinsic pathway

This pathway is regulated by the pro- and anti-apoptotic Bcl-2 family proteins and involves mitochondrial depolarization, cytochrome c release from the mitochondrial intermembrane into the cytoplasm, formation of apoptosome and the subsequent activation of caspases [162]. The Bcl-2 family proteins consist of three subclasses: the pro-survival proteins with strong homology to Bcl-2, e.g. $BclX_L$; the pro-apoptotic proteins Bax and Bak with structural similarity to Bcl-2 and the 'BH3-only' pro-apoptotic proteins e.g. PUMA. BH3 domain is the domain required for the pro-apoptotic activity and heterodimerization and is present in all members [163,164].

Several members from all three groups are targets of p53. *Bax* is one of the first genes of the Bcl-2 family identified, whose expression was shown to be activated by p53 in response to cellular stress [165]. Upon induction Bax undergoes a conformational change, forms homodimer and translocates to the mitochondrial membrane where it promotes cytochrome c release leading to activation of caspase-9 [166]. Bax-deficient mice exhibit accelerated tumor growth and substantial decrease in p53-mediated apoptosis [167]. Accordingly, Bax can also mediate p53-independent tumor suppression [168].

The BH3-only protein PUMA, a powerful mediator of cell death is upregulated by p53 in response to various types of stress [169,170]. PUMA requires Bax for induction of apoptosis and has been demonstrated to promote translocation and oligomerization of Bax resulting in effective cell death while bax deficient cells are resistant to PUMA-mediated apoptosis [171]. Recent report suggests that PUMA upon induction can couple the nuclear and cytoplasmic apoptotic function of p53 by liberating p53 from the anti-apoptotic $BclX_L$ in the cytoplasm whereupon freed p53 localizes to the mitochondria to directly induce apoptosis in a transcription-independent manner [172]. This mitochondrial apoptotic function of p53 is further described below. Other p53-upregulated pro-apoptotic targets involved in the intrinsic pathway are the BH3-only protein, Noxa, Bid that via direct interaction triggers functional activation of Bax, and APAF-1 required for apoptosome formation [173-176]. Interestingly, Bid has been reported to be the link between the extrinsic and intrinsic pathway since caspase-8 involved in the death receptor signaling can induce Bid cleavage and the resulting truncated Bid induces Bax activation [177](Fig. 5). Hence, by inducing transcription of Bid, p53 can promote convergence of the intrinsic and extrinsic pathways. Moreover, p53 induces caspase-6 expression via a response element within the third intron of the *caspase-6* gene that plays an important role in p53-induced neuronal cell death [178,179].

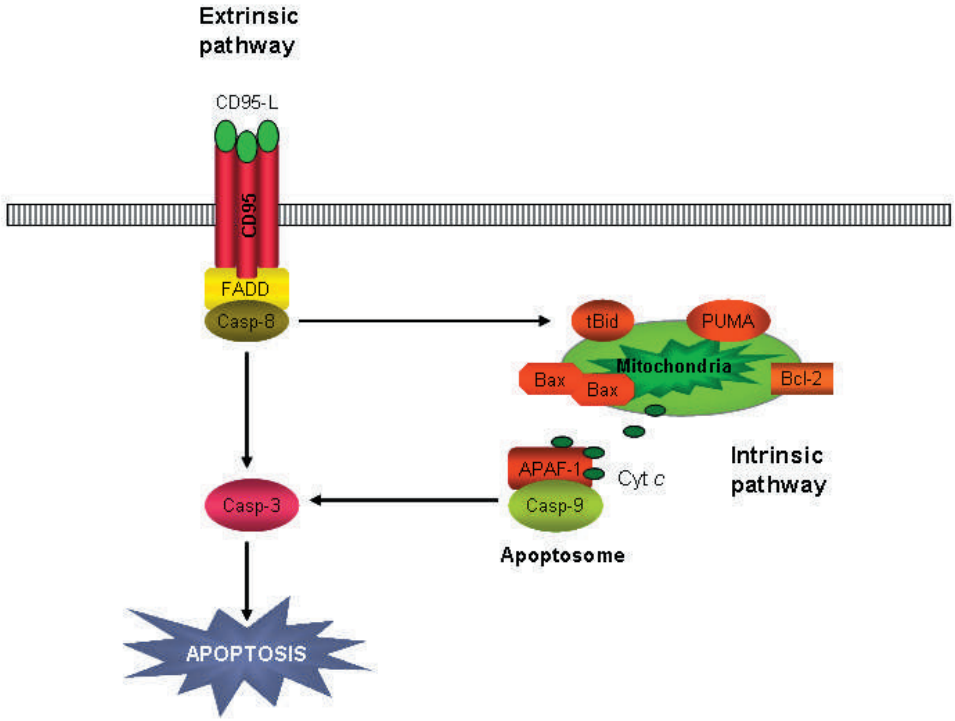

Figure 5. Model of the extrinsic and intrinsic apoptotic pathways. The p53 targets are shown in red.

The anti-apoptotic member of this pathway, Bcl-2 is also a transcriptional target of p53, a target that is repressed upon p53 activation. Bcl-2 functions as an oncogene when overexpressed in tumors and has metastatic potential in mice [180,181]. Moreover, the data showing that Bcl-2 overexpression counteracts p53-dependent transrepression and apoptotic response but not p53-mediated transactivation, is one of the first studies emphasizing the role of p53-dependent transrepression in apoptotic response [182].

Senescence

Cellular senescence is a program that is triggered by normal cells in response to different types of stress such as telomere shortening, oxidative stress and DNA damage. Upon entering senescence, cells cease to divide and undergo a number of morphological and metabolic changes [183]. Although detailed description of its *in vivo* manifestation is lacking, cellular senescence is considered to have a key role in tumor suppression and organismal aging. Both p53 and RB pathways are active during senescence and inactivation of p53 in MEFs is sufficient to prevent senescence [184]. p53-induced p21 seems to be important for activation of the RB pathway and for triggering cellular senescence in response to DNA damage and telomere uncapping [185]. Telomere shortening can be prevented by activation or overexpression of the human telomerase catalytic subunit, hTERT that facilitates tumorigenesis.

overexpression of the human telomerase catalytic subunit, hTERT that facilitates tumorigenesis. Interestingly, hTERT is downregulated by p53 and overexpressed hTERT exhibits anti-apoptotic activity [186](see below). This suggests that the two anti-tumor events senescence and apoposis are linked via p53 and p53-regulated proteins. Moreover, recent evidence suggest that restoration of p53 function in sarcomas leads to tumor regression via cell cycle arrest with features of cellular senescence [187] .

DNA repair

Damage to DNA triggers signaling that involves activation of multiple factors that act in parallel to repair the damage. Involvement of p53 in both base excision repair (BER) and nucleotide excision repair (NER) has been demonstrated. p53 enhances NER mainly by induction of target genes such as *GADD45*, a gene induced by IR in cells with wt p53 cells [188]. Regulation of the BER by p53 is on the other hand, correlated with its ability to directly associate with AP endonuclease (APE) and DNA polymerase β (DNA pol β). The interaction between DNA pol β and the abasic DNA sites is further stabilized by p53 [189]. Moreover, following γ-irradiation p53 induction is accompanied by increased activity of the 3-methyladenine DNA glycosylase (3-MeAde), the first enzyme acting in the BER pathway [190].

Inhibition of angiogenesis and metastasis

The switch to angiogenic phenotype i.e. ability to form new blood vessels is a requirement for tumors to expand and metastasize. p53 inhibits this process through several factors. Angiogenesis is triggered by hypoxic conditions that activates HIF-1 transcription factor which in turn induces expression of its major target VEGF, the most potent endothelial mitogen that directly participates in angiogenesis [191]. The alpha subunit of HIF-1 (HIF-α) that is upregulated by hypoxia is targeted for MDM2-dependent proteosomal degradation mediated by p53 [192]. Moreover, p53 has been shown to downregulate expression of *VEGF* and upregulate the anti-angiogenic *Tsp-1* suppressed in a variety of tumors [193,194]. p53 also positively regulates the expression of the *Nm23-H1* metastasis suppressor gene to decrease the invasive potential of hepatocellular carcinoma cells [195]. Furthermore, cell invasiveness is controlled largely by the matrix metalloproteinases (MMPs) that promote degradation of extra cellular matrix. Both MMP-1 and MMP-13 are transcriptionally repressed by activated p53 [196,197].

RNA processing

p53 has been shown to induce the growth inhibitory zinc finger protein, Wig-1 that preferentially binds double stranded (ds) RNA with structural similarity to siRNA and miRNA [198,199]. Although the biological relevance of the RNA-binding property of Wig-1 is unknown a function in ribosome biogenesis and posttranslational RNA interference has been proposed. Double stranded RNA-binding proteins (dsRBPs) are involved in various cellular processes including cell growth control, gene expression and RNA localization and knockout of several dsRBPs in mice has generated embryonic lethal phenotype [200,201].

p53-mediated transrepression

The role of p53 as a transcription factor comprises transrepression of subsets of genes involved in various cellular functions. In fact, a global transcription analysis of p53-regulated genes in ovarian cancer cells revealed that a large number of the p53-regulated genes are repressed via p53-DNA-binding upon p53 expression [202]. Several p53-repressed genes have been identified. Repression of these genes is important for various p53-mediated cellular processes. G2 arrest does not only require upregulation of p21 and 14-3-3σ but also rely on p53-dependent repression of cyclinB and cdc2. Likewise p53 is thought to prevent multi drug resistance by inhibition MDR1 expression. Overexpression of *MDR1* encoding the transmembrane glycophosphoprotein, P-glycoprotein mediates resistance to chemotherapeutic agents and other apoptotic inducers [203]. p53-dependent repression of specific targets has been mostly associated to apoptotic response.

In addition to previously mentioned Bcl-2, the insuline like growth factor 1 receptor (IGF-1R), a survival factor highly expressed in tumors is repressed upon induction of p53 [204]. Similarly, the putative caspase inhibitor, Survivin is also a target for p53-mediated downregulation [205]. Elevated expression of Survivin is found in multiple tumor types including gastric carcinoma and neuroblastoma and is correlated with poor prognosis. Overexpression of Survivin inhibits p53-induced apoptosis. Other genes negatively regulated by p53 are for example *Map4*, *stathmin*, *VEGF*, *PTGFβ*, *WT-1*, *hTERT* and the list keeps growing. Anti-apoptotic activity is the common trait of most of these p53-regulated targets.

Mechanisms of repression

In contrast to p53-activated genes that almost exclusively require p53 binding to their consensus sequence, negative regulation by p53 can be mediated by different mechanisms [206]. Moreover, binding of p53 to novel DNA sequences in some repressed promoter has been shown [207]. Downregulation by p53 probably occurs by any of the three mechanisms that transcriptional repressors are thought to function through.

The first comprises interference with other activators at the target promoter. In this model, transcriptional repression by p53 falls into two categories. In the first category, downregulation takes place through p53 binding to the consensus DNA elements. For instance, repression of the alpha-fetoprotein gene (AFP) mediated by p53 results in inhibition of the activating transcription factor HNF-3. Competitive binding to the AFP promoter containing overlapping binding sites for p53 and HNF-3 results in displacement of HNF-3 and net repression by p53 [208]. However, repression via noncompetitive binding, where p53 interferes with the function of an activator, but not its DNA binding has been shown for repression of Bcl-2 [209]. In the second category, repression occurs in the absence of apparent p53 binding to the consensus sites. Downregulation of hTERT occurs in this manner by physical interaction of p53 with in this case with the coactivator Sp1 and consequent inhibition of Sp1 without p53 DNA binding, resulting in hTERT downregulation [186]. Recent analysis

using ChiP on ChiP assay revealed direct binding of p53 to the DNA-binding NF-Y complex for repression of target genes [210].

The second mechanism of repression involves direct interference with the basal transcriptional machinery. p53 represses some promoters without affecting specific activators or their binding to DNA. Repression of cyclin B is not affected by progressive deletions of the promoter or by mutations in the binding sites of other regulators e.g. Sp1. p53 acts through the basal promoter to inhibit transcription. Wild type p53 has been shown to interact with TBP and certain TAFs and disrupt pre-initiation complex assembly [211-213].

The third mechanism confers alteration of the chromatin structure by recruitment and association of histone deactylases (HDAC) and the corepressor mSin3a with p53 in the target promoters. Inhibition of HDAC by the HDAC inhibitor, TSA abrogates p53-mediated repression of *Map4* and histone acetylation is decreased in the *Survivin* promoter in the presence of p53 [138,205]. Moreover, in response to hypoxia that has been shown to preferentially induce p53-dependent transrepression, interaction between p53 and mSin3a is promoted [214]. Thus, association of p53 and mSin3a is considered to be the basis for downregulation of a subset of p53-repressed genes.

Both the N-terminus and C-terminus regions as well as the PRD play important role in p53-mediated transrepression. Mutation in the N-terminal Ser25/Arg26 renders p53 unable to repress Map4 [138]. Abrogation of the Ser386 phosphorylation in the C-terminus diminishes p53-mediated repression of SV40 early promoters and c-fos without affecting p53-mediated activation [66]. Deletion of the PRD impairs p53 transrepression function and is required for p53 and mSin3a interaction [27,139]. The different regions are likely to be required for repression of different targets owing to the distinct mechanisms that p53 acts through as a repressor. Although the physiological significance of p53-mediated transrepression is still unclear, this activity of p53 is important for induction of apoptosis during specific cellular stress conditions such as hypoxia.

Transcription-independent function

Despite the fact that the role of p53 as a transcription factor is the most studied function, compelling evidence suggests that p53 has additional transcription-independent (TI) pro-apoptotic function. This non-transcriptional activity of p53 is a subject of active debate and increasing attention [215]. The first study that demonstrated a potential TI function of p53 comes from analysis of the temperature sensitive p53 mutant (p53Val135) that upon shift to a permissive temperature induces p53-mediated apoptosis by upregulation of p53-targets. However, inhibition of transcription or translation in these cells using drugs that blocks these processes did not prevent apoptosis [216]. Subsequently, the TI pro-apoptotic function of p53 has been confirmed by others [154,217]. Recent reports have described at least some of the mechanisms of this particular function involving the intrinsic apoptotic pathway.

Distinct pools of p53 protein exist in different intracellular locations. In response to a wide range of apoptotic stimuli a fraction of p53 can rapidly translocate into the mitochondrial outer membrane triggering cytochome *c* release and caspase-3 activation [218]. Closer look into this matter revealed that p53 physically interacts with certain Bcl-2 family members including Bcl-2 and Bcl-X_L through its DBD and disrupts stabilization of the mitochondrial membrane mediated by these anti-apoptotic proteins [219]. p53 mutants deficient in Bcl-X_L binding are incapable of triggering cytochrome *c* release. Under certain conditions mitochondrial p53 also interacts with pro-apoptotic Bak liberating it from its inihibitor Mcl-1 and promoting its pro-apoptotic activity [220]. In addition, cytosolic p53 can directly activate pro-apoptotic Bax and p53-dependent Bax activation is not impaired by blocking transcription nor by using an endogenous transcription-inactive mutant p53 further verifying the TI p53-mediated induction of apoptosis [221,222].

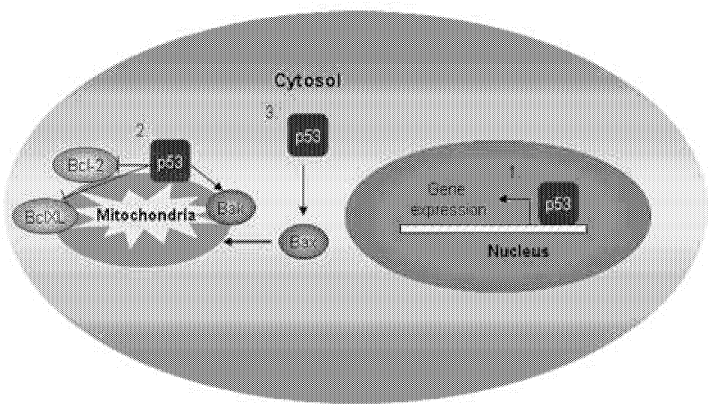

Figure 6. Model of transcription-dependent and independent mechanisms of p53-mediated apoptosis. 1. p53 induces expression of pro-apoptotic Bcl-2 family members (e.g *PUMA*, *Bax*, *Noxa*) that execute apoptotic response. 2. It also translocates to the mitochondria to regulate Bcl-2 proteins (e.g. BclXL, Bcl-2, Bak) and triggers cell death. 3. Cytosolic p53 can directly activate Bax and thereby induce apoptosis.

In summary, p53 seems to promote apoptosis by three mechanisms, 1) by transcriptional regulation of apoptosis-related genes in the nucleus, 2) by activating cytosolic Bax in the cytoplasm and 3) through inhibition of the anti-apoptotic Bcl-XL and activation of pro-apoptotic Bak directly at the mitochondria (Fig. 5). Which way p53 chooses to trigger apoptosis may dependent on the mode of p53 activation, for example, by specific modification that either facilitates its transcription activity or directs p53 out of the nucleus to the mitochondria or stabilizes cytoplasmic p53. The levels of the different cellular p53 pools and its nucleo-cytoplasmic shuttling can also have an impact on which apoptotic procedure is applied. However, *in vivo* kinetics analysis has shown that in mice upon γ-irradiation p53 rapidly accumulates in the mitochondria of radiosensitive tissue such as spleen, thymus and testis and triggers a first wave of caspase-3 activation followed by a significantly later p53-transcription-dependent second wave [223]. This implies that the TI pro-apoptotic function of p53 may be the acute response upon certain damages.

The observed TI activity of p53 most certainly adds a new dimension to its apoptotic role, the most pivotal pre-requisite for tumor suppression. It is therefore, plausible that p53 may regulate other cellular events independently of transcription. It is possible that p53 can alter stabilization and/or activation of some targets without affecting their transcription, through regulating their posttranslational modifications. This may be mediated by direct interaction of p53 with the target proteins inducing certain conformational changes or by recruiting them to e.g. kinases, phosphatases, ligases or other modifiers that alter their translational status. However, it is also plausible that such modification may be secondary effects of p53-regulated primary targets. Furthermore, the cytoplasmic and mitochondrial p53 may affect other cellular proteins to promote apoptosis. Taken together, one can assume that the current view that p53's role in tumor suppression relies primarily on its function as a transcription factor, is likely to go through a major transition. Only time can tell what other surprises this tumor suppressor will exhibit.

p53 and cancer therapy

The requirement of p53 inactivation for tumor cell survival makes it an attractive target for cancer therapy. Recent studies suggest that restoration of p53 function alone causes regression of various established tumors *in vivo* confirming that it indeed represents an effective therapeutic strategy to treat cancer [187,224]. Many scientists are devoted to develop strategies for reinstalling the p53 tumor suppressor function. These include wild type p53 gene therapy, reactivation of mutant p53 and relief of wt p53 from overexpressed MDM2.

Gene therapy

The *p53* gene is mutated in almost 50% of all human tumors. Several approaches have been taken to reintroduce wt *p53* into tumor cells using gene transfer. In most cases an adenoviral vector (Adp53) has been used to insert an intact cDNA copy of the *p53* gene [225]. Although the outcome of these trials has not been that promising, stabilized tumor growth and even tumor regression has been observed in some treated patients. Treatment of head and neck cancer with Adp53 gene therapy has shown clinical effect in half of the patients and has advanced into phase III trials in USA [226]. Several tumor promoting genes including *VEGF, MMP and MDR1* have been shown to be repressed upon Adp53 gene delivery [227]. Another approach utilizes a mutant adenovirus lacking the E1B 55K gene, ONYX-015 that replicates and lyses tumor cells lacking wt p53 [228]. Upon systemic administration ONYX-015 inhibits tumor growth in animals *in vivo*, and its anti-tumor effect is enhanced by combination treatment with chemotherapeutic drugs [229].

Reactivation of mutant p53

The missense p53 mutations primarily affect p53 interaction with DNA as these mutations most commonly occur in the DNA-binding core domain leading to defective activation/repression of target genes. Several small molecular compounds able to restore the wild type function of mutant p53 have been identified by screening of chemical libraries of low molecular weight compounds. An example of such compound is PRIMA-1. Treatment of tumor cells carrying mutant p53 e.g. the R175H with PRIMA-1 efficiently induces apoptosis in a mutant p53-dependent manner [230]. The conformation of mutant p53 is altered by PRIMA-1 treatment that promotes p53-mediated transcriptional activation of target promoters such as *PUMA*, *MDM2* and *p21*, possibly by facilitating mutant p53 DNA binding. Moreover, nucleolar translocation is critical for mutant p53 reactivation since PRIMA-1^{MET} treatment results in a striking redistribution of mutant p53 to nucleoli, together with the PML body-associated proteins PML, CBP and Hsp70 [231]. Systemic administration of PRIMA-1 inhibits growth of human tumor xenografts in mice without any detected toxicity. Another molecule designated CP-313198 activates p53 also by restoring DNA binding of mutant p53 core domain in vitro [232]. CP-313198 treatment protects p53 from MDM2-mediated degradation whereupon high levels of transcriptionally active p53 accumulate and induce expression of members of both extrinsic and intrinsic apoptotic pathways [233]. Tumor growth of human melanoma and colon carcinoma is suppressed by CP-313198 in mice [234].

Rescue of wt p53 from its inhibitor MDM2

In the other 50% tumor cells carrying wt p53, inactivation of p53 occurs by various mechanisms. One strategy employed in some tumors is overexpression of MDM2 that constitutively targets p53 for proteosomal degradation. A clever approach to prevent this is to block the interaction between p53 and MDM2. Several potent small molecules capable of inhibiting this binding have been identified. One such compound that efficiently induces apoptosis in acute myeloid leukemia (AML) is Nutlin-3a. In AML, p53 is inactivated by overexpressed MDM2. Nutlin-3a acts synergistically with the Bcl-2 antagonist, ABT-737 to induce Bax activation and mitochondrial apoptosis in AML cells [235]. Nutlin-3a triggers both transcription-dependent and transcription-independent pathways to induce p53-mediated apoptosis. Similarly, RITA was selected by the basis of its capacity to kill cancer cells carrying wt p53 and has been shown to disrupt the interaction between p53 and MDM2 and induce apoptosis [236]. In mice, RITA treatment was shown to suppress growth of tumors with wt p53 cells. Several p53 targets such as GADD45 and PUMA are induced upon RITA treatment. RITA is thought to bind p53 N-terminus liberating it from MDM2 binding.

A plethora of active compounds are under investigation for reinstalling p53 signaling pathway in tumor cells and many of them have potentials to be anti-cancer drugs in future. The novel approaches mentioned above confirm that p53 pathway indeed is a promising target for tailored cancer therapy

ROLE OF PHOSPHORYLATION

Paper IV in this thesis deals with p53-dependent phosphorylation and therefore a few words on phosphorylation is included in this section. Protein phosphorylation is the most commonly occurring posttranslational modification that regulates key cellular processes such as cell division, metabolism, survival and apoptosis. As an example the cell cycle phases are driven by multiple cyclin/CDK-dependent phosphorylation processes. Entry into mitosis is dependent on the synthesis of cyclinB, its association to CDK1, and the activation of the CDK1/cyclinB complex by regulatory phosphorylations [237]. Exit from mitosis involves inactivation of CDK1 through cyclinB destruction mediated by the APC.

Phosphorylation is an ATP-dependent event catalyzed by kinases that by adding a phosphate group to a specific aminoacid residue of target proteins alter their activity. Kinases can act as receptors on the cell membrane or as intracellular signal mediators [238]. Activation of a cell surface transmembrane receptor kinase by its ligand or other stimulus triggers a cascade of intracellular kinase interactions that lead to changes in gene transcription and cell response. Phosphorylation leads to either stabilization and/or activation or destabilization/inactivation of proteins. As discussed above p53 function is regulated by phosphorylation. It is phosphorylated at multiple sites in its N- and C-terminal regions in response to cellular stress, resulting in p53 stabilization and activation of DNA binding. For example Jun NH_2-terminal kinase or JNK-mediated phosphorylation of p53 stabilizes p53 and p53-induced transcription and apoptosis following DNA-damage by UV-irradiation [239,240].

Knowing the complex and diverse nature of p53 function it is possible that activated p53 launches phosphorylation (or other modifications) of some proteins through direct interaction or via intermediaries such as primary targets. Phosphorylation of such targets may be cruical for proper p53-mediated cellular response. It is thus important to investigate the possibility whether p53 can mediate changes in the phosphorylation state of target proteins to further elucidate its tumor suppressive character.

TELOMERASE

Immortalization

Immortalization is an essential requirement for tumor development that involves activation of telomerase or hTERT [3]. Normal human cells are mortal, undergo successive telomere shortening and lack telomerase activity. By contrast, majority of human cancer cells are immortal, have stable telomeres and express telomerase. Human cells possess a limited replicative lifespan and enter senescence after a certain number of doublings [241]. However, this state of irreversible growth arrest can be circumvented through inactivation of the p53 and RB tumor suppressors by introduction of oncoproteins such as HPV E6 and SV40 LT-antigen allowing continued proliferation with further telomere shortening. These post-senescence cells after progression through additional generations reaches a second proliferative barrier referred to as crisis. Cells at this point suffer from gross chromosomal disarray including end-to-end fusions as a result of extensive telomere loss and are destined to die. However, occasionally few cells (1 in 10^7) acquire the ability to escape crisis and multiply without limit through activation of telomerase and hence become immortalized, a common trait of most human tumors [242] (Fig 7). In fact, 90% of all human tumors express high levels of telomerase.

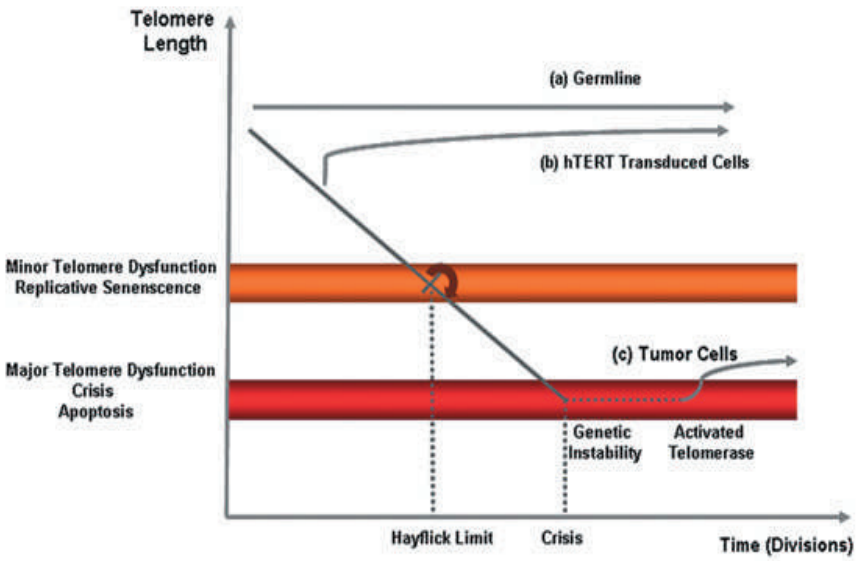

Figure 7. Telomere shortening during cell division. (a) Stable telomere length in germline and (b) in hTERT transfected cells. (c) Progressive telomere shortening leading to crisis which is rescued by activation of hTERT in tumor cells.

Telomerase in length maintenance

Telomeres

Human telomeres consist of tandem repeats of the hexameric DNA sequence TTAGGG, ranging from 15 kb at birth to less than 5kb in chronic diseases. Telomeres are dynamic nucleoprotein complexes that cap the ends of linear eukaryotic chromosomes [243]. The role of telomeres for chromosome stability was reported already in the early 1940s by experiments in maize [244]. Telomeres have two important duties. First, they protect the chromosome ends from destructive nucleases and other damaging events e.g. end-joining. Second they enable the ends to be completely replicated. Due to the nature of lagging-strand DNA synthesis, conventional DNA polymerases are unable to completely replicate the ends of chromosomes resulting in loss of 50-200 bp of telomeric DNA per round of DNA replication. This dilemma has been solved in most organisms by the telomerase complex that adds telomeric repeats onto the chromosome ends.

Telomerase

Telomerase was first discovered in the unicellular ciliate, Tetrahymena where it was shown to use an unusual mode of DNA synthesis for polymerization of telomeric DNA [245,246]. It is a ribonucleoprotein enzyme that consists of two major components, a reverse transcriptase catalytic subunit (TERT) and an RNA subunit (TR). The intrinsic RNA subunit functions as a template for synthesis of telomeric DNA by TERT directly onto the 3' ends of the chromosomes. The enzyme is expressed in early embryogenesis and in adults in male germline but is almost undetectable in most normal somatic cells except in proliferative cells of renewing tissue where its expression is tightly regulated [247,248]. However, recent evidence shows that a small amount of the enzyme activity is detectable also in normal cells as they enter S-phase but disappears when the cells go into G2-phase. This low amount of the protein is proposed to be sufficient to repair any telomeric damage [249].

The human gene, *hTERT* consists of 16 exons and 15 introns spanning around 35 kb. Characterization of *hTERT* promoter has revealed the presence of binding motifs for several transcription factors such as Myc, NFκB, Sp1, and estrogen receptor suggesting multiple levels of regulation of hTERT expression [250].

Telomerase in cancer

An association of hTERT function with tumor progression was evident by the finding that it is activated in immortalized and cancer cells [251]. It is now well established that 90% of all human tumors have express high levels of hTERT [252]. The most apparent evidence of its role in tumorigenesis comes from the observations that introduction of hTERT into normal human telomerase-negative cells extends their life span whereas inhibition of hTERT limits the growth of human cancer cells [253,254]. Several mouse model studies have provided valuable insights into the role of telomerase in telomere maintenance and cancer. Lack of telomerase activity in the first generation of telomerase knockout

(KO) mice with long telomeres, as mice have significantly longer telomeres than humans results in lower incidence of skin tumors than in wt mice following skin carcinogenesis. The subsequent fifth generation mice with short telomeres show much higher resistance to tumorigenesis possibly due to activation of p53 tumor suppressive actions [255]. This implicates that telomerase may promote tumorigenesis independently of telomere length.

What is the mechanism of hTERT overexpression or activation? Several mechanisms have been reported. Amplification of the *hTERT* gene has been found in 20% of primary tumors and 40% of human cancer derived cell lines [256]. Previous work showed a strong correlation between telomerase activity and the levels of hTERT mRNA [257]. Tumor cells utilize various strategies to activate the transcription of *hTERT* involving several viral oncoproteins and cellular transcription factors consistent with previously demonstrated presence of binding sites for such factors in the *hTERT* promoter. In ovary epithelial cells, the ligand-dependent transcription factor estrogen receptor α (ERα), in the presence of its ligand 17β-estradiol (E2) activates transcription from the *hTERT* promoter through binding to the estrogen receptor element (ERE) in the *hTERT* regulatory region and induces telomerase activity [258]. Others have shown that in human keratinocytes expression of the HPV E6 oncoprotein induces hTERT transcription through a minimal E6-responsive region in the *hTERT* promoter which is coordinated with E6-mediated telomerase activity [259]. A following study demonstrated that the E6-responsive minimal region also contains Myc/Max (Myc-Max dimers induce transcription to promote cell cycle in proliferating cells) binding E-box elements and that E6 and Myc interact *in vivo* to cooperatively activate the *hTERT* promoter [260]. The Myc antagonist Mad1 (Max-Mad1 dimers inhibit E-box-driven transcription, cell cycle progression and promote differentiation) suppresses E6-mediated *hTERT* transactivation and this repression is rescued by overexpressing Myc. Furthermore, Myc/Max complex activates the *hTERT* promoter in proliferating cells and in contrast Max/Mad1 binds to the E-box elements to repress *hTERT* transactivation in the same cells induced to differentiate [261]. Thus the reciprocal E-box occupancy by Myc and Mad1 accounts for *hTERT* activation or repression in proliferating versus differentiated cells. Although the mechanism of *hTERT* repression in normal cells is largely unknown contribution of histone deacetylation has been suggested. Reduced histone acetylation of the *hTERT* promoter in differentiated cells and inhibition of deacetylases leading to activation of hTERT expression in resting cells is implicative of a universal mechanism of telomerase inactivation during differentiation and development of normal human somatic cells [261].

p53 and Telomerase

The connection between telomere shortening, telomerase and p53 has been established by various publications. Telomere loss induces cellular senescence through activation of the p53 and RB pathways since cell division beyond this senescence program requires viral oncoprotein-mediated inactivation of these tumor suppressors [262]. Critically short telomeres, in mice lacking telomerase activity by inactivation of the TR component of the telomerase complex has been shown to trigger p53-dependent DNA-damage responses e.g. cell cycle arrest and apoptosis. The adverse effects of telomere shortening are rescued upon p53 knockdown and p53 deficiency further cooperates with telomere dysfunction to accelerate tumorigenesis [263]. In addition, the increased cancer incidence in transgenic mice with constitutively expressing hTERT is aggravated by p53 mutation confirming that upregulation of telomerase cooperates with p53 loss to promote tumorigenesis [255].

p53 downregulates hTERT

Early work showed that abrogation of p53 function by introduction of mutant p53 induces telomerase reactivation and immortalization of human mammary epithelial cells (HMECs) [264]. As mentioned above HPV E6 that promotes degradation of p53 can also activate telomerase expression. Thus, inhibition of telomerase is likely to be critical for p53 tumor suppressor function. Recent research on the relationship between p53 and hTERT, two key regulators of cancer has generated substantial amount of information about their function. Adenoviral expression of p53 downregulates hTERT at mRNA level and represses telomerase activity [265,266]. In parallel, both exogenous and endogenous p53 after activation have shown to inhibit *hTERT* transcription. This repression is mediated by wt p53 since expression of His-273 mutant does not affect the *hTERT* promoter activity [186]. The mechanism of p53-mediated downregulation of hTERT has been also analyzed. Like many other p53-repressed genes *hTERT* lacks p53 consensus-binding sites but harbors several binding sites for Sp1 transcription factor. Xu et al showed that p53 forms complex with Sp1 to inhibit binding of Sp1 to the *hTERT* promoter and thereby blocks *hTERT* transactivation. A recent work demonstrated that p21 is required for p53-dependent *hTERT* repression [267]. Knockdown of p21 was shown to be sufficient to abolish p53-mediated transrepression of *hTERT*. Furthermore, E2F and RB are also involved in p53-dependent *hTERT* downregulation and since p21 is the obvious link between these proteins it is not surprising that p21 plays an essential role in this process. However, in the previous study by Xu et al, p21 was found to be dispensable for p53-mediated repression of *hTERT*. It was suggested that the downstream elements of the p53/p21/Rb/E2F pathway may be defective in "p21-independent-repression" cells where high levels of p53 utilizes other mechanisms such as Sp1 binding to inhibit hTERT expression. However, the effect of p53 loss on telomerase activity has been demonstrated in HMECs. As expected, p53 deficiency in the HMEC sub-cell lines results in rapid activation of telomerase and full immortality [268].

Anti-Apoptotic Function of hTERT

Several lines of evidence suggest that hTERT has an additional anti-apoptotic function in addition to its ability to maintain telomeres. The anti-apoptotic role has been demonstrated by antisense knockdown of hTERT that results in enhanced mitochondrial dysfunction and apoptosis by different stimuli in pheochromocytoma cells [269]. Overexpression of Bcl-2 and inhibition of caspase-3 protects these cells against apoptosis induced by hTERT inhibitors implying that the site of hTERT anti-apoptotic function is upstream of caspase activation. However, this anti-apoptotic activity was correlated to its telomerase activity as antisense-mediated inhibition of hTERT reduced telomerase activity. Likewise, stress-induced apoptosis is suppressed in fibroblasts expressing hTERT unlike their normal counterpart [270].

An anti-apoptotic function of hTERT independent of its enzymatic activity has been demonstrated by the usage of telomerase activity-null hTERT mutant. Downregulation of wt hTERT causes apoptosis without affecting the telomere length whereas introduction of the hTERT mutant rescues the apoptotic response triggered by wt hTERT downregulation [271]. Similarly, others have shown that mouse TERT (mTERT) expression in MEFs confers resistance to stress-induced p53-dependent apoptosis without affecting differentiation [272]. Telomerase has been shown to perturb both the intrinsic mitochondrial apoptotic pathway and the extrinsic death receptor pathway [273,274]. The latter was shown to occur independently of telomere maintenance. Furthermore, hTERT incapable of telomere maintenance has been shown to promote tumorigenesis in mice suggesting that hTERT indeed confers an additional function required for tumor development that does not rely on its ability to stabilize chromosome ends [275].

What is the nature of this anti-apoptotic behavior of hTERT? Although the answer is currently unknown, there are a few implications. One explanation is that overexpression of hTERT alters the gene expression pattern granting cells with growth advantageous properties [276]. The authors showed that the expression of growth-suppressor genes including TNF-related apoptosis-inducing ligand (TRAIL) and interleukin 1 receptor antagonist (IL-1Ra) is decreased upon hTERT expression in HMECs. Similarly, the expression of certain growth-promoting genes such as epidermal growth factor receptor (*EGFR*) and fibroblast growth factor (*FGF*) is enhanced. Telomerase-dependent expression of EGFR stimulates proliferation of cells grown in minimal medium and this increased proliferative phenotype is reversed by inhibition of EGFR expression. Thus, hTERT appears to stimulate cell proliferation by regulating growth-promoting genes and this proliferative advantage cannot be attributed to hTERT telomere maintenance activity alone.

Another clue comes from the observation that hTERT prevents mitochondrial dysfunction and caspase activation. Overexpression of Bcl-2 increases hTERT expression and activity and protects cells from apoptosis induced by Bcl-2 inhibitors. Likewise overexpression of hTERT abrogates apoptotic response and siRNA knockdown of hTERT markedly increases Bcl-2-dependent apoptosis [277]. Moreover, the catalytically inactive hTERT behaves in a similar manner suggesting an anti-apoptotic role of hTERT involving the mitochondria. Interestingly, a mitochodrial localization sequence in the N-terminal region of hTERT has been shown to target ectopic hTERT to the mitochondria and induce mitochondrial

DNA (mtDNA) damage and apoptosis upon hydrogen peroxide treatment [278]. These data suggest that hTERT has a role at the mitochondria. Recent data shows that siRNA inhibition of endogenous hTERT expression facilitates the pro-apoptotic Bax conformational activation and mitochondrial apoptosis induced by a variety of stimuli [273]. Based on these data it is assumable that hTERT acts directly at the mitochondria to suppress mitochondrial apoptotic pathway by interference with pro-apoptotic Bcl-2 family members. Thus hTERT may favor cell growth by various mechanisms including stabilizing telomeres, modulating gene expression and suppressing apoptosis (Fig. 8).

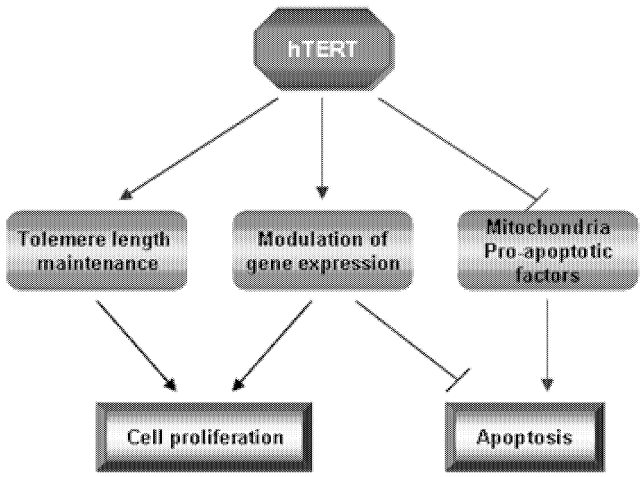

Figure 8. The growth promoting and anti-apoptotic activities of hTERT.

Telomerase and Cancer Therapy

The fact that the vast majority of tumors express high levels of activated telomerase and the emerging evidence on its anti-apoptotic role affecting tumor suppressor pathways, such as the p53 pathway makes telomerase an interesting target for development of anticancer drug. In fact elevated levels of hTERT RNA or protein is recognized as a potential tumor marker [279,280]. Multiple approaches towards targeting telomerase have been under taken. Most of these strategies rely on telomere shortening in tumor cells during the treatment. Attempts for interrupting the telomere length maintenance have been directed against both RNA and the catalytic subunit of telomerase as well as telomere structures.

Antisense therapy against the hTR has proven to be successful in some tumor cells. Introduction of an antisense vector shortens the telomeres leading to death of cervical cancer cells and increases the susceptibility of cisplatin-induced apoptosis in glioma cells [278,281]. Antisense treatment has been shown to trigger induction of two distinct pathways, apoptosis and differentiation [282]. Treatment of tumors grown in nude mice with the antisense oligonucleotide attenuates survival of the tumor cells. Furthermore, combined treatment with hTR antisense oligonucleotide and recombinant adenovirus bearing p53 in glioma cells with endogenous mutant p53, shows additive effect on tumor suppression inducing caspase-dependent apoptosis and cell growth arrest [283].

Certainly, hTERT has also been subjected for targeted therapy. A dominant-negative mutant telomerase has shown to shorten telomeres and induce apoptosis and reduce tumorigenesis in mice [254,284]. Adenoviral delivery of anti-hTERT ribozymes, small catalytically active RNA molecules that cleave their RNA substrate in a sequence-dependent manner in ovarian cancer cells results in immediate apoptosis without causing telomere shortening [285]. Small molecules and natural compounds have been shown to act as potent telomerase inhibitors [286,287]. As most attempts of telomerase inhibition involve the slow process of telomere shortening, long treatment time before therapeutic effect is achieved is required. This problem can be solved by combining telomerase inhibitors with DNA-damaging chemotherapy.

CLOSING REMARKS

The proper function of p53 is vital for tumor suppression. The fact that p53 is the most commonly mutated gene in human cancers is a clear manifestation of its central role in cancer pathogenesis. p53 responds to many distinct sensors of physiological distress and therefore loss of p53 during the tumorigenesis process plays a role in many of the steps. By eliminating p53, pre-malignant cells not only liberate themselves from its apoptotic effects but also facilitate angiogenesis and metastasis.

Following p53's discovery nearly 40,000 reports on p53 have been published in the last 25 years. In spite of the continuous flow of information about p53 our knowledge of this protein is quite superficial. Characterization of p53 target genes along with analyses of its posttranslational modification accounts for the majority of information known about p53. Much is still needed to be explored. The most perplexing question remains unanswered as outlined in "The biology of Cancer" (by Weinberg et al). Why has a single protein been entrusted with so many vital alarm functions by the mammalian cell? As loss of p53 function renders the cell insensitive to a whole range of conditions that normally halt cell growth or trigger apoptosis. Although there is no clear cut answer to this question, delineation of p53 function is absolutely essential for understanding the mechanisms of tumor suppression by p53 with the hope of tailoring the proper therapeutics to combat cancer in the near future.

AIMS OF THE THESIS

The basic aims of this thesis were to study the relationship between p53 and its target hTERT and to analyze the p53-dependent proteome. Identification of novel p53 targets and delineation of such targets is most important for the understanding of p53 as regulator of cell growth and survival.

SPECIFIC AIMS:

- **Paper I:** To investigate the effects of constitutive hTERT expression on p53-induced apoptosis.
- **Paper II:** To characterize the mechanism of the anti-apoptotic function of hTERT.
- **Paper III:** To determine global p53-dependent protein expression.
- **Paper IV:** To analyze p53-dependent phosphorylation of proteins.

RESULTS AND DISCUSSION

PAPER I - Analysis of the effects of hTERT expression on p53-induced apoptosis.

p53 transactivates and represses a growing number of target genes. We have previously shown that the catalytic subunit of telomerase is downregulated at the mRNA level by activated p53 [186]. This downregulation of hTERT was attributed specifically to wt and not mutant p53 function. Others have shown similar effects on hTERT by expression of adenoviral p53.

In this paper we further investigated the consequences of constitutive expression hTERT on p53-induced cell apoptosis and whether this effect is dependent on telomerase activity. Two different cell lines, BL41 Burkitt lymphoma cells carrying a temperature sensititve mouse p53 (BL41-tsp53) that has a mutant conformation at 37°C and wild type conformation at 32°C, and the colon carcinoma line HCT116 wtp53 and its isogenic p53 null cells were used.

First, we confirmed downregulation of endogenous hTERT at protein level both in BL41-tsp53 cells and HCT116 cells by Western blotting. Activation of wtp53 in the BL41-tsp53 cells by temperature shift to 32°C led to reduced levels of hTERT protein whereas p21 was upregulated in these cells. Likewise activation of p53 in the HCT116 wtp53$^{+/+}$ cells but not in the p53$^{-/-}$ cells by treatment with the DNA-damaging agent mitomycin C (MMC) resulted in downregulation of hTERT in the HCT116 wtp53$^{+/+}$ cells. Thus, activation of p53 suppresses expression of hTERT.

In order to study the effects of constitutive expression of hTERT on p53-induced apoptosis, the BL41-tsp53 and HCT116 wtp53$^{+/+}$ and p53$^{-/-}$ cells were transfected with an empty vector or an hTERT or a Bcl-2 expressing vector. The Bcl-2 vector served as a positive control for anti-apoptotic activity. In addition, HCT116 cells were transfected with a catalytically inactive dominant negative hTERT, DN-hTERT for analysis of the effect of hTERT telomerase activity. Three clones of BL41-tsp53 cells overexpressing hTERT and one expressing Bcl-2 were selected for further investigation. hTERT and Bcl-2 positive clones were verified by PCR using construct specific primers. Constitutive expression of hTERT and Bcl-2 was confirmed by immunoblotting. To study the effects of hTERT expression on p53-induced cell death, the transfected BL41-tsp53 cells were incubated at 32°C for 24 hours whereupon cell death was analyzed by trypan blue staining. Only 25% of the control cells were alive after 24 hours of p53 activation whereas 50% of the hTERT expressing cells were alive at this time point. Most of the Bcl-2 expressing cells were still alive. FACS analysis of propidium iodide (PI) stained cells confirmed this result, showing a marked decrease in the sub-G1 population among hTERT and Bcl-2 transfectants as compared to the control cells. TUNEL staining was performed as complementary analysis and showed that the parental BL41 cells with endogenous mutant p53 and BL41-tsp53 control cells presented much higher fraction of apoptotic cells than the hTERT expressing cells. As expected the Bcl-2 expressing were resistant to p53-induced apoptosis. These results demonstrate that constitutive expression of hTERT partially attenuates p53-induced apoptosis.

Next, the effect of constitutive hTERT expression on endogenous wild type p53-induced apoptosis was examined using the HCT116. Pools of hTERT expressing HCT116 wtp53$^{+/+}$ and p53$^{-/-}$ cells were analyzed. FACS analysis of sub-G1 cells following activation of endogenous p53 by treatment with MMC or 5-flurouracil (5-FU), both DNA-damaging agents was performed. We found that the wtp53$^{+/+}$ control cells showed higher amount of cell death (up to 42%) was detected and while the wt hTERT expressing cells were much resistant to p53-induced cell death showing only 21% cell death. Strikingly, the DN-hTERT expressing cells also showed reduced cell death. TUNEL staining of these cells verified this anti-apoptotic activity of hTERT. As usual the Bcl-2 expressing cells were almost completely resistant. In all the wtp53 null pools similar amount of cell death was detected, suggesting that hTERT expression specifically antagonizes p53-mediated cell death.

Moreover, a colony formation assay was performed to determine the ability of hTERT and DN-hTERT to promote cell survival upon p53-activation by 5-FU treatment. The HCT116 wtp53$^{+/+}$ cells expressing these proteins or Bcl-2 formed significantly more colonies than vector-transfected cells.

Taken together these data suggest that hTERT has an additional anti-apoptotic function that counteracts p53-induced apoptosis and promotes cell survival. Our results are consistent with several other studies showing an anti-apoptotic role of hTERT [270,271]. This function can be separated from hTERT telomerase activity as the DN-hTERT was equally efficient in this regard. Therefore, the ability of p53 to downregulate hTERT may be critical for the p53-dependent elimination of tumor cells that already express elevated levels of hTERT.

PAPER II - Characterization of hTERT anti-apoptotic function.

The nature of the anti-apoptotic function of hTERT remains unknown. Previous data has only showed that ectopic expression of hTERT can alter the gene expression pattern to facilitate cell growth.

In this paper we sought to analyze the mechanisms of hTERT's anti-apoptotic activity in p53-mediated apoptosis. We induced p53 activation in HCT116 wtp53$^{+/+}$ cells expressing wt hTERT and Bcl-2 and in control cells by 24 hours treatment with MMC to investigate the effects on the intrinsic mitochondrial pathway. In order to localize the site of hTERT's actions, we first analyzed caspase activity, the most downstream event in the apoptosis cascade, upon induction of p53 by MMC. As expected FACS analysis of the hTERT expressing cells showed much lower caspase activity upon p53 activation than in the control cells. The mitochondrial membrane permeabilization (MMP) was investigated in the next step which was also inhibited in the hTERT expressing cells. Immunoflourescence staining and Western blotting of cytochrome *c* release in the cytosol showed low levels of cytochrome *c* in the cytosolic fraction of hTERT expressing cells after MMC treatment. Bcl-2 cells were highly resistant at all steps. Thus, hTERT antagonizes the mitochondrial activity.

To determine whether hTERT expression affects p53-mediated transcriptional activation of target genes mRNA expression of five p53-targets including p21, MDM2, Bax, PUMA and Wig-1 was analyzed by real time PCR (RT-PCR) upon p53 activation for 8, 16 and 24 hours. Expression of these targets was basically uninfluenced in the hTERT expressing cells although upregulation of Bax was slightly inhibited at 24 hours MMC treatment.

To examine the effect of hTERT upstreams of mitochondria, we analyzed the induction of several pro-apoptotic Bcl-2 family proteins including PUMA, Bax and Bak. Protein expression analysis by Western blotting showed similar upregulation of all three proteins in the different HCT116 cell transfectants. Bcl-2 expression was detected only in the Bcl-2 transfected cells. Since activation of pro-apoptotic Bax/Bak is a key step for induction of MMP and cyt c release, we investigated whether Bax or Bak activation was affected by hTERT. Interestingly, immunostaining of Bax using an antibody specific against activated Bax showed much lower amount of activated Bax in the hTERT expressing cells upon MMC treatment. We also immunoprecipitated activated Bax from the different HCT116 cells with an antibody specific to active Bax and assessed the amount of bound Bax with a polyclonal Bax antibody. The level of active Bax was significantly lower in cells expressing exogenous hTERT than in the control cells. Next, we examined the mitochondrial membrane localization of Bax and Bak upon p53 induction and detected lower levels of active Bax at the membrane fraction in hTERT expressing cells whereas active Bak was present at similar levels in all cells. FACS analysis of cells with activated Bax showed 21% active-Bax positive cells among hTERT expressing cells whereas in the control cells more than 36% were positive for active Bax further confirming inhibition of Bax activation in the hTERT expressing cells.

In order to verify, that inhibition of Bax activation was caused by telomerase expression, we downregulated hTERT, using two different siRNAs. Both siRNAs significantly increased activation of Bax. Caspase activity was also elevated.

Finally, since to hTERT has been shown to repress p53-dependent as well as p53-independent apoptosis we tested whether hTERT prevents cell death mediated by other stimuli including staurosporine (STS), serum starvation (SS) and hydrogen peroxide (H_2O_2). FACS analysis of sub-G1 cells showed that HCT116 cells expressing exogenous hTERT were more resistant to apoptosis induced by not only MMC but also serum starvation (SS) and hydrogen peroxide (H_2O_2). However, only MMC induced p53 and its target MDM2.

Our results strongly suggest that expression of hTERT has an inhibitory effect on activation of pro-apoptotic Bax (Fig. 9). This is in line with a recent report that showed inhibition of hTERT by siRNA promotes Bax activation by DNA damaging drugs [273]. Thus, hTERT may interfere with the activation of pro-apoptotic proteins to block apoptosis. The presence of a mitochondrial localization signal at the N-terminal region of hTERT suggests that hTERT can execute its anti-apoptotic activity directly at the mitochondria [278]. It is plausible that hTERT suppresses Bax activation by direct interaction or by binding to inhibitors of Bax that facilitate mitochondrial stabilization. Another possibility is the observed ability of hTERT to modulate the gene expression profile in favor of cell growth.

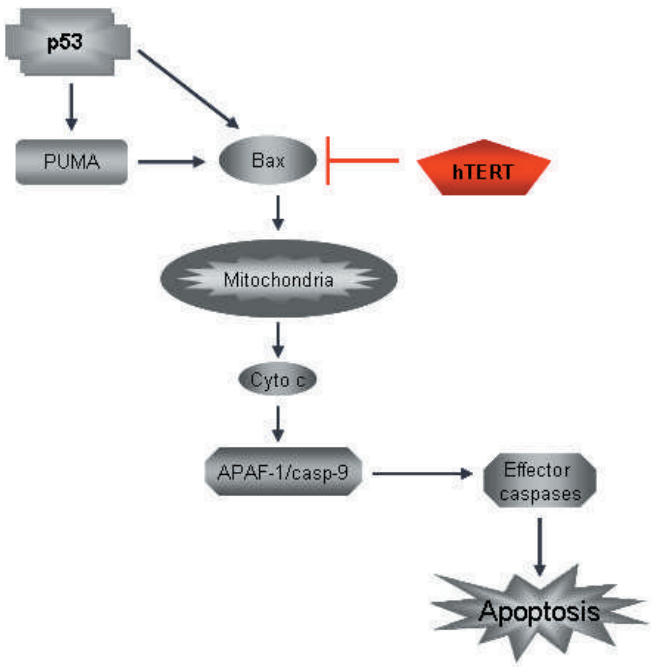

Figure 9. Model of hTERT's anti-apoptotic role. hTERT expression blocks the mitochondrial apoptosis induced by p53 through preventing Bax activity.

PAPER III - Analysis of the p53-regulated proteome upon DNA damage

Most of the current knowledge on p53 tumor suppressor function derives from identification and characterization of its target genes. Numerous target genes have already been characterized and depiction of many of these genes has provided much insight into p53-mediated cellular responses. Novel target identification studies have been concentrated mostly on p53-dependent mRNA expression using microarray techniques for analysis of p53-transactivation or repression function. However, since the function of protein-coding genes is carried out by the protein product, it is important to study p53-dependent expression at the protein level. This may reveal novel p53 targets, such as targets that are regulated by transcription-independent mechanisms.

In this study we examined the p53-regulated proteome to identify novel p53 targets at protein level using 2D gel electrophoresis (2DE) and mass spectrometry. The colon carcinoma HCT116 wtp53$^{+/+}$ and the isogenic p53 null cells were treated with MMC for 8, 16 and 24 hours to induce DNA-damage-mediated p53 activation. Upregulation of p53 and several p53 targets including p21, MDM2 and Wig-1 and downregulation of hTERT in the p53$^{+/+}$ but not p53$^{-/-}$ verified activation of the p53-pathway. Higher fraction of apoptotic cells was detected in the p53$^{+/+}$ cells.

Total protein was analyzed by 2DE and 5,789 detectable protein spots were separated using four overlapping micro pH-ranges. Gel matching by PDQuest software revealed differential expression of 115 proteins in a p53-dependent manner. A minimum of 1.5-fold increase or decrease in expression level over at least two time points in comparison to the protein expression level in the p53$^{-/-}$ and statistical significance in the Mann-Whitney test defined spots for identification. We identified 55 of the p53-regulated proteins by mass spectrometry. Forty-two of these proteins showed upregulation and 13 were downregulated upon p53 activation. We found p53-binding motif in both upregulated and downregulated proteins. However, 18 induced and 5 repressed proteins lacked any p53-consensus site suggesting indirect or transcription-independent effects of p53. Among the identified targets we found proteins involved in various cellular functions including apoptosis, cell cycle arrest, DNA-repair, metastasis inhibition, mRNA-processing and translation.

In order to confirm the 2DE data we analyzed the expression pattern of five upregulated proteins, including eukaryotic translation factor 5A (eIF5A), heterogenous nuclear ribonucleoprotein C1/C2 (hnRNP C1/C2), heterogenous nuclear ribonucleoprotein K (hnRNP K), lamin A/C, and metastasis-inhibitor factor Nm23-H1and two downregulated proteins peroxiredoxin II (Prx II) and tryptophanyl-tRNA synthetase (TrpRS), by Western blotting and real-time PCR (RT-PCR). Protein expression of all the upregulated proteins, Nm23, eIF-5A, Lamin A/C, hnRNP K, hnRNP C1C2 was in consistent with the 2DE data. Likewise, analysis of mRNA expression by RT-PCR showed p53-dependent transcriptional activation of Nm23, eIF-5A, Lamin A/C and hnRNP K. However, transcription of hnRNP C1C2 was repressed in both cells after 16- and 24 hours of MMC treatment suggesting that the observed increase in protein level was independent of transcriptional regulation by p53. Similarly, discordant regulation between mRNA levels and protein levels of TrpRS and PrxII was observed. Downregulation of TrpRS was verified at the protein level by Western blotting. Analysis of mRNA levels, however, showed slight downregulation in both cells upon p53 activation. As for PrxII no

change was detected neither at protein nor at mRNA levels except for a minor increase after 16 hours of MMC treatment in the $p53^{+/+}$ cells although a significant reduction of protein levels was observed by 2DE . These data suggest a p53-dependent regulation at the protein level.

The protein expression of several of these targets was analyzed in the wt p53 carrying MCF7 and U2OS cells and showed a similar pattern. In order to examine whether the protein expression was p53-dependent or a result of induction of apoptosis, the HCT116 cells were treated with staurosporine to induce p53-independent apoptosis. Immunoblotting of Nm23-H1, eIF-5A, Lamin A/C and hnRNP C1C2 showed no significant changes in protein levels by STS treatment. Treatment with the caspase inhibitor zVAD prior to MMC treatment to block apoptosis did not alter the p53-dependent expression of Nm23-H1, eIF-5A, Lamin A/C and hnRNP C1C2, although eIF-5A was induced in lesser extent further confirming that the protein expression was dependent of p53 activation and not apoptosis *per se*.

Some of the identified proteins in this study have previous connection to p53. Overexpression of eIF-5A causes increased expression of p53 targets as well as p53-mediated apoptosis [288]. Our observation on induction of eIF-5A both at mRNA and protein levels and the presence of a p53-binding motif in the eIF-5A promoter strongly suggest that it is a bona fide p53 target gene.

The hnRNP K, implicated in multiple cellular activities has recently been shown to be required for p53-dependent transcription [55]. We identified hnRNP K as a p53-induced gene. It is thus possible that hnRNP K upon induction by p53 acts as a coactivator of p53 in a positive feedback loop. Interestingly, hnRNP isoform a was downregulated in $p53^{+/+}$ cells upon MMC treatment suggesting that this isoform may have an reduced stability or altered mobility as a result of posttranslational modification. Phosphorylation of hnRNP K has been repoted to abrogate its binding to poly(C). p53 may be involved in regulation of this isoform regulating it posttranslationally .

The Lamin A/C proteins encoded by *LMNA* are the major component of nuclear lamina. Mutations in the *LMNA* gene have been linked to premaure aging symdromes such as the Hutchinson-Gilford progeria syndrome [289]. Recent report suggests that accumulation of unprocessed lamin A causes increased DNA damage and chromosome aberrations as a result of defective DNA repair [290]. Such defects are characteristics of progeroid syndrome. Intranuclear lamin scaffolds seem to play an important role in proper assembly of DNA repair machinery upon DNA damage. Disruption of the lamin network results in defective transcription and replication. We observed strong upregulation of lamin A/C by p53 activation and identified a p53 consnsus site the *LMNA* gene suggesting that p53-dependent upregulation of lamin A/C may contribute to DNA repair process.

The Nm23, also known as metastasis inhibition factor is a nucleoside diphosphate kinase. Reduced expression of Nm23 seems to be related to increased metastatic potential in a variety of tumor types [291]. Others have shown that *Nm23-H1* is induced by p53 [195]. We detected significant upregulation of Nm23 at mRNA and protein levels further confirming a role of p53 in metastasis inhibition.

The hnRNP C1/C2 are the major pre-RNA-binding proteins involved in mRNA maturation and have no previous link to p53. We identified hnRNP C1/C2 to be induced upon p53 activation at protein

level whereas the mRNA levels were reduced in a p53-independent manner. Moreover, the hnRNP C1/C2 promoter lacks p53-consensus motifs suggesting that it is not a classical p53-target. However, the levels of one of the five hnRNP C1/C2 spots, designated hnRNP C1/C2a in 2DE analysis was reduced in the $p53^{+/+}$ cells after DNA damage. This could also be a result of posttranslational modification that alters its stability. Moreover, hnRNP C1/C2 is cleaved by caspase-3 that we found to be induced in $p53^{+/+}$ cells. p53 may influence mRNA processing by regulating hnRNP C1/C2.

Among the downregulated targets, PrxII, was downregulated based on our 2DE data, but we were unable to detect any significant p53-dependent repression by immunoblotting. It is plausible that p53 down-regulates specific isoforms and/or posttranslationally modified forms of PrxII and not the entire pool. p53 overexpression induces ROS production, resulting in oxidative degradation of mitochondrial components and cell death. PrxII null MEFs show elevated levels of reactive oxygen species (ROS) and cellular senescence suggesting that p53-mediated downregulation of Prx II has a role in the generation of ROS and p53-dependent apoptosis [292]. We did not observe any repression of Prx II at mRNA level, indicating that this gene is not a direct transcriptional target of p53.

The full-length TrpRS is thought to function primarily in translation whereas a truncated form, mini-TrpRS, inhibits retinal angiogenesis [293]. We observed downregulation of the full-length TrpRS by 2DE and Western blot analysis after p53 activation but no changes were found by RT-PCR indicating that p53 downregulates TrpRS independently of transcription possibly by posttranslational modifications. There is no report about p53 effect on translation involving downregulation of TrpRS.

Our analysis of the p53-regulated proteome has revealed fifty-five putative p53 protein targets. These proteins are involved in various cellular processes. Lack of putative p53-binding motifs in the promoters of several identified targets suggests transcriptional-independent regulation by p53. This work demonstrates the importance of protein expression profiling as a tool to identify and characterize downstream p53 targets as transcription-independent targets will remain undiscovered in a microarray analysis. Our findings support the notion that the p53 tumor suppressor regulates cell growth and survival and other cellular processes at multiple levels via both transcription-dependent and transcription-independent mechanisms. This study also shows that global proteome analysis is suitable for identification of novel p53 targets and suggests that p53 is involved in posttranslational modification of many proteins.

PAPER IV - Analysis of p53-dependent protein phosphorylation.

Our previous analysis of p53-dependent protein expression profiling revealed that multiple putative p53-targets are regulated post-transcriptionally. Others have shown that p53 can induce apoptosis independent of transcriptional activation, by direct interaction with apoptosis-related proteins. It is possible that p53 influences posttranslational modifications of a number of proteins to regulate their stability and/or function at the protein level.

In this study we analyzed p53-dependent phosphorylation of target proteins using the fluorescent phosphoprotein dye (Pro-Q Diamond or Pro-QD) and 2DE-based polypeptide separation and mass spectrometry. The HCT116 wtp53$^{+/+}$ and p53$^{-/-}$ cells were treated with MMC for 16 hours to activate p53. Upregulation of p53 protein level and phosphorylation of p53 at Ser15, 20, 37 and 392 was detected in the wtp53$^{+/+}$ cells after MMC treatment by Western blotting and immunostaining. Upregulation of p53 targets p21 and MDM2 was verified by Western blot analysis. Increased cell death of the wtp53$^{+/+}$ cells was observed by FACS analysis of casapase activity.

Total protein of HCT116 wtp53$^{+/+}$ and p53$^{-/-}$ cells treated with MMC for 0 and 16 hours was separated by 2DE and stained for phosphoproteins using Pro-QD staining solution. More than 3,000 protein spots were separated using two overlapping micro pH ranges (pH ranges 4.7-5.9 and 5.5-6.7). We detected changes in phosphorylation in total of 279 proteins in MMC-treated wild type p53 and/or p53 null cells by Pro-QD staining. Forty-four of these protein spots were detected exclusively in the p53$^{+/+}$ cells upon p53 activation. The gels were then restained with SR staining solution and the interesting spots were excised and identified by mass spectrometry. Identity of 21 phosphorylated proteins in the wtp53$^{+/+}$ cells were obtained. We identified proteins that showed increased or decreased phosphorylation without any changes in total expression levels upon p53 activation, and proteins that showed p53-dependent changes in both total levels and phosphorylation.

In paper III we identified Annexin 1 (ANXA1) to be upregulated upon activation of p53. Here, we detect both upregulation and phosphorylation of ANXA1 in a p53-dependent manner. ANXA1 is a calcium-dependent phospolipid-binding protein that has anti-inflammatory functions and overexpression of ANXA1 promotes apoptosis associated with caspase-3 activation [294]. Recent data suggest that ANXA1 can mediate 'eat-me' signal on apoptotic cells surface and is required for efficient clearance of apoptotic cells [295]. p53-dependent phosphorylation of ANXA1 may regulate the function of the protein. One obvious possibility is that ANXA1 is involved in removal of apoptotic cells upon induction of p53.

Also the metastasis inhibition factor, Nm23A encoded by *NME1* has been identified by us and others to be induced by p53. In this study we detected both p53-dependent upregulation and phosphorylation of the Nm23A protein. Contradicting data exists on the relationship between mutations in the *NME1* gene and causation of metastasis of different tumor types. Interestingly, *in vivo* Nm23 serine phosphorylation levels showed direct correlation with suppression of the tumor metastatic potential of transfected murine melanoma cells, suggesting that phosphorylation of Nm23 is indeed required for its

ability to inhibit metastasis [296]. It is possible p53 may regulate metastasis through posttranslational modifications of this target, in addition to inducing overall expression levels.

In the previous study we observed transcription-independent downregulation of PrxII upon activation of p53. Here, we observed phosphorylation of PrxII suggesting that its downregulation in wtp53$^{+/+}$ cells upon p53 activation could be regulated by phosphorylation Overexpression of PrxII has proliferative and anti-apoptotic properties [297]. It is possible that p53-mediated inhibition of PrxII function in eliminating peroxides generated during metabolism is importatant for induction of apoptosis.

The DJ-1 protein was phosphorylated upon induction of p53 in the wtp53$^{+/+}$ cells without any detectable change in the net protein levels. Although the function of the DJ-1 protein is largely unknown, overexpression of DJ-1 has been correlated with negative regulation of the tumor suppressor PTEN and increased cell survival [298]. In a recent report, knockdown of DJ-1 in zebrafish model of Parkinson's disease resulted in increased expression of p53 and Bax, suggesting that absence of DJ-1 activates the death pathway [299]. Our finding that DJ-1 is phosphorylated upon activation of p53 raises the possibility that p53-dependent phosphorylation of DJ-1 inhibits its function and thus facilitates apoptosis.

Our data presented in this paper suggests that activation of p53 by treatment with MMC induces changes not only in total expression levels but also in phosphorylation of a number of proteins. This effect could be a transcription-dependent secondary effect of p53-mediated activation of protein kinases. Alternatively, p53 could stabilize such kinases by transcription-independent mechanisms, for instance via protein-protein interaction. In paper III, we identified the mitogen-activated protein kinase 8, MAPK8 or JNK1 to be upregulated by p53. This kinase is activated by various cellular stresses. Its activation by tumor-necrosis factor alpha (TNFα) is required for TNFα-induced apoptosis and JNK1 is also important for UV-induced phosphorylation of p53 and p53-mediated apoptosis [240,300]. Therefore, it is possible that JNK1 is responsible for p53-dependent phosphorylation of specific proteins. Additional unknown p53-induced kinases or regulators of kinases can also affect phosphorylation of these proteins.

In summary, we have shown that p53 is able to mediate phosphorylation of a number of proteins involved in various cellular activities. This could explain the non-transcriptional regulation of proteins reported in paper III. Phosphorylation of such targets may be critical for proper p53-mediated cellular response. Our findings add new insights to the current understanding of p53's capabilities and based on our data, we propose an evolving model for p53 functions as shown in Fig. 10. Additional work is required to elucidate the mechanisms underlying this new role of p53, and determine if p53-dependent phosphorylation can be exploited for novel anti-cancer therapeutic strategies.

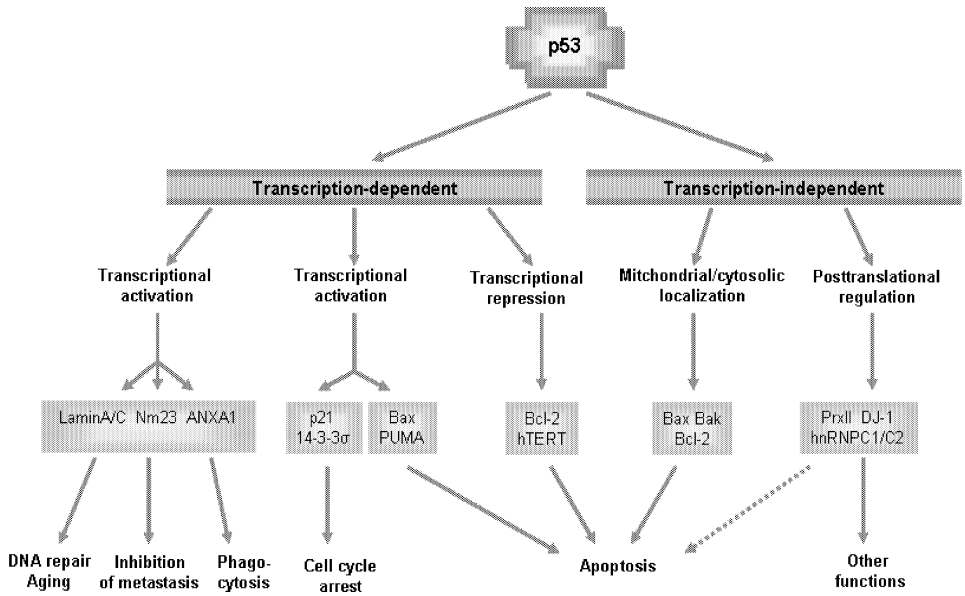

Figure 10. Evolving model for transcription-dependent and independent functions of p53.

CONCLUSIONS

PAPER I – Analysis of the effects of hTERT expression on p53-induced apoptosis.

- Endogenous hTERT protein is downregulated after activation of both exogenous and endogenous wt p53.
- Constitutive expression of hTERT inhibits both exogenous and endogenous p53-mediated cell death.
- hTERT exerts its anti-apoptotic function independently of its function in telomere length maintenance.
- hTERT promotes colony formation in spite of activation of endogenous p53.
- p53-mediated downregulation of hTERT may be critical for the p53-dependent elimination of tumor cells that already express elevated levels of hTERT.

PAPER II – Characterization of hTERT anti-apoptotic function.

- Constitutive expression of hTERT blocks apoptosis upstream of the mitochondria
- hTERT does not affect p53-dependent transcription of target genes.
- hTERT inhibits activation but not upregulation of Bax.
- Inhibition of hTERT by siRNA induces Bax activation and cell death.
- hTERT blocks p53-induced mitochondrial apoptosis by inhibition of Bax activation
- hTERT antagonizes both p53-dependent and independent apoptosis.

PAPER III – Analysis of p53-regulated proteome upon DNA damage.

- Fifty-five differentially expressed proteins upon wt p53 activation were identified by 2DE and mass spectrometry.

- Forty-two are upregulated and 13 are downregulated in a p53-dependent manner. The proteins are involved in multiple cellular processes.

- Eighteen among the induced and 5 of the downregulated proteins lack p53-consensus sites in their promoter, indicative of transcription-independent regulation by p53.

- Differential expression is mediated by p53 activation and is not a result of induction of apoptosis per se.

- p53 regulates protein expression via both transcription dependent and independent mechanisms.

PAPER IV– Analysis of p53-dependent protein phosphorylation.

- Forty-four proteins are phosphorylated after activation of wt p53 by DNA damage.

- Some proteins show changes in total protein levels as well as phosphorylation after p53 activation whereas others are only phosphorylated without any net change in total levels.

- p53 regulates a subset of proteins posttranslationally by phosphorylation.

- This is an additional strategy by which p53 regulates expression/activity of proteins.

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THE END

…or as they say, only the end of the beginning…

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