REGULATION OF p53-DEPENDENT TRANSCRIPTION

Arvi Jõers

Department of Cell Biology, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy (in Cell Biology) on May 18, 2004 by the Council of the Institute of Molecular and Cell Biology, University of Tartu.

Opponent: Klas Wiman, PhD, Professor of Molecular Cell & Tumor Biology, Dept. of Oncology-Pathology, Karolinska Institute, Stockholm, Sweden.

Commencement: Room No 217, Riia 23, Tartu on June 28, 2004 at 11.00

The publicationof this dissertation is granted by the University of Tartu

© Arvi Jõers

CONTENTS

LIST OF ORIGINAL PUBLICATIONS 4
LIST OF ABBREVIATIONS 5
INTRODUCTION 6
1. REVIEW OF LITERATURE 7
  1.1. p53 is a tumour suppressor protein 7
  1.2. p53 is a transcriptional activator 8
  1.3. Signalling to p53 10
  1.4. p53 target genes and their action 12
    1.4.1. Cell cycle inhibitors 12
    1.4.2. Apoptosis promoting genes 13
  1.5. Choosing between cell cycle arrest and apoptosis 15
  1.6 p53 and *in vivo* tumour models 16
  1.7 p53-independent effects of genotoxic stress on cell proliferation 17
  1.8 Tumour therapy through the p53 18
AIMS OF THE STUDY 20
2. RESULTS AND DISCUSSION 21
  2.1. The activities of monomeric p53 can be inhibited by mutated p53 without heterooligomerisation (I) 21
  2.2. Screening for p53 activators 21
  2.3. DNA damage dose-dependent responses of p53 pathway (II, III) 24
    2.3.1 p53 protein accumulation in response to genotoxic stress (II, III) 24
    2.3.2 Transactivation activity of p53 is uncoupled from its protein accumulation and can be regulated by on/off manner (II, III) 25
    2.3.3 p53-dependent transcription follows the stochastic model for gene activation 28
CONCLUSIONS 30
REFERENCES 31
KOKKUVÕTE 47
ACKNOWLEDGEMENTS 49
CURRICULUM VITAE 50
ELULOOKIRJELDUS 51

LIST OF ORIGINAL PUBLICATIONS

Current thesis is based on the following original publications which will be referred to by their Roman numerals

I **Jõers A**., Kristjuhan A., Kadaja L. and Maimets T. (1998) Tumour associated mutants of p53 can inhibit transcriptional activity of p53 without heterooligomerization. *Oncogene* **17**: 2351 – 2358.

II Jaks V., **Jõers A**., Kristjuhan A. and Maimets T. (2001) p53 protein accumulation in addition to the transactivation activity is required for p53-dependent cell cycle arrest after treatment of cells with camptothecin. *Oncogene*. **20**: 1212-1219.

III **Jõers A**., Jaks V., Kase J. and Maimets T. (2004) p53-dependent transcription can exhibit both on/off and graded response after genotoxic stress. *Oncogene* Accepted for publication.

LIST OF ABBREVIATIONS

Arf	alternative reading frame
Arg	arginine
ASPP	apoptosis stimulating protein of p53
ATM	ataxia-telangiectasia mutated
ATR	ATM and Rad3 related
Bax	bcl-2-associated protein X
Bcl-2	B-cell lymphoma protein 2
BH3	bcl-2 homology 3
cdc	cell division cycle control protein
Cdk	cyclin-dependent kinase
Chk	checkpoint kinase
DNA-PK	DNA-associated protein kinase
ES cells	embryonic stem cells
EGFP	enhanced green fluorescent protein
Gadd45	growth arrest and DNA-damage-inducible protein 45
HAUSP	herpesvirus-associated ubiquitin-specific protease
His	histidine
IL-3	interleukine 3
IR	ionising radiation
Leu	leucine
MAP4	microtubules-associated protein 4
mdm2	mouse double minute 2
MEF	mouse embryonic fibroplast
Miz	myc-interacting zinc-finger 1
MMTV	mouse mammary tumor virus
mSIN3a	mammalian sin3a
Noxa	nicotine adenine dinucleotide
p53AIP1	p53-regulated apoptosis-inducing protein 1
PERP	p53 apoptosis effector related to PMP-22
PIG-3	p53-induced gene 3
PIP_3	phosphatidylinositol triphosphate
PKB	protein kinase B
PRIMA-1	p53 reactivation and induction of massive apoptosis
PTEN	phosphatase and tensin homolog
Pu	purine
PUMA	p53 upregulated modulator of apoptosis
Py	pyrimidine
ROS	reactive oxygen species
SV-40	simian virus 40
SWI/SNF	switch/sucrose non-fermenting
TFIID	transcription factor II D
Trp	tryptophan
UV	ultra-violet light
WAF1	wild-type p53-activated fragment 1

INTRODUCTION

Multicellular organisms must keep their cell number under control. Failure to do so can lead to many pathologies, from which the tumours are by far the most life-threatening. There are many mechanisms evolved during the evolution that can avoid the unwanted proliferation of individual cells. Among others the tumour suppressor p53 has received a special attention over the last 15 years.

p53 is not necessary for normal cell cycle or development. However, it becomes a very important player, if cell faces stressful conditions. Originally described as an important protein in DNA damage response pathway, p53 has also been shown to react in the case of other stress signals. If p53 is absent or inactive, the unwanted proliferation of cells is not avoided and this leads quickly to the development of tumours.

The range of p53 studies is wide. It covers protein crystallography and chemistry, molecular interactions, cell biology, developmental biology, tumour development and epidemiology to name just a few. Hopefully my results, summarised in this study, will also contribute to the understanding of function of this interesting molecule and of pathways it is involved.

1. REVIEW OF LITERATURE

1.1. p53 is a tumour suppressor protein

The function of p53 is well defined by knockout studies. p53 deficient mice develop normally (with some exceptions, see below) and are phenotypically indistinguishable from their wt littermates. However, all p53 knockout mice will get tumours before 6 months of age (Donehower et al., 1992). 70% of these are lymphomas and 20% are sarcomas. This defines p53 as a *bona fide* tumour suppressor and indicates that some other pathway cannot compensate for the loss of p53. The tumour spectrum may differ in different strains of mice (Blackburn and Jerry, 2002), but lymphomas remain the prominent type of tumours in p53 knockout mice. Accordingly, the analysis of normal bone marrow cells in p53-negative mice revealed a 20-fold elevation of spontaneous stable chromosomal aberrations compared to wt (Bouffler et al., 1995). Genomic instability is also detected in other tissues (Fukasawa et al., 1997). This led to the defining the p53 as a "guardian of the genome" (Lane, 1992).

Although not detected in the first knockout study, the developmental defects in p53-/- embryos are present at higher frequencies. Considerable number of female knockout mice exhibit exencephaly (brain develops outside the scull) and subsequent unencephaly (lack of brain) (Armstrong et al., 1995). Female mice are also born in smaller numbers than expected. Also the teratogen induced *in utero* death and resorption of embryos are more frequent with p53 -/- genotype (Nicol et al., 1995). In adult knockout mice the liver (Dumble et al., 2001) and the eyes (Ikeda et al., 1999) show some abnormalities, whereas the development of skeletal muscles is completely normal (White et al., 2002). p53 seems to have role in suppression of developmental abnormalities in general but not in some specific developmental process.

p53 heterozygous (+/-) mice exhibit an elevated tumour incidence compared to the wt, but they survive longer than knockouts (Donehower et al., 1992). Approximately half of the spontaneous tumours in p53 +/- mice retain their wt allele regardless of tumour type (Venkatachalam et al., 1998). Wt p53 is functional in these tumours, this indicates that its level of expression level might not be high enough to effectively suppress tumour development. Another support for haploinsufficiency comes from study of p53 activity during embryogenesis. It is much lower than expected in p53 heterozygous mice (Gottlieb et al., 1997). These facts indicate that p53 is normally present in cells at limiting amounts as further reduction of its level leads to the impairment of its activity. This hypothesis is further supported by results from Serrano and colleagues. They generated and described a "super p53" mouse carrying a third copy of wt p53 controlled by endogenous promoter (Garcia-Cao et al., 2002). These mice are more resistant to tumours than wt mice and exhibited elevated p53 activity in response to irradiation. It would be interesting to analyse the p53 status in the few tumours developed in these mice.

p53 functions as a tumour suppressor also in humans. Its inactivation by mutations is a most common genetic alteration in human tumours - approximately 50% of tumours contain the mutant p53 (Hollstein et al., 1994). Most of these are missense mutations, leading only to one amino acid change. Distribution of mutations along the p53 molecule is highly non-random, they are clustered in the central part of the molecule

and 40% of mutations localise to only 3 "hot spot" amino acids. Because the p53 is a tetramer (Friedman et al., 1993) the mutant form of the molecule can heterodimerise with wt and disrupt the activity of the latter. This dominant negative effect is thought to be the main reason for high number of missense mutations. Indeed, tumour-associated mutant p53 is able to accelerate tumour incidence at the background of wt protein, but not in p53 knockout background (Harvey et al., 1995; Hegi et al., 2000). Some mutants are described as "gain-of-function" mutants, being able to enhance the tumourigenesis even in the absence of wt p53 (Dittmer et al., 1993; Hsiao et al., 1994). Whether this is true also *in vivo* remains to be analysed.

p53 can be used also as a prognostic indicator of tumour development. Mutations in coding sequence of p53 gene are associated with poor prognosis in breast (Bergh et al., 1995; Kovach et al., 1996), liver (Honda et al., 1998) and colon (Pricolo et al., 1997) carcinoma, in some lymphomas (Rodriguez et al., 1991) and leukaemias (Hsiao et al., 1994). The information about the p53 status should help in choosing the most appropriate scheme for tumour treatment. Germline p53 mutations in humans leads to the Li-Fraumeni syndrome that is characterised by early onset of tumours (Akashi and Koeffler, 1998).

1.2. p53 is a transcriptional activator

The transcriptional activation function is the best-characterised biochemical activity of p53. p53 can bind sequence-specifically to DNA and activate transcription if the binding site is in vicinity of basal promoter (El-Deiry et al., 1992; Funk et al., 1992). The DNA binding site for p53 is defined quite loosely - only 4 out of 20 nucleotides are absolutely fixed. The consensus site contains two copies of motif 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3', separated by 0-13 nucleotides. p53 binding sites occurring in human and mouse genomes are derivatives of this sequence and almost always contain some discrepancy from the consensus. More than 70 known or potential p53 binding sites are described (Qian et al., 2002), but only one of them is a perfect match with the p53 consensus DNA binding site. The high variation in binding site sequences is accompanied with very different binding efficiencies. In context of the same heterologous promoter two thirds of them are not capable of mediating p53-dependent transcriptional activation, whereas at least some of them can do this in their natural context (Qian et al., 2002).

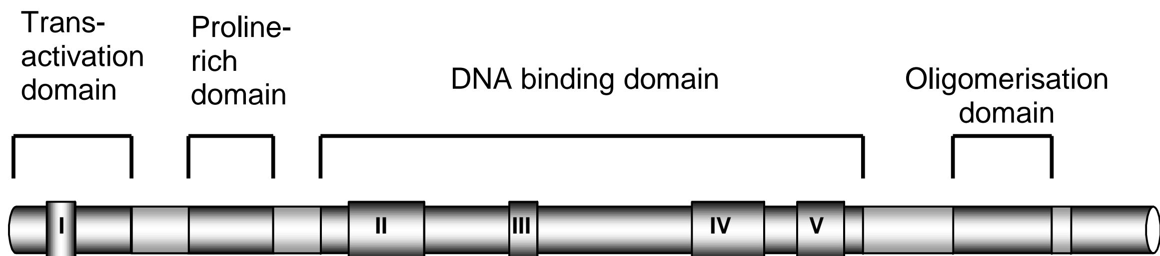


Figure 1. Schematic representation of the p53 molecule. The five conserved regions are indicated by Roman numerals.

p53 binds to DNA through its central domain (amino acids 100-300) (Bargonetti et al., 1993; Halazonetis and Kandil, 1993; Pavletich et al., 1993; Wang et al., 1993) (Figure 1). The structure of this domain consists of a β-sandwich, comprising of two antiparallel β sheets that serve as a scaffold for the structural elements at the DNA-protein interface (Cho et al., 1994). Four evolutionary conserved regions comprise two large loops (L2 and L3) and the loop-sheet-helix motif that have critical roles in providing the structure, surfaces and residues that actually contact DNA. The conformation of DNA binding domain is further stabilised by zinc, chelation of zinc forces the p53 to take "mutant" conformation that is unable to bind DNA (Verhaegh et al., 1998; Verhaegh et al., 1997).

The N-terminal part of the p53 (amino acids 1 - 40) functions as a transactivation domain - when fused to DNA binding domain of the GAL4 protein it can activate transcription from promoters containing GAL4 binding site (Fields and Jang, 1990). This domain can bind components of TFIID (Liu et al., 1993; Lu and Levine, 1995; Thut et al., 1995) and contain two amino acid residues, Leu 22 and Trp 23 of human p53, critical for transactivation function of p53 (Lin et al., 1994). In addition, the proline-rich region seems to be important for transactivation *in vivo*, as p53 with amino acids 75-91 deleted is transactivation deficient in knock-in mice (Frank Toledo, unpublished results). p53 also binds several subunits of the human chromatin remodelling factor SWI/SNF (Lee et al., 2002).

Transactivation capability seems to be a critical property of p53 to function as a tumour suppressor. Amino acids Leu 25 and Trp 26 of mouse p53 (equivalent to human Leu 22 and Trp 23) are absolutely necessary for transcription activation function. If these residues are mutated in endogenous p53 (knock-in experiments), the mice are tumour prone similarly to p53 knockouts. Both induced and spontaneous tumours arise with similar frequencies, the tumour spectrum is the same and isolated cells in culture behave like p53-/- cells (Chao et al., 2000; Jimenez et al., 2000)(Geoff Wahl, unpublished results).

Another strong evidence arguing for importance of transactivation function in tumour suppression comes from the analysis of mutations of p53 in human tumours. Majority of mutations occurs in conserved regions II - V that are important in DNA binding. Mutational hotspots are directly involved in protein-DNA contact formation (Arg248) or maintaining the proper conformation (His175) (Cho et al., 1994).

In addition to transcriptional activation, p53 can also repress the transcription from several target genes. These include stathmin (Ahn et al., 1999), MAP4 (Murphy et al., 1996), survivin (Hoffman et al., 2002), cyclin B1 (Innocente et al., 1999) and cdc2 (Taylor et al., 2001). How exactly p53 represses the target promoters is not clear, but unusual binding site for p53 seems to be responsible in the case of survivin. 3-nucleotide insertion between two pentameric DNA sequences in p53 binding site defines a new function - p53 can bind to it, but can't activate transcription. Mere binding seems to block the influence of other transactivators (Hoffman et al., 2002). However, recent experiments suggest that transcriptional repression function of p53 needs p21/WAF1 to be present in cells. According to these results the target gene repression is not direct effect of p53, but mediated through its positively regulated target gene (see below).

Although other activities than transactivation have been assigned to p53 (Cox et al., 1995; Dudenhoffer et al., 1998; Mummenbrauer et al., 1996), their roles in tumour suppression *in vivo* remains to be clarified.

1.3. Signalling to p53

p53 becomes active in transcription only in response to certain signals. These include DNA damage (Maltzman and Czyzyk, 1984), aberrant expression of oncogenes (de Stanchina et al., 1998; Zindy et al., 1998), depletion of ribonucleotides (Linke et al., 1996), hypoxia (Graeber et al., 1994) and other types of stress. In all these cases p53 itself does not detect the stress directly, but receives the information through the signalling pathways. The activation of p53 involves two types of events - the p53 protein level rises in the cell and the transactivation activity of p53 is enhanced. The first is due to the blocked degradation of the protein (Maltzman and Czyzyk, 1984) and enhanced translation of p53 mRNA (Fu et al., 1996). Changes in transactivation activity are accompanied with changes in posttranslational modifications of p53 protein.

It is not clear how cells initially sense the DNA damage. Inhibition of RNA polymerase II is suggested as a trigger for p53 in response to UV and different chemicals (Ljungman et al., 1999). Indeed, the p53 accumulation correlated well with inhibition of mRNA synthesis, but not with DNA strand brakes formation. Ionising radiation, however, induced p53 without any effect on mRNA synthesis, indicating that multiple mechanisms exist to activate p53 in response to DNA damage. Recent discovery suggests that disruption of nucleolus is the key event in stabilising the p53 (Rubbi and Milner, 2003), as p53 is not accumulating even in the case of large amount of DNA damage when nucleolus is intact.

One of the earliest events in response to ionising radiation (IR) is the activation of ATM kinase. Among other targets it can phosphorylate the serine 15 (S15) in human p53 *in vitro* (Banin et al., 1998; Canman et al., 1998) and p53 phosphorylation in response to IR is delayed in cells were ATM is defective. In addition, ATM can phosphorylate another kinase Chk2, which in turn is capable of phosphorylating p53 (serine 20) and seems to be necessary for IR induced apoptosis (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000). However, in some human cells the presence of Chk2 is not necessary for p53 phosphorylation and activation (Ahn et al., 2003; Jallepalli et al., 2003). ATM related kinase ATR is involved in activation of p53 in response to UV either directly (Lakin et al., 1999; Tibbetts et al., 1999) or through Chk1 kinase (Shieh et al., 2000). Also serines 6, 9 and 46 as well as threonine 18 can get phosphorylated in response to DNA damage (Appella and Anderson, 2001). However not all the phosphorylated serines are absolutely necessary for p53 response. Missense mutation in endogenous p53 gene that changes serine 23, the murine equivalent of human serine 20, to alanine does not affect p53 accumulation or p53-dependent apoptosis in ES cells or in thymocytes (Wu et al., 2002). Similar change at serine 18 partially abolishes p53-dependent transactivation, but has no influence on spontaneous tumorigenesis (Chao et al., 2003).

Phosphorylation of p53 at S15 and S20 disrupts its association with mdm2 protein (Shieh et al., 1997). In normal cells mdm2 binds to N-terminus of p53 and inhibits the transcriptional activity of the latter (Oliner et al., 1993). In addition mdm2 binding

promotes nuclear export and degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). Mdm2 has an E3 ubiquitin ligase activity and it is able to drive the ubiquitinylation and subsequent degradation of the p53 protein. p53 regulation seems to be the main activity of mdm2, as the lethality of mdm2 knockout mice can be rescued by p53 deficiency (mdm2-/- p53-/- mice are like p53-/- mice) (Montes de Oca Luna et al., 1995). Mdm2 is a p53 target gene itself and accumulates in response to p53 activation (Barak et al., 1993; Juven et al., 1993). Because p53 is phosphorylated at critical sites, mdm2 cannot initiate the degradation of p53. As soon as nonphosphorylated p53 appears, either by dephosphorylation or *de novo* synthesis, it is rapidly degraded because of high levels of mdm2. This mechanism probably ensures the quick turnoff of p53 response. Mdm2 regulates p53 activity not only to response to genotoxic stress, but also in homeostatic tissues without any DNA damage. Mice with a hypomorphic allele of mdm2 have elevated levels of p53 activity (Mendrysa et al., 2003) and these mice are small and radiosensitive.

In addition to p53 stability its transactivation activity is also subject to regulation by posttranslational mechanisms. Phosphorylation and acetylation of several residues in p53 C-terminus are associated with this process (Appella and Anderson, 2001). By classical model the C-terminus of p53 inhibits the sequence-specific DNA binding and modifications like phosphorylation and acetylation can alleviate this inhibition. However, recent results challenge this model. When assayed on chromatin templates, the C-terminus is actually necessary for DNA binding and acetylation of p53 is completely dispensable for transcription activation from *in vitro* chromatin templates (Espinosa and Emerson, 2001). Instead, acetytransferase p300 acetylates nucleosomal histones in p53-dependent manner. *In vivo* the p53-dependent transcription, but not DNA binding is dependent on p53 acetylation (Barlev et al., 2001). Acetylation seems to regulate cofactor recruitment rather than DNA binding of p53. In addition acetylation may affect p53 degradation because p53 acetylation and ubiquitinylation are alternative modifications working on the same amino acids and therefore one type of modification excludes the other (Jin et al., 2002; Li et al., 2002; Sabbatini and McCormick, 2002).

In addition to DNA damage, hypoxia can induce p53 accumulation (Graeber et al., 1994). The protein kinase ATR is responsible for p53 phosphorylation and accumulation under the hypoxic conditions (Hammond et al., 2002), but reactive oxygen species (ROS) are also important in p53 stabilisation (Chandel et al., 2000). Accumulated p53 mainly associates with transcriptional repressor mSIN3a, indicating that transcriptional repression may be the dominant activity of p53 in the case of hypoxia (Koumenis et al., 2001).

p53 can react to aberrant expression of oncogenes. The central protein in this signalling pathway is Arf/p19 (Arf/p14 in humans). How exactly Arf senses the proliferative signal is not known, but E2F family transcription factors are important in this process (Bates et al., 1998). Arf can bind mdm2 and inhibit its ubiquitin ligase activity (de Stanchina et al., 1998; Kamijo et al., 1998; Zhang et al., 1998; Zindy et al., 1998). This leads to the p53 accumulation and transcriptional activation of its target genes. Arf seems to play some role in DNA damage pathway also, as the response of Arf-/- cells to damage is something between wt and p53-/- cells (Khan et al., 2000). Despite of that lots of p53-dependent responses are preserved in Arf-/- cells, arguing against the dominant role of Arf in DNA damage response. Arf-/- mice are tumour prone, indicating

the importance of oncogene activation pathway *in vivo*. p53-/- Arf-/- mice have a tumour incidence similar to the p53-/- mice (Moore et al., 2003). When mouse embryo fibroblasts (MEF) are immortalised in cell culture they lose either p53 or Arf expression (Kamijo et al., 1997), but almost never both. This places p53 and Arf to the same tumour suppression pathway.

Overexpression of myc oncogene can induce the DNA damage and activate p53 without Arf (Vafa et al., 2002).

1.4. p53 target genes and their action

p53 executes its growth inhibitory function through its target genes. More than hundred p53 target genes are described, but in lots of cases their role in p53 pathway remains to be determined. Most of the target genes with known function can be divided into two big classes: cell cycle inhibitory genes and inducers of apoptosis. mdm2 is the most well-known member of the third class of target genes - it is a negative regulator of p53 (Barak et al., 1993; Juven et al., 1993)

1.4.1. Cell cycle inhibitors

Waf1

Waf1 (p21/CIP) was one of the first p53 target genes described (El-Deiry et al., 1993). There are two p53 binding sites in waf1 promoter, 1.3 and 2.2 kb upstream from transcription start site. Waf1 is a 21 kDa protein capable of inhibiting Cdk2-cyclinE complex and stopping the cell cycle progression from G1 to S phase. MEFs from waf1 -/- mouse have almost completely lost their ability to arrest in G1 phase in response to IR, indicating that waf1 is the main mediator of this p53-dependent process (Deng et al., 1995). However, they possess some residual G1 arrest, if compared with p53 -/- MEFs, indicating that there are some waf1 independent mechanisms for p53 to induce the G1 arrest. Waf1 is not required for p53-dependent mitotic spindle checkpoint or p53-dependent apoptosis in mouse thymocytes. This data clearly defines waf1 as a mediator of p53-dependent G1 arrest.

Waf1 deficient mice do get tumours, but later than p53-/- mice do (Martin-Caballero et al., 2001). The average age of tumour development for waf1-/- mice is 16 months whereas p53-/- mice get tumours at the age of 6 months. Waf1 knockout mice develop mostly sarcomas, but the T-cell lymphomas, that are the main tumour type in p53 deficient mice, are completely absent. In contrast to p53 knockouts the waf1-/- mice are not susceptible to radiation induced tumourigenesis. These differences indicate that although waf1 has some role in tumour suppression there must be another pathways for p53 to suppress tumourigenesis. This is further supported by the fact that waf1 is not mutated in human tumours (Shiohara et al., 1994; Shiohara et al., 1997).

14-3-3σ

14-3-3σ is a member of a family of proteins that regulate cellular activity by binding and sequestering phosphorylated proteins. 14-3-3σ is induced after genotoxic

stress in p53-dependent manner, also p53 overexpression induces 14-3-3σ (Hermeking et al., 1997). This induction seems to be specific to epithelial cells, as 14-3-3σ is not induced in fibroblasts or endothelial cells. Epithelial cells arrest mainly in G2 in response to genotoxic stress. In the absence of 14-3-3σ cells show initial arrest, but fail to maintain it. Cells proceed to mitosis and die due to the mitotic catastrophe (Chan et al., 1999). The role of 14-3-3σ is probably to sequester cdc2-cyclinB complex to cytoplasm thereby avoiding it to enter the nucleus and induce mitosis.

14-3-3σ is frequently silenced in human cancers because of the methylation of its promoter region (Ferguson et al., 2000; Iwata et al., 2000; Suzuki et al., 2000).

GADD45

Gadd45 was identified as a p53 target gene already more than 10 years ago (Kastan et al., 1992), but the exact role of this protein is still unclear. Gadd45 deficient mice born normally and show no spontaneous tumour formation during their lifetime (Hollander et al., 1999). However the cells from these mice show centrosome abnormalities and genomic instability. If irradiated, gadd45-/- animals develop tumours with decreased latency compared to wt. Thymic apoptosis is normal in these mice, but they show deficiency in G2 checkpoint in splenic lymphoblasts. The global genomic repair of UV induced lesions is impaired in gadd45-/- MEFs to the similar extent it is impaired in p53-/- MEFs (Smith et al., 2000). Also the stabilisation of p53 is greatly abolished in Gadd45-/- MEFs in response to UVB treatment (Jin et al., 2003). This suggests that Gadd45 is part of some positive feedback loop in p53 stabilisation pathway. Interestingly, the gadd45-/- mice exhibit a low frequency of exencephaly like p53-/- mice (Hollander et al., 1999). Although the exact biochemical activity of gadd45 is still not clear, it certainly has a role in p53 tumour suppression pathway.

1.4.2. Apoptosis promoting genes

PTEN

PTEN is a phosphatase capable of removing the phosphate group from the lipid second messenger PI(3,4,5)P_3 (Maehama and Dixon, 1998). By this the PTEN blocks the signalling pathways employing this molecule. Among others the Akt/PKB protein kinase depends on PI(3,4,5)P_3. Akt can promote cell survival in many ways being well-established antiapoptotic factor. It can phosphorylate mdm2 and promote the nuclear localisation of mdm2 (Mayo et al., 2002). Induction of PTEN expression leads to the inhibition of Akt activity and apoptosis in many cells, loss of PTEN function results in an increased PI(3,4,5)P_3 levels and hyperactivation of Akt pathway (Stambolic et al., 1998). PTEN itself is a tumour suppressor gene, as it is frequently mutated in human tumours (Cantley and Neel, 1999) and PTEN -/+ mice get different tumours (Di Cristofano et al., 1999) (PTEN-/- mice are not viable).

Wt p53 is a direct transcriptional activator of PTEN gene (Stambolic et al., 2001). It binds to DNA consensus element in PTEN promoter and this is necessary for p53-

dependent regulation of PTEN. Especially noteworthy is the fact that p53-dependent apoptosis is severely impaired in PTEN -/- fibroblasts. This can be compensated by introducing the PTEN gene under the control of the fragment of its p53-dependent promoter, but no compensation is achieved if p53 binding site in mutated in front of the PTEN gene. PTEN is one of the few examples among apoptosis-related p53 target genes whose deletion really impairs the p53-dependent apoptosis. Also the fact that mutations in p53 and PTEN are mutually exclusive in breast carcinoma puts them to the same tumour suppression pathway (Kurose et al., 2002).

Noxa

Noxa is a small protein that contains BH3 (Bcl-2 homology 3) domain as an only recognisable motif. IR induces it in thymocytes undergoing p53-dependent apoptosis, but not in p53-/- thymocytes that do not die after the IR. (Oda et al., 2000). Noxa protein localises into mitochondria and its overexpression can initiate apoptosis. Noxa gene has a p53 binding site in its promoter and critical nucleotides in this binding site are necessary for p53-dependence. Antisense oligonucleotide for noxa gene blocks its expression and can attenuate the p53-dependent apoptosis in response to p53 overexpression and IR. However, the p53-dependent apoptosis in most cell types is intact in noxa-/- mice (Villunger et al., 2003). The only exemption seems to be apoptosis in MEFs induced by etoposide. Another report (Shibue et al., 2003) argues for more widespread role for noxa in p53-dependent apoptosis. This indicates that p53 induces apoptosis at least partly by inducing noxa expression.

PUMA

PUMA is also a BH3-only protein that, like noxa, localizes into mitochondria and can initiate apoptosis. PUMA is a p53 target gene (Nakano and Vousden, 2001; Yu et al., 2001) and a potential mediator of p53-dependent apoptosis. When PUMA expression is suppressed by antisense oligonucleotide, the p53-dependent apoptosis is reduced although not eliminated. In some PUMA-/- cells p53-dependent apoptosis is attenuated, as well as p53-independent cell death (Jeffers et al., 2003; Villunger et al., 2003). This again indicates for existence of many parallel pathways for p53-dependent apoptosis.

p53AIP1

p53AIP1 is a protein without any detectable homology to known proteins. It is strongly induced by p53 and also by genotoxic stress in p53-dependent manner (Oda et al., 2000). It localizes to mitochondria and can induce apoptosis by altering the membrane potential in mitochondria. p53AIP1 expression is elevated later than the expression of waf1 and mutating the serine 46 in p53 to alanine abolishes the p53AIP1 induction. Blocking p53AIP1 expression by antisense oligonucleotides almost completely inhibits apoptosis induced by p53 overexpression. This underlines the importance of p53AIP1 in p53-dependent apoptosis.

Bax

Bax was the first apoptosis promoting gene described as a p53 target gene (Miyashita and Reed, 1995). Despite its wide acceptance as a mediator of p53-dependent apoptosis there are evidence that bax is not a universal mediator of p53 induced apoptosis. In many cases bax is not induced when other p53 target genes are and bax may not be a p53 target gene in mouse at all (Schmidt et al., 1999). Thymocyte apoptosis, known to be a p53-dependent process, is intact in bax-deficient mice (Knudson et al., 1995). Recently the new p53 binding site was identified in the first intron of human and murine bax gene (Thornborrow et al., 2002), but whether bax is a true p53 target gene is still an open question.

PIG3

PIG3 was identified as a gene induced after p53 overexpression in cells committed to undergo apoptosis (Polyak et al., 1997). It is though to participate in metabolism of reactive oxygen species (ROS). In addition to originally described p53-responsive element in PIG3 promoter, a new p53-binding DNA sequence was recently identified (Contente et al., 2002). It consists of microsatellite DNA and is polymorphic. This suggests that there is a considerable variation in PIG3 induction among people.

p53 seems to be able to induce apoptosis also directly by enhancing the cytochrome C release from mitochondria. Recent reports (Chipuk et al., 2004; Chipuk et al., 2003; Dumont et al., 2003; Mihara et al., 2003) show that p53 can bind to Bcl-XL and release Bax from inhibition by Bcl-X. This leads to the pore formation in mitochodrial membrane and cytochrome C release. Some p53 mutants, that are unable to activate transcription, are capable of initiating this mitochondrial arm of apoptosis, whereas tumour-associated point mutants are not. Does transcription-independent apoptosis play a role in the tumour supression function of p53 remains to be seen.

1.5. Choosing between cell cycle arrest and apoptosis

Whether the cell undergoes cell cycle arrest or apoptosis in response to p53 activation is a complex question and depends on many factors. Cell type, severity of damage and presence of survival factors are just a few conditions influencing the output.

The first possibility is that p53 can somehow discriminate between binding sites in front of cell cycle arrest genes and binding sites in front of the apoptosis genes. Indeed, when analysed in the context of heterologous promoter in yeast assay, most binding sites from cell cycle genes can support p53 dependent transactivation, whereas the most binding sites from apoptosis genes can't (Qian et al., 2002). This indicates some general difference in functioning of these two classes of binding sites. p53 might also use help of specific cofactors in binding to the right promoters. ASPP1 and ASPP2 are two closely related proteins capable of influencing the promoter binding choice of p53 (Samuels-Lev et al., 2001). Coexpression of p53 and either ASPP proteins can markedly stimulate p53-dependent transcription from the promoters of apoptosis-related genes bax and PIG-3. At the same time ASPP expression had no effect on p21 or mdm2 promoter. p53-dependent apoptosis was also enhanced in ASPP expressing cells.

p53 family members p63 and p73 seem to have similar cofactor activities. Their expression is necessary for p53-dependent activation of proapoptotic genes PERP and bax, but not cell cycle gene waf1 or mdm2 (Flores et al., 2002). In p63-/- p73-/- cells the p53-dependent apoptosis is absent (but not in single knockouts). Chromatin immunoprecipitation experiments showed that p63 binds to p53 target promoters. This binding is dependent on p53 in the case of waf1 and mdm2, but independent of p53 in the case of apoptotic genes noxa, bax and PERP. These experiments clearly define ASPP, p63 and p73 as p53 transcriptional cofactors that are able to direct the binding site preference of p53. Transcription factor Brn-3a can antagonise the apoptotic effect of p53 but co-operates with p53 to induce cell cycle arrest by driving the selective p53 target gene activation (Budram-Mahadeo et al., 2002).

Upstream activators of p53 can also direct the choice between cell cycle arrest and apoptosis. In mouse thymocytes deficient in ATM kinase the p53-dependent cell cycle block is absent, but p53 dependent apoptosis is functional (Barlow et al., 1997). Interestingly the accumulation of p53 is also impaired suggesting that the stability and transcriptional activity of p53 protein are regulated separately. If another kinase, the DNA-PK, is inactivated the p53 looses its apoptotic activity, but preserves the G1 arrest function (Wang et al., 2000). Chk2 -/- fibroblasts have a very similar phenotype (Jack et al., 2002). These results indicate that factors upstream from p53 can determine the path the p53 response should take. Indeed, phosphorylation of specific residues in p53 can influence its DNA binding site preference (Wang and Prives, 1995). Whether differential phosphorylation also determines the pathway choice *in vivo* remains to be clarified.

p53-mediated growth arrest versus apoptosis can be modulated by growth factors. Irradiation of murine hematopoietic cell line Baf-3 in the presence of interleukin-3 (IL-3) leads to G1 arrest, in the absence of IL-3 irradiation induces apoptosis (Canman et al., 1995). Both these processes are p53-dependent. Removal of IL-3 is accompanied with p53-independent downregulation of waf1. Similar cytokine-dependent modulation of cell cycle arrest versus apoptosis is described also for another system (Lin and Benchimol, 1995).

Transcription factor Myc together with its partner protein Miz can also determine the choice between p53-dependent apoptosis *versus* growth arrest (Seoane et al., 2002). They can bind to waf1 promoter and abolish both p53-dependent and -independent waf1 expression. Myc overexpression can convert cell cycle arrest to apoptosis in irradiated cells. Interestingly, the levels of two apoptotic p53 target genes PUMA and PIG-3 do not change in response to myc overexpression. This suggests that p53 activates both types of genes in either case and apoptosis/cell cycle block decision is made downstream of p53.

It is not clear whether all possibilities mentioned above take part of the decision process at the same time or do they have some sort of cell type specificity. In any case different cellular pathways are involved in apoptosis/cell cycle arrest decision and it would be naïve indeed to assign such a central task to p53 alone.

1.6 p53 and *in vivo* tumour models

As mentioned above, p53 can induce cell cycle arrest and apoptosis. In addition, p53 is also involved in other processes, including DNA repair, senescence, angiogenesis and the surveillance of genomic integrity (Evan and Vousden, 2001). In the case of

mutated p53 all these functions will be compromised. However, only some of them might be actually selected against during the tumour development whereas the loss of others may be just a consequence of p53 inactivation. This issue has been addressed using experimental lymphoma model, where the tumour is initiated by myc overexpression. It turned out that disruption of apoptosis by overexpression of bcl-2 completely alleviates pressure to inactivate p53 during lymphomagenesis (Schmitt et al., 2002). Also p53 +/- bcl-2 cells have an *in vivo* growth advantage over the p53+/- cells but not over the p53-/- cells, indicating that during the lymphoma formation and growth the apoptotic function of p53 is the dominant activity that is selected against. However, p53 and p16-dependent senescence and not apoptosis play the dominant role in tumour suppression during chemotherapy (Schmitt et al., 2002). Because the same tumour model was used in these studies it is clear that different p53 functions are important at different stages of tumour development.

Importance of apoptosis is underlined also by the fact that some p53 mutants, isolated from human tumours, can still activate cell cycle block but are deficient in apoptosis induction (Aurelio et al., 2000; Rowan et al., 1996). Mice who express a truncated large T antigen from SV-40 virus in their choroid plexus develop slowly growing benign tumours with high apoptosis rate (Symonds et al., 1994). In the p53+/- background local fast-growing nodules with little apoptosis appear and p53 is lost in these cells.

In different tumour models the cell cycle inhibitory function of p53 also seems to be important. Transgenic mice expressing an activated allele of Ha-ras under the control of the MMTV promoter in the salivary and mammary glands develop tumours in both tissues (Hundley et al., 1997). Crossing this transgene onto a p53 -/- background accelerated tumour growth, but levels of apoptosis were low and constant in all tumours regardless of tumour type. Instead, a higher growth rate and increased aneuploidy was evident in p53 -/- background.

When amino acid 172 is changed from arginine to proline in mouse p53 genomic locus the apoptotic activity of p53 is also lost, but cell cycle arrest function is retained, although in diminished manner. These mice do get tumours, but slower than p53 -/- mice (Liu et al., 2004), indicating there are some role for cell cycle inhibitory function of p53 in tumour development.

1.7 p53-independent effects of genotoxic stress on cell proliferation

Not all genotoxic stress-induced processes are p53-dependent. Rapid downregulation of cyclinE-cdk2 activity in response to DNA damage occurs even in cells lacking p53 (Rotman and Shiloh, 1999). Signalling starts with ATM/ATR and Chk2/Chk1. Chk2 and Chk1 kinases phosphorylate cdc25A and target it to degradation (Bartek and Lukas, 2001). Cdc25A is a protein phosphatase that normally removes the inhibiting phosphates from cdk2. When cdc25A is absent, the cdk2 stays phosphorylated at threonine 14 and tyrosine 15 and remains inactive. Another G1 checkpoint involves rapid degradation of cyclin D1 (Agami and Bernards, 2000). This leads to a release of waf1 from cdk4 to inhibit cdk2. Both these responses are rapid, but transient. They are later replaced by delayed and more sustained p53-dependent response. As a pure speculation one can imagine that weak DNA damage provokes only a transient response

during which DNA reparation can take place. More severe damage could lead to the accumulation of p53 and sustained cell cycle arrest or apoptosis.

If cell cycle arrest is not permanent, the cells have to get back to cycle. Little is known about this step, but recovery from UV induced cell cycle block needs c-jun activity (Shaulian et al., 2000). UV, but not IR, induces c-jun. C-jun can repress the waf1 transcription in p53-dependent manner and accordingly c-jun -/- cells have higher waf1 expression levels and exhibit decreased clonogenic survival. When expressed constitutively the c-jun prevents the UV induced cell cycle arrest. Interestingly, c-jun expression only affects the outcome of UV exposure and has no influence on the IR induced cell cycle arrest.

1.8 Tumour therapy through the p53

Because the inactivation of p53 by mutation is so common in human tumours, considerable effort have been made to restore the p53 activity in cells with mutant p53. Hopefully it would be enough to initiate proliferation block or apoptosis. The most straightforward method is the overexpression of wt p53 in tumour cells, but the main obstacle here is the delivery and these problems are beyond the purpose of this overview.

PRIMA-1 is a low molecular weight compound isolated as mutant p53 reactivator (Bykov et al., 2002). It can restore the transactivation function of His-175 mutant, induce the expression of endogenous p53 target genes and induce apoptosis in mutant p53-dependent manner. PRIMA-1 has no effect on wt p53. It can suppress the growth of human tumour xenographs in mouse and this suppression is dependent of mutant p53 expression. The mechanism behind the PRIMA-1 mediated mutant p53 reactivation is not known, but it induces the conformational change in p53 DNA-binding domain.

Another promising compound, CP-31398, has the ability to stabilise the wt conformation of mutant p53 protein (Foster et al., 1999). Incubation of mutant p53 expressing cells with this compound leads to the induction of p53 target genes. CP-31398 was able to suppress the tumour formation in mouse.

Both these compounds are also cytotoxic in p53 independent way. Whether the *in vivo* effects of these drugs rely more on the activation of mutant p53 or some other effect remains to be seen. Indeed, the connection between p53 stabilisation and cytotoxity of CP-31398 is challenged by the work showing that this compound is quite cytotoxic without any p53 (Rippin et al., 2002). Yet another compound, ellipticine, was recently described also capable of reactivating tumour-associated point mutants of p53 (Peng et al., 2003).

In vitro the DNA binding activity of mutant p53 can be induced also by short peptides, derived from p53 C-terminus (Selivanova et al., 1997), but they are not tested in mouse tumour models. The second-site suppressor mutations can also, at least partially, restore specific DNA binding of mutant p53 (Brachmann et al., 1998; Wieczorek et al., 1996), but the therapeutic usefulness of this scheme is hard to imagine.

Adenoviruses induce the p53 response through the action of their E1A gene product. This response is suppressed by another adenoviral protein E1B 54K. In the absence of E1B 54K, the virus can only replicate in p53 deficient cells. This simple, but elegant, scheme led to the development of therapeutic virus strain ONYX-015 that can only replicate in cells lacking the wt p53 activity and this leads to the lysis of cells

(Bischoff et al., 1996). Although this approach has earned some criticism (Edwards et al., 2002) the clinical trials look promising and had entered the phase III (Biederer et al., 2002; Nemunaitis et al., 2000).

During the conventional radiation and chemotherapy, normal cells are also affected. The treatment results in p53 activation and subsequent arrest or apoptosis in these cells leading to the hair loss, cell death in intestine and compromised immunity. Pifithrin α can temporarily suppress the p53 activity and therefore allows to use higher doses of radiation or chemotherapy (Komarov et al., 1999). It effectively protected mice from otherwise lethal doses of radiotherapy.

Many tumours retain the wt p53 gene, but its function is still compromised. One reason for that is the overexpression of mdm2, the negative regulator of p53 (Freedman et al., 1999). The peptide, capable of blocking mdm2-p53 interaction by binding to the mdm2, can induce the p53 response at least in cell culture (Bottger et al., 1997). Low-molecular weight compound with similar properties have been recently described (Vassilev et al., 2004). It also shows its effect in human tumour xenografts in nude mice. The problem with this approach is that it does not discriminate between normal and tumour cells.

AIMS OF THE STUDY

p53 can bind DNA and activate transcription from near-by promoters. This function is critical for p53 to carry out its tumor suppressor role. The objective of this study was to characterize the regulation of p53 transactivating activity. More specifically the aims of the present study were:

1. To characterize the mechanisms the mutated p53 can inhibit the transactivation activity of the wt p53.

2. To analyze the differences in p53 response pathway in low *versus* high level of DNA damage.

3. To clarify the relationship between the p53 protein accumulation and its transcriptional activity regulation in response to genotoxic stress.

4. To analyze the p53 response to genotoxic stress at single cell level

2. RESULTS AND DISCUSSION

2.1. The activities of monomeric p53 can be inhibited by mutated p53 without heterooligomerisation (I)

During the oncogenic transformation process, cells must get rid of the p53 growth suppressing activity. It is often achieved by point mutations occurring in the DNA binding domain of p53 in one allele. Although during the tumour progression the second allele is usually also lost, the coexpression of mutated and wt p53 proteins is enough to inhibit the wt p53 activity. It has been shown that mutated p53 can drive wt into mutant conformation (Milner and Medcalf, 1991) and the transactivation function of wt p53 is inhibited by mutated p53 (Kern et al., 1992). Heterooligomerisation between wt and mutated p53 is usually believed to be behind the wt p53 inactivation.

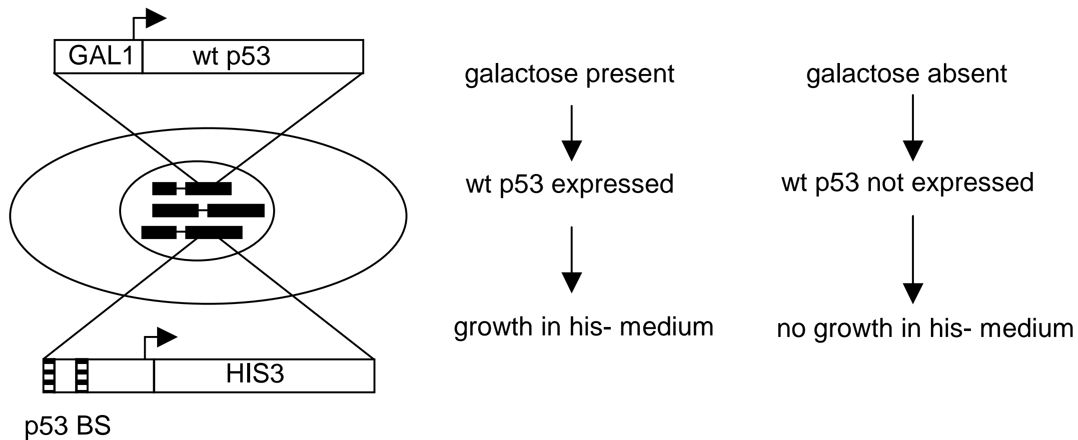
Here we show that mutated p53 can inhibit the activities of p53 also by other mechanisms (we use human p53 in our experiments). Monomeric p53, from where the oligomerisation domain (amino acids 324-355) is deleted, can activate transcription from p53-dependent promoters. Coexpression with p53, bearing the tumour-associated point mutation, inhibits the transactivation activity of the monomeric p53 (I, fig.2 and 3). Deletion of either N-terminal transactivation domain or C-terminal regulatory domain does not affect the ability of the mutated p53 to inactivate monomeric p53. If both these domains are absent, the mutated p53 looses its ability to inhibit the monomeric p53. At the same time none of the mutated p53 forms showed any suppression of p53-independent transcription (I, fig.4). This indicates that (1) suppression of transcription is limited to p53-specific promoters and (2) cellular viability is not compromised. Mutated p53 can also inhibit the growth suppression activity of monomeric p53 (I, fig.8). At the same time neither the expression nor the nuclear localisation of monomeric p53 is altered by coexpression with mutated p53 (I, fig.5 and 6). In addition, different point mutated p53 proteins form tetramers in cells, but fail to heterooligomerise with monomeric p53 (I, fig 7).

From this data we conclude that mutated forms of p53 can sequester some important cofactor necessary for p53-dependent transcription. This putative cofactor must be p53-specific, as the p53-independent transcription is not affected. Many p53 cofactors are identified, including ref-1 (Jayaraman et al., 1997), HMG-1 (Jayaraman et al., 1998), p33/ING1 (Garkavtsev et al., 1998) and others (see Literature overview). Mutated p53 could also saturate the capacity of some modifying enzyme and leave the monomeric p53 depleted from this necessary modification. Despite the exact nature of this putative cofactor, it should enhance the transcriptional activity of p53. This assumption became the basis of our strategy to identify p53 cofactors.

2.2. Screening for p53 activators

We decided to take advantage of the powerful methods of yeast genetics and set up a screen for p53 activators in *Saccharomyces cerevisiae*. p53 can function as a transcriptional activator in yeast (Scharer and Iggo, 1992). We constructed a yeast strain where the wt p53 is under the control of GAL1 promoter. This allowed us to regulate the

p53 expression by changing the concentration of galactose in the growth medium. We also cloned the yeast selectable marker gene under the control of p53-dependent promoter and integrated this construct into the yeast genome. In this way we obtained the yeast strain whose growth in selective media was dependent on the p53 activity (figure 2A). Then we determined the test growth medium where p53 is expressed, but not at levels high enough to support the yeast growth under the selective conditions (figure 2B). The activity of p53 is not sufficient in these conditions. After transfection with cDNA library we looked for the growth of yeast colonies on the test medium. These should have an elevated p53-dependent transcription, hopefully because of some p53 cofactor is expressed from the library plasmid.



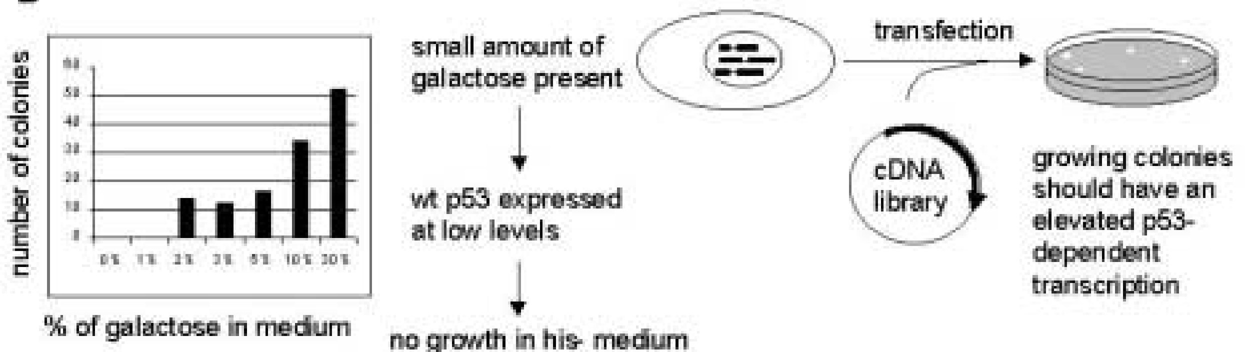


Figure 2. **A** – Schematic representation of yeast strain designed for screening of p53 activators. Yeast growth in selective medium is dependent on p53 expression, which in turn can be regulated by amount of galactose in growth medium. **B** – Screening strategy for p53 activators. The left panel indicates that yeast growth is dependent on galactose concentration (i.e. p53 expression level).

We used two different cDNA libraries - one derived from 9.5-10.5 dpc (days *post coitum*) mouse embryos and another from human thymus. In both cases we obtained a couple of hundred colonies growing on test medium. Next we tested their ability to grow on the selective media without p53 expression (without galactose present). All colonies growing without p53 expression were considered to be false positives and excluded from further analysis. More than 90% were eliminated by this step. We then purified the plasmids from remaining yeast colonies, transformed them to bacteria, purified and transformed the plasmids back to the original yeast test strain. None of the transformants were able to repeat the original phenotype. This indicates that the growth of colonies on the test medium was not dependent on cDNA expression. Instead the growing yeasts probably acquired some mutation, enabling them to grow on selective media.

There are several possible explanations for our failure to identify p53 cofactors. The easiest one is to blame a cDNA library - maybe it was not good enough to contain a putative full length cofactor. Another possibility is that the DNA binding site for p53 is "too good" and one can't make binding more efficient. Putative cofactors may need a special DNA sequence in promoter to assist p53 to activate transcription. This DNA element might be missing in constructed yeast promoter.

We set up a very similar screen to identify low molecular weight compounds capable of reactivating the p53 bearing the tumour-associated point mutation. In this case, the yeast strain was engineered to express a mutated p53 (arg273 to his). Screening conditions allowed quite high expression level for mutated p53 (figure 3). We used previously described assay format (Young et al., 1998) where the yeast cells are plated at low densities in top agar onto test medium. No growth of yeast cells occurs because the marker gene is not expressed. Then we applied 2 μl of testable compounds, dissolved in DMSO, to the surface of agar plates. If some compounds are capable of entering the cells and activating the mutant p53, the growth of yeast cells should be visible. We tested 7000 low molecular weight compounds obtained from National Cancer Institute, Bethesda, Maryland, USA (http://dtp.nci.nih.gov). Although 3 compounds induced the growth of the test strain, they did so also without p53 expression and therefore we discarded these compounds as false positives. The screen failed to find any compounds capable of restoring the transactivation function of mutant p53. As an assay, based on the human cell growth inhibition, was used to identify the mutant p53 reactivating compound from the same library (Bykov et al., 2002), our set-up was faulty in some stage. The yeast cell wall, that might be an unpenetrable barrier for many chemicals, is the most likely problem with this type of screen.

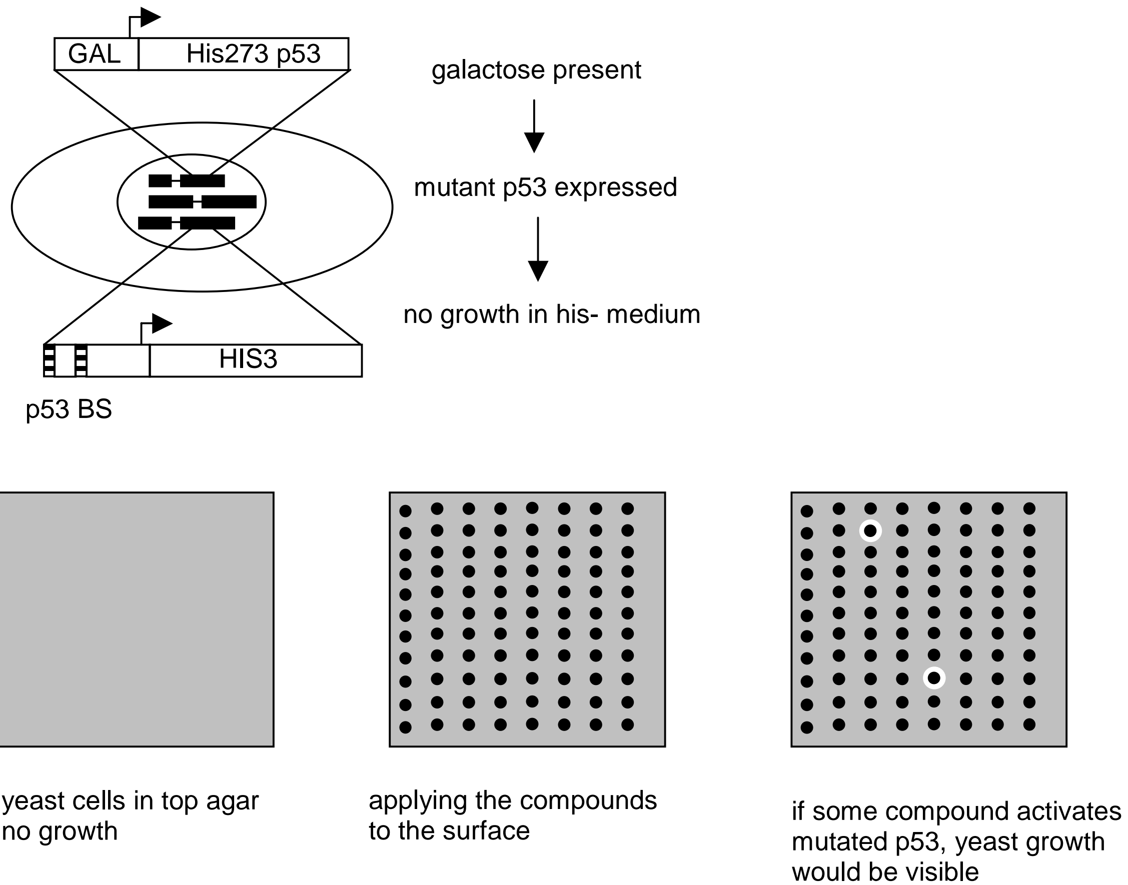


Figure 3. Screening strategy for mutant p53 activating compounds. Despite high p53 expression the yeast cells can't grow, because mutated p53 is unable to activate transcription. If some compound is capable of entering the cells and restoring the transactivation function of mutant p53, the yeast would grow around the application spot.

2.3. DNA damage dose-dependent responses of p53 pathway (II, III)

The p53-dependent pathways in cells are activated in response to various forms of genotoxic stress. The strength of the stress can vary from barely detectable to lethal. It is obvious that the cellular responses must be different for stresses with different severity. How the p53 activity is regulated in response to varying the stress strength was the question addressed in following experiments.

2.3.1 p53 protein accumulation in response to genotoxic stress (II, III)

In normal cells and in most cell lines containing wt p53, the DNA damage induces accumulation of p53 protein (Deptala et al., 1999; Nelson and Kastan, 1994). This happens due to the block of its degradation (Maltzman and Czyzyk, 1984) and enhanced translation (Fu et al., 1996). We used different concentrations of camptothecin

(CPT), a DNA damaging drug (Avemann et al., 1988; Ryan et al., 1991), to induce DNA damage with variable severity in four cell lines carrying wt p53 (II, fig. 1). Low concentrations of CPT do not induce any changes in p53 levels, except in LoVo cells, which are very sensitive to CPT. p53 starts to accumulate if the concentration of CPT is higher than some threshold. After a while it reaches the plateau - adding more CPT does not result in higher p53 expression level anymore. So the concentration window exists, where adding more CPT results in higher p53 expression. Interestingly the exact position of this window is different in all cell lines tested. This shows that dose-dependent relationship between DNA damaging agent and p53 expression levels holds only in the certain concentration range and this range is different in different cell lines. If the concentration of DNA damaging agent is below the lower threshold, p53 does not accumulate and if it is above the higher threshold, there is no further increase in p53 level.

Inside this concentration window the submaximal p53 expression occurs. The western blot measures only population average, so the question of distribution inside the population arises. Whether all cells accumulate p53 at equal level or maybe some cells do it maximally but in others the p53 level is unchanged? Higher doses of DNA-damaging agent may increase the p53 expression levels equally in all cells, or alternatively the number of cells with maximal p53 expression level increases. To discriminate between these options, we stained the p53 in individual cells and analysed them in flow cytometer. Cell staining profiles clearly indicate that p53 expression is homogenous and dose-dependent (III, fig. 2). This is true for CPT and also for mitomycin C (MMC). The fact that p53 is expressed in every cell at the same level also indicates that all cells have experienced more or less equal amount of DNA damage.

Taken together we may say that p53 protein accumulates, if the concentration of the DNA damaging agent exceeds a certain threshold, which is different in different cells. All cells in population accumulate p53 dose-dependently, but only until the levels of p53 reach the plateau. Further increase in concentration of DNA damaging agent has no influence on p53 protein level.

2.3.2 Transactivation activity of p53 is uncoupled from its protein accumulation and can be regulated by on/off manner (II, III)

Transactivation activity is essential for p53 to function as a tumour suppressor (Jimenez et al., 2000)(G. Wahl, personal communication). To get insight into the regulation of p53 transactivation activity, we measured different p53 target gene mRNA levels in response to wide range of CPT treatment (II, fig. 2, 3). mRNA levels of target genes are elevated already at CPT concentrations that do not cause any changes in p53 protein level. This indicates the presence of so called latent p53 pool that can be activated in the case of DNA damage. In NIH 3T3 fibroblasts, the increase in concentration of CPT is accompanied by further increase in some, but not all target gene mRNA levels (II, fig. 2). Same treatment did not cause any activation of p53 target genes in p53-negative cell line 10(1). The fact that low level of DNA damaging agent induces p53-dependent transcription indicates that signals for p53 activation and accumulation have different sensitivities for DNA damage.

Next we asked whether all cells in population regulate p53-dependent transcription similarly. To monitor p53 transcriptional activity at the single cell level, we integrated a reporter construct into the genome of NIH 3T3 cells. In this construct the p53 DNA binding site from mdm2 gene is cloned in front of the basic promoter from adenovirus ML gene and this hybrid promoter drives the expression of the Enhanced Green Fluorescent Protein (EGFP). The resulting reporter cell line is clonal: the copy number and integration site is identical for all cells in population. After treatment with DNA damaging agents the EGFP accumulates in these cells indicating the activation of p53 (III, fig. 3). However, the activation pattern is different from p53 protein accumulation. Only certain subpopulation of cells become EGFP-positive, whereas others remain EGFP-negative. Higher concentration of both CPT and MMC and longer treatment leads to the higher percentage of EGFP positive cells. At the same time stronger damage does not lead higher expression among EGFP-positive cells: the EGFP-positive cells are with the same intensity after both 1 μM and 7 μM CPT treatment (III, fig. 3). This indicates that p53-dependent transcription in response to genotoxic stress is regulated in on/off manner and damage intensity changes the distribution of cells between EGFP-positive and -negative subpopulation. This distribution is independent from p53 protein levels as both EGFP+ and EGFP- populations express p53 protein at the same level (III, fig. 3 and fig. 7A).

More than a hundred p53 target genes are known, each of them having a unique promoter structure. To get insight, how p53 regulates the promoters of its natural target genes, we made cell lines where the expression of EGFP is controlled by Waf-1 or PIG3 promoters, both being established as the transcriptional targets of p53 (El-Deiry et al., 1993; Polyak et al., 1997). Two analysed PIG3 cell lines exhibited the on/off type transcriptional activation in response to genotoxic stress (III, fig. 4A). This indicates that both artificial and natural p53 promoters can respond in a similar way. In contrast, the Waf-1 promoter is regulated in graded manner after genotoxic stress (III, fig 4B). The cells stain homogeneously for EGFP and the staining intensity is dose-dependent. This shows that at least two types of p53-dependent promoters exist which differ from each other in their regulation pattern (graded or on/off).

In order to get mechanistic insight into the on/off *versus* graded activation of promoters we made several constructs where elements from artificial (on/off responder) and Waf-1 (graded responer) promoters were combined. Waf-1 promoter contains at least two p53 binding sites to which p53 protein can bind *in vivo*. We inserted both of these in front of the artificial basal promoter and combined Waf-1 basal promoter with p53 binding sites from mdm2 promoter. In this way we could test the elements of Waf-1 promoter separately. All promoter constructs were inserted in front of the EGFP gene and we used them to create stable cell lines (using NIH 3T3 cells). All three cell lines, containing different element from Waf-1 promoter in their reporter constructs, behaved in on/off manner after genotoxic stress (III, fig 5). This indicates that none of the sequence elements analysed is able to ensure the graded response.

The transcriptional activity of p53 leads to the elevation of the protein levels of its target genes. To follow the expression pattern of endogenous Waf1 and mdm2 proteins we induced the genotoxic stress with CPT or MMC in different cell lines and stained the cells for these proteins with appropriate antibodies. In Arf-/- MEF (data not shown) and NIH 3T3 cell lines both Waf1 and mdm2 proteins followed the gradual model of

induction (III, fig. 6A). This result is in good correlation with EGFP experiments, as the reporter construct with Waf1 endogenous promoter in NIH 3T3 based cell line also exhibited gradual behavior. In contrast, in response to MMC treatment endogeneous Waf1 is induced in on/off manner in MCF7 cells (III, fig. 6C), while p53 protein accumulates homogenously (III, fig. 6B and fig. 7B). There are two populations already in untreated cells and induction of Waf1 clearly follows the binary model. Subsequently we created a stable cell line using MCF7 cells and Waf1 promoter in front of EGFP gene (clone MCF7 A5). EGFP in this cell line is also induced in on/off manner (III fig. 6D), mimicking the induction of Waf1 protein. These results indicate that endogeneous p53 targets can also be induced by both gradual and binary mode.

Several conclusions can be drawn from these results. First, they again uncouple p53 protein accumulation from its transcriptional activity. The mere accumulation of p53 protein is not enough to ensure the activation of p53-dependent transcription. Secondly, the p53-dependent transcription can be regulated in both graded and on/off manner. There seems to be a need for special DNA sequence in order to ensure the graded type of promoter activation, as short versions of Waf1 promoter have lost their gradual behaviour. Different behaviour of Waf1 promoter in NIH 3T3 (gradual) and MCF7 (on/off) cells indicates for a need of the special *trans*-factor for gradual promoter activation. This putative factor must be absent or inactive in MCF7 cells.

The property to activate transcription in on/off manner is not unique to p53. The two models– graded versus binary – have been studied earlier in other systems. Fiering and colleagues have demonstrated that after stimulation of TCR pathway in Jurkat T cells the NF-AT and NF-κB- dependent transcription is regulated by on/off pattern (Fiering et al., 1990). In yeast cells, the GAL4-dependent transcription from *GAL1* promoter occurring in response to changes in sugar composition of the growth medium is also regulated by binary mode (Biggar and Crabtree, 2001). These studies, however, did not address the expression pattern of the transcriptional activator itself leaving open the possibility that the differences in steady-state level of transcriptional activator can cause the on/off type response. We show here that the binary transcriptional regulation occurs in the presence of homogeneously expressed p53 protein and, therefore, this possibility is excluded.

It has been shown that the same promoter can be regulated in either graded on binary manner. Artificial tet-dependent promoter responds gradually to altered activity of either tet-activator or tet-repressor (Rossi et al., 2000). However, when both modulators are co-expressed, the transactivation is regulated in binary manner. In this example doxycyclin controls the DNA binding activity of both the activator and repressor in opposite ways and the binary regulation is apparently achieved due to the highly cooperative binding of them to the multiple binding sites in the promoter. There is no multimerised p53-binding sites in our reporter constructs. Although it cannot be excluded, there are no data available about any repressors binding both PIG3 promoter and artificial p53-dependent promoter we used. It is therefore unlikely that the on/off regulation described by us is due to interplay between p53 and a repressor competing for the same DNA sequence.

p53 might need a specific modification for transactivation initiation from PIG3 but not from waf-1 promoter in NIH 3T3 cells. Indeed, serine-18 (equivalent to human serine-15) phosphorylation displays heterogenity among induced cells, but this does not

correlate with EGFP staining (data not shown). It still may be that p53 acquires a specific modification needed for transactivation from PIG3 promoter in only subset of cells. Indeed, PIG3 and Waf-1 promoters have different requirements for p53 activity as the p53 lacking the proline-rich domain can activate transcription from Waf-1 promoter but not from PIG3 promoter (Venot et al., 1998).

2.3.3 p53-dependent transcription follows the stochastic model for gene activation

On/off type, or binary, regulation of transcription has been described before (Biggar and Crabtree, 2001; Fiering et al., 1990) and it might be relatively common type of regulation, because only few works have looked at transcription in single cells. The ratio of GFP-positive and -negative cells, which seems to be determined by severity of DNA damage, may relay on purely deterministic mechanisms. In that case the cell population is consisting of different subpopulations and each of them has different threshold for p53-dependent transactivation. Although genetically identical, cells in culture differ from each other in their position in cell cycle. It could be that promoters exhibiting on/off activation pattern are activated only in some specific cell cycle phase and this is the main reason for their binary behaviour. We tested it by staining the cells for their DNA content and analysed the cell cycle profile separately in EGFP+ and EGFP- cells. It turned out that EGFP expression can be induced in all cell cycle phases (III, fig. 8A) and therefore the on/off regulation is not due to the activation taking place only in some specific cell cycle phase.

After removal of the damaging agent EGFP+ and EGFP- cells behave differently (III, fig. 8B). EGFP- cells transiently accumulate in S and G2 phase of the cell cycle and then resume their normal cycle distribution. EGFP+ cells, on the contrary to EGFP- cells, accumulate in G2/M and do not go through mitosis. This indicates that p53 transcriptional activity defines 2 subpopulations of cells with distinct behaviour after genotoxic stress.

Alternatively the transactivation competent p53 can initiate transcription with certain probability. This is higher if the damage-related signal is either stronger or lasts longer. In this case the severity of genotoxic stress determines only the probability for transactivation, but the choice to activate transcription in particular cell is stochastic. The latter hypothesis is supported by reports describing stochastic steps in transcription activation in both prokaryotic (Elowitz et al., 2002) and eukaryotic (de Krom et al., 2002; Graubert et al., 1998) cells.

We analysed whether the cell decision to initiate the EGFP expression is influenced by its previous decision. To do that we induced the genotoxic stress in on/off responder cell line and sorted the EGFP+ and EGFP- subpopulations into separate pools. After growing we subjected both populations to the second treatment and followed their EGFP expression profile. The original EGFP+ and EGFP- populations behaved indistinguishably from each other (III, fig. 9) - their sensitivity to cpt treatment was the same and they followed the same expression pattern. This result supports the stochastic model for transcriptional activation.

The gradually changing concentration of DNA damaging agent must be converted into discrete decisions by cells - arrest or proliferation, death or survival. Our results

suggest that p53 transcriptional activity may be involved in generation of heterogeneity in otherwise homogenous population. This means that in the case of DNA damage of intermediate strength, in some cells the p53-dependent transcription is not activated. They are functionally "p53-negative" and in danger to accumulate mutations. Thus, some cells do not react to DNA damage “properly” even before the mutations in their DNA have occurred: the sensitivity of activation of p53-dependent pathway seems to be insufficient to ensure transcriptional activation of p53 in every single cell. This hypothesis is supported by the fact that tumours developing in p53+/- mice retain the functional wt p53 allele in 50% of cases (Venkatachalam et al., 1998). It seems that p53 is not active enough to block the uncontrolled proliferation in these cases. In p53+/- embryos the transcriptional activity of p53 is indeed reduced and is much weaker than in wt embryos (Gottlieb et al., 1997). At the light of our results the lower p53 expression level would reduce the probability to activate transcription and despite of intact p53 pathway the target genes are not activated in many cells.

CONCLUSIONS

The knowledge I acquired during these studies can be summarised as follows.

1. Mutant p53 can inhibit the transcriptional activation activity of p53 without heterooligomerisation. The most probable reason is the sequestering of some p53-specific cofactor by mutant p53.

2. p53 protein accumulation is homogenous in cell population and depends on the dose of damaging agent. This dose-dependency is observable only in the certain concentration range of DNA damaging agent.

3. p53 transcriptional activity is regulated separately from the protein accumulation and is more sensitive to DNA damage than the latter.

4. In single cells the p53 dependent transcription can be regulated either gradually or in on/off manner. The latter regulation type results in heterogeneity among isogenic cells.

5. p53 transcriptional activity defines two subpopulations of cells. According to that some cells do not activate p53 pathway in response to DNA damage and are in danger to accumulate mutations.

In addition I learned that the screening projects are high-risk projects. If you find nothing then all your effort is wasted and all you learn is this bitter piece of knowledge. Don't let some screening to be your only project in graduate school.

The three papers discussed here also represent the development of my understanding of adequate model systems in molecular cell biology: from transient overexpression to activation of endogenous p53, from population average to single cell analysis. I have been trying to move towards less manipulation and more observation of processes in the cell.

REFERENCES

Agami, R., and Bernards, R. (2000). Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell *102*, 55-66.

Ahn, J., Murphy, M., Kratowicz, S., Wang, A., Levine, A. J., and George, D. L. (1999). Down-regulation of the stathmin/Op18 and FKBP25 genes following p53 induction. Oncogene *18*, 5954-5958.

Ahn, J., Urist, M., and Prives, C. (2003). Questioning the role of checkpoint kinase 2 in the p53 DNA damage response. J Biol Chem *278*, 20480-9.

Akashi, M., and Koeffler, H. P. (1998). Li-Fraumeni syndrome and the role of the p53 tumor suppressor gene in cancer susceptibility. Clinical Obstetrics And Gynecology *41*, 172-99.

Appella, E., and Anderson, C. W. (2001). Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem *268*, 2764-72.

Armstrong, J., Kaufman, M., Harrison, D., and Clarke, A. (1995). High-frequency developmental abnormalities in p53-deficient mice. Curr. Biol. *5*, 931-6.

Aurelio, O. N., Kong, X. T., Gupta, S., and Stanbridge, E. J. (2000). p53 mutants have selective dominant-negative effects on apoptosis but not growth arrest in human cancer cell lines. Mol Cell Biol *20*, 770-8.

Avemann, K., Knippers, R., Koller, T., and Sogo, J. (1988). Camptothecin, a specific inhibitor of type I DNA topoisomerases, induces DNA breakage at replication forks. Mol Cell Biol *8*, 3026-3034.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science *281*, 1674-7.

Barak, Y., Juven, T., Haffner, R., and Oren, M. (1993). mdm2 expression is induced by wild type p53 activity. EMBO J. *12*, 461-8.

Bargonetti, J., Manfredi, J. J., Chen, X., Marshak, D. R., and Prives, C. (1993). A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. Genes & Dev. *7*, 2565-74.

Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. (2001). Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol Cell *8*, 1243-54.

Barlow, C., Brown, K., Deng, C., Tagle, D., and Wynshaw-Boris, A. (1997). Atm selectively regulates distinct p53-dependent cell-cycle checkpoint and apoptotic pathways. Nat. Genet. *17*, 453-6.

Bartek, J., and Lukas, J. (2001). Pathways governing G1/S transition and their response to DNA damage. FEBS Lett *490*, 117-22.

Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998). p14ARF links the tumour suppressors RB and p53. Nature *395*, 124-5.

Bergh, J., Norberg, T., Sjogren, S., Lindgren, A., and Holmberg, L. (1995). Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. Nat Med *1*, 1029-34.

Biederer, C., Ries, S., Brandts, C. H., and McCormick, F. (2002). Replication-selective viruses for cancer therapy. J Mol Med *80*, 163-75.

Biggar, S. R., and Crabtree, G. R. (2001). Cell signaling can direct either binary or graded transcriptional responses. EMBO J *20*, 3167-76.

Bischoff, J., Kirn, D., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J., Sampson-Johannes, A., Fattaey, A., and McCormick, F. (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science *274*, 373-6.

Blackburn, A. C., and Jerry, D. J. (2002). Knockout and transgenic mice of Trp53: what have we learned about p53 in breast cancer? Breast Cancer Res *4*, 101-11.

Bottger, A., Bottger, V., Sparks, A., Liu, W. L., Howard, S. F., and Lane, D. P. (1997). Design of a synthetic Mdm2-binding mini protein that activates the p53 response in vivo. Curr Biol *7*, 860-9.

Bouffler, S., Kemp, C., Balmain, A., and Cox, R. (1995). Spontaneous and ionizing radiation-induced chromosomal abnormalities in p53-deficient mice. Cancer Res. *55*, 3883-9.

Brachmann, R., Yu, K., Eby, Y., Pavletich, N., and Boeke, J. (1998). Genetic selection of intragenic suppressor mutations that reverse the effect of common p53 cancer mutations. EMBO J. *17*, 1847-59.

Budram-Mahadeo, V., Morris, P. J., and Latchman, D. S. (2002). The Brn-3a transcription factor inhibits the pro-apoptotic effect of p53 and enhances cell cycle arrest by differentially regulating the activity of the p53 target genes encoding Bax and p21(CIP1/Waf1). Oncogene *21*, 6123-31.

Bykov, V. J., Issaeva, N., Shilov, A., Hultcrantz, M., Pugacheva, E., Chumakov, P., Bergman, J., Wiman, K. G., and Selivanova, G. (2002). Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. Nat Med *8*, 282-8.

Canman, C., Gilmer, T., Coutts, S., and Kastan, M. (1995). Growth factor modulation of p53-mediated growth arrest versus apoptosis. Genes & Dev. *9*, 600-11.

Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677-9.

Cantley, L. C., and Neel, B. G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A *96*, 4240-5.

Chan, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1999). 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. Nature *401*, 616-20.

Chandel, N. S., Vander Heiden, M. G., Thompson, C. B., and Schumacker, P. T. (2000). Redox regulation of p53 during hypoxia. Oncogene *19*, 3840-8.

Chao, C., Hergenhahn, M., Kaeser, M. D., Wu, Z., Saito, S., Iggo, R., Hollstein, M., Appella, E., and Xu, Y. (2003). Cell type- and promoter-specific roles of Ser18 phosphorylation in regulating p53 responses. J Biol Chem *278*, 41028-33.

Chao, C., Saito, S., Kang, J., Anderson, C. W., Appella, E., and Xu, Y. (2000). p53 transcriptional activity is essential for p53-dependent apoptosis following DNA damage. EMBO J *19*, 4967-75.

Chehab, N. H., Malikzay, A., Appel, M., and Halazonetis, T. D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev *14*, 278-88.

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science *303*, 1010-4.

Chipuk, J. E., Maurer, U., Green, D. R., and Schuler, M. (2003). Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. Cancer Cell *4*, 371-81.

Cho, Y., Gorina, S., Jeffrey, P. D., and Pavletich, N. P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science *265*, 346-55.

Cox, L. S., Hupp, T., Midgley, C. A., and Lane, D. P. (1995). A direct effect of activated human p53 on nuclear DNA replication. EMBO J. *14*, 2099-105.

de Krom, M., van de Corput, M., von Lindern, M., Grosveld, F., and Strouboulis, J. (2002). Stochastic patterns in globin gene expression are established prior to transcriptional activation and are clonally inherited. Mol Cell *9*, 1319-26.

de Stanchina, E., McCurrach, M. E., Zindy, F., Shieh, S. Y., Ferbeyre, G., Samuelson, A. V., Prives, C., Roussel, M. F., Sherr, C. J., and Lowe, S. W. (1998). E1A signaling to p53 involves the p19(ARF) tumor suppressor. Genes Dev *12*, 2434-42.

Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell *82*, 675-84.

Deptala, A., Li, X., Bedner, E., Cheng, W., Traganos, F., and Darzynkiewicz, Z. (1999). Differences in induction of p53, p21WAF1 and apoptosis in relation to cell cycle phase of MCF-7 cells treated with camptothecin. Int J Oncol *15*, 861-871.

Di Cristofano, A., Kotsi, P., Peng, Y. F., Cordon-Cardo, C., Elkon, K. B., and Pandolfi, P. P. (1999). Impaired Fas response and autoimmunity in Pten+/- mice. Science *285*, 2122-5.

Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A., Moore, M., Finlay, C., and Levine, A. (1993). Gain of function mutations in p53. Nat. Genet. *4*, 42-6.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A. J., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-21.

Dudenhoffer, C., Rohaly, G., Will, K., Deppert, W., and Wiesmuller, L. (1998). Specific mismatch recognition in heteroduplex intermediates by p53 suggests a role in fidelity control of homologous recombination. Mol Cell Biol *18*, 5332-42.

Dumble, M. L., Knight, B., Quail, E. A., and Yeoh, G. C. (2001). Hepatoblast-like cells populate the adult p53 knockout mouse liver: evidence for a hyperproliferative maturation-arrested stem cell compartment. Cell Growth Differ *12*, 223-31.

Dumont, P., Leu, J. I., Della Pietra, A. C., 3rd, George, D. L., and Murphy, M. (2003). The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet *33*, 357-65.

Edwards, S. J., Dix, B. R., Myers, C. J., Dobson-Le, D., Huschtscha, L., Hibma, M., Royds, J., and Braithwaite, A. W. (2002). Evidence that replication of the antitumor

adenovirus ONYX-015 is not controlled by the p53 and p14(ARF) tumor suppressor genes. J Virol *76*, 12483-90.

El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. Nat. Genet. *1*, 45-9.

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-25.

Elowitz, M. B., Levine, A. J., Siggia, E. D., and Swain, P. S. (2002). Stochastic gene expression in a single cell. Science *297*, 1183-6.

Espinosa, J. M., and Emerson, B. M. (2001). Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. Mol Cell *8*, 57-69.

Evan, G. I., and Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. Nature *411*, 342-8.

Ferguson, A. T., Evron, E., Umbricht, C. B., Pandita, T. K., Chan, T. A., Hermeking, H., Marks, J. R., Lambers, A. R., Futreal, P. A., Stampfer, M. R., and Sukumar, S. (2000). High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. Proc Natl Acad Sci U S A *97*, 6049-54.

Fields, S., and Jang, S. K. (1990). Presence of a potent transcription activating sequence in the p53 protein. Science *249*, 1046-9.

Fiering, S., Northrop, J. P., Nolan, G. P., Mattila, P. S., Crabtree, G. R., and Herzenberg, L. A. (1990). Single cell assay of a transcription factor reveals a threshold in transcription activated by signals emanating from the T-cell antigen receptor. Genes Dev *4*, 1823-34.

Flores, E. R., Tsai, K. Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F., and Jacks, T. (2002). p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. Nature *416*, 560-4.

Foster, B. A., Coffey, H. A., Morin, M. J., and Rastinejad, F. (1999). Pharmacological rescue of mutant p53 conformation and function. Science *286*, 2507-10.

Freedman, D. A., Wu, L., and Levine, A. J. (1999). Functions of the MDM2 oncoprotein. Cell Mol Life Sci *55*, 96-107.

Friedman, P., Chen, X., Bargonetti, J., and Prives, C. (1993). The p53 protein is an unusually shaped tetramer that binds directly to DNA. Proc. Natl. Acad. Sci. USA *90*, 3319-23.

Fu, L., Minden, M., and Benchimol, S. (1996). Translational regulation of human p53 gene expression. EMBO J. *15*, 4392-401.

Fukasawa, K., Wiener, F., Vande Woude, G., and Mai, S. (1997). Genomic instability and apoptosis are frequent in p53 deficient young mice. Oncogene *15*, 1295-302.

Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. Mol. Cell. Biol. *12*, 2866-71.

Garcia-Cao, I., Garcia-Cao, M., Martin-Caballero, J., Criado, L. M., Klatt, P., Flores, J. M., Weill, J. C., Blasco, M. A., and Serrano, M. (2002). "Super p53" mice exhibit enhanced DNA damage response, are tumor resistant and age normally. EMBO J *21*, 6225-35.

Garkavtsev, I., Grigorian, I. A., Ossovskaya, V. S., Chernov, M. V., Chumakov, P. M., and Gudkov, A. V. (1998). The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. Nature *391*, 295-8.

Gottlieb, E., Haffner, R., King, A., Asher, G., Gruss, P., Lonai, P., and Oren, M. (1997). Transgenic mouse model for studying the transcriptional activity of the p53 protein: age- and tissue-dependent changes in radiation-induced activation during embryogenesis. EMBO J. *16*, 1381-90.

Graeber, T., Peterson, J., Tsai, M., Monica, K., Fornace, A. J., and Giaccia, A. (1994). Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol. Cell. Biol. *14*, 6264-77.

Graubert, T. A., Hug, B. A., Wesselschmidt, R., Hsieh, C. L., Ryan, T. M., Townes, T. M., and Ley, T. J. (1998). Stochastic, stage-specific mechanisms account for the variegation of a human globin transgene. Nucleic Acids Res *26*, 2849-58.

Halazonetis, T. D., and Kandil, A. N. (1993). Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. EMBO J. *12*, 5057-64.

Hammond, E. M., Denko, N. C., Dorie, M. J., Abraham, R. T., and Giaccia, A. J. (2002). Hypoxia links ATR and p53 through replication arrest. Mol Cell Biol *22*, 1834-43.

Harvey, M., Vogel, H., Morris, D., Bradley, A., Bernstein, A., and Donehower, L. (1995). A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice. Nat. Genet. *9*, 305-11.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-9.

Hegi, M. E., Klein, M. A., Ruedi, D., Chene, P., Hamou, M. F., and Aguzzi, A. (2000). p53 transdominance but no gain of function in mouse brain tumor model. Cancer Res *60*, 3019-24.

Hermeking, H., Lengauer, C., Polyak, K., He, T., Zhang, L., Thiagalingam, S., Kinzler, K., and Vogelstein, B. (1997). 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell *1*, 3-11.

Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science *287*, 1824-7.

Hoffman, W. H., Biade, S., Zilfou, J. T., Chen, J., and Murphy, M. (2002). Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem *277*, 3247-57.

Hollander, M. C., Sheikh, M. S., Bulavin, D. V., Lundgren, K., Augeri-Henmueller, L., Shehee, R., Molinaro, T. A., Kim, K. E., Tolosa, E., Ashwell, J. D., Rosenberg, M. P., Zhan, Q., Fernandez-Salguero, P. M., Morgan, W. F., Deng, C. X., and Fornace, A. J., Jr. (1999). Genomic instability in Gadd45a-deficient mice. Nat Genet *23*, 176-84.

Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R., and Harris, C. C. (1994). Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res. *22*, 3551-5.

Honda, K., Sbisa, E., Tullo, A., Papeo, P., Saccone, C., Poole, S., Pignatelli, M., Mitry, R., Ding, S., Isla, A., Davies, A., and Habib, N. (1998). p53 mutation is a poor prognostic indicator for survival in patients with hepatocellular carcinoma undergoing surgical tumour ablation. Br J Cancer *77*, 776-82.

Hsiao, M., Low, J., Dorn, E., Ku, D., Pattengale, P., Yeargin, J., and Haas, M. (1994). Gain-of-function mutations of the p53 gene induce lymphohematopoietic metastatic potential and tissue invasiveness. Am J Pathol *145*, 702-14.

Hsiao, M., Yu, A., Yeargin, J., Ku, D., and Haas, M. (1994). Nonhereditary p53 mutations in T-cell acute lymphoblastic leukemia are associated with the relapse phase. Blood *83*, 2922-30.

Hundley, J., Koester, S., Troyer, D., Hilsenbeck, S., Subler, M., and Windle, J. (1997). Increased tumor proliferation and genomic instability without decreased apoptosis in MMTV-ras mice deficient in p53. Mol. Cell. Biol. *17*, 723-31.

Ikeda, S., Hawes, N. L., Chang, B., Avery, C. S., Smith, R. S., and Nishina, P. M. (1999). Severe ocular abnormalities in C57BL/6 but not in 129/Sv p53-deficient mice. Invest Ophthalmol Vis Sci *40*, 1874-8.

Innocente, S. A., Abrahamson, J. L., Cogswell, J. P., and Lee, J. M. (1999). p53 regulates a G2 checkpoint through cyclin B1. Proc Natl Acad Sci U S A *96*, 2147-52.

Iwata, N., Yamamoto, H., Sasaki, S., Itoh, F., Suzuki, H., Kikuchi, T., Kaneto, H., Iku, S., Ozeki, I., Karino, Y., Satoh, T., Toyota, J., Satoh, M., Endo, T., and Imai, K. (2000). Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. Oncogene *19*, 5298-302.

Jack, M. T., Woo, R. A., Hirao, A., Cheung, A., Mak, T. W., and Lee, P. W. (2002). Chk2 is dispensable for p53-mediated G1 arrest but is required for a latent p53-mediated apoptotic response. Proc Natl Acad Sci U S A *99*, 9825-9.

Jallepalli, P. V., Lengauer, C., Vogelstein, B., and Bunz, F. (2003). The Chk2 tumor suppressor is not required for p53 responses in human cancer cells. J Biol Chem *278*, 20475-9.

Jayaraman, L., Moorthy, N. C., Murthy, K. G., Manley, J. L., Bustin, M., and Prives, C. (1998). High mobility group protein-1 (HMG-1) is a unique activator of p53. Genes & Dev. *12*, 462-72.

Jayaraman, L., Murthy, K. G., Zhu, C., Curran, T., Xanthoudakis, S., and Prives, C. (1997). Identification of redox/repair protein Ref-1 as a potent activator of p53. Genes & Dev. *11*, 558-70.

Jeffers, J. R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K. H., Han, J., Chittenden, T., Ihle, J. N., McKinnon, P. J., Cleveland, J. L., and Zambetti, G. P. (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell *4*, 321-8.

Jimenez, G. S., Nister, M., Stommel, J. M., Beeche, M., Barcarse, E. A., Zhang, X. Q., O'Gorman, S., and Wahl, G. M. (2000). A transactivation-deficient mouse model provides insights into Trp53 regulation and function. Nat Genet *26*, 37-43.

Jin, S., Mazzacurati, L., Zhu, X., Tong, T., Song, Y., Shujuan, S., Petrik, K. L., Rajasekaran, B., Wu, M., and Zhan, Q. (2003). Gadd45a contributes to p53 stabilization in response to DNA damage. Oncogene *22*, 8536-40.

Jin, Y., Zeng, S. X., Dai, M. S., Yang, X. J., and Lu, H. (2002). MDM2 inhibits PCAF (p300/CREB-binding protein-associated factor)-mediated p53 acetylation. J Biol Chem *277*, 30838-43.

Juven, T., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993). Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene. Oncogene *8*, 3411-6.

Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell *91*, 649-59.

Kamijo, T., Weber, J., Zambetti, G., Zindy, F., Roussel, M., and Sherr, C. (1998). Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. Proc Natl Acad Sci U S A *95*, 8292-7.

Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J. J. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell *71*, 587-97.

Kern, S. E., Pietenpol, J. A., Thiagalingam, S., Seymour, A., Kinzler, K. W., and Vogelstein, B. (1992). Oncogenic forms of p53 inhibit p53-regulated gene expression. Science *256*, 827-30.

Khan, S. H., Moritsugu, J., and Wahl, G. M. (2000). Differential requirement for p19ARF in the p53-dependent arrest induced by DNA damage, microtubule disruption, and ribonucleotide depletion. Proc Natl Acad Sci U S A *97*, 3266-71.

Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A., and Korsmeyer, S. J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science *270*, 96-9.

Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V., and Gudkov, A. V. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science *285*, 1733-7.

Koumenis, C., Alarcon, R., Hammond, E., Sutphin, P., Hoffman, W., Murphy, M., Derr, J., Taya, Y., Lowe, S. W., Kastan, M., and Giaccia, A. (2001). Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transactivation. Mol Cell Biol *21*, 1297-310.

Kovach, J., Hartmann, A., Blaszyk, H., Cunningham, J., Schaid, D., and Sommer, S. (1996). Mutation detection by highly sensitive methods indicates that p53 gene mutations in breast cancer can have important prognostic value. Proc. Natl. Acad. Sci. USA *93*, 1093-6.

Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kurose, K., Gilley, K., Matsumoto, S., Watson, P. H., Zhou, X. P., and Eng, C. (2002). Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinomas. Nat Genet *32*, 355-7.

Lakin, N. D., Hann, B. C., and Jackson, S. P. (1999). The ataxia-telangiectasia related protein ATR mediates DNA-dependent phosphorylation of p53. Oncogene *18*, 3989-95.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15-6.

Lee, D., Kim, J. W., Seo, T., Hwang, S. G., Choi, E. J., and Choe, J. (2002). SWI/SNF complex interacts with tumor suppressor p53 and is necessary for the activation of p53-mediated transcription. J Biol Chem *277*, 22330-7.

Li, M., Luo, J., Brooks, C. L., and Gu, W. (2002). Acetylation of p53 Inhibits Its Ubiquitination by Mdm2. J Biol Chem *277*, 50607-11.

Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes & Dev. *8*, 1235-46.

Lin, Y., and Benchimol, S. (1995). Cytokines inhibit p53-mediated apoptosis but not p53-mediated G1 arrest. Mol. Cell. Biol. *15*, 6045-54.

Linke, S., Clarkin, K., Di Leonardo, A., Tsou, A., and Wahl, G. (1996). A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. Genes & Dev. *10*, 934-47.

Liu, G., Parant, J. M., Lang, G., Chau, P., Chavez-Reyes, A., El-Naggar, A. K., Multani, A., Chang, S., and Lozano, G. (2004). Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nat Genet *36*, 63-8.

Liu, X., Miller, C. W., Koeffler, P. H., and Berk, A. J. (1993). The p53 activation domain binds the TATA box-binding polypeptide in Holo-TFIID, and a neighboring p53 domain inhibits transcription. Mol. Cell. Biol. *13*, 3291-300.

Ljungman, M., Zhang, F., Chen, F., Rainbow, A. J., and McKay, B. C. (1999). Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene *18*, 583-92.

Lu, H., and Levine, A. J. (1995). Human TAFII31 protein is a transcriptional coactivator of the p53 protein. Proc. Natl. Acad. Sci. USA *92*, 5154-8.

Maehama, T., and Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem *273*, 13375-8.

Maltzman, W., and Czyzyk, L. (1984). UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell. Biol. *4*, 1689-94.

Martin-Caballero, J., Flores, J. M., Garcia-Palencia, P., and Serrano, M. (2001). Tumor susceptibility of p21(Waf1/Cip1)-deficient mice. Cancer Res *61*, 6234-8.

Mayo, L. D., Dixon, J. E., Durden, D. L., Tonks, N. K., and Donner, D. B. (2002). PTEN protects p53 from Mdm2 and sensitizes cancer cells to chemotherapy. J Biol Chem *277*, 5484-9.

Mendrysa, S. M., McElwee, M. K., Michalowski, J., O'Leary, K. A., Young, K. M., and Perry, M. E. (2003). mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. Mol Cell Biol *23*, 462-72.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003). p53 has a direct apoptogenic role at the mitochondria. Mol Cell *11*, 577-90.

Milner, J., and Medcalf, E. A. (1991). Cotranslation of activated mutant p53 with wild type drives the wild-type p53 protein into the mutant conformation. Cell *65*, 765-74.

Miyashita, T., and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell *80*, 293-9.

Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature *378*, 203-6.

Moore, L., Venkatachalam, S., Vogel, H., Watt, J. C., Wu, C. L., Steinman, H., Jones, S. N., and Donehower, L. A. (2003). Cooperativity of p19ARF, Mdm2, and p53 in murine tumorigenesis. Oncogene *22*, 7831-7.

Mummenbrauer, T., Janus, F., Muller, B., Wiesmuller, L., Deppert, W., and Grosse, F. (1996). p53 Protein exhibits 3'-to-5' exonuclease activity. Cell *85*, 1089-99.

Murphy, M., Hinman, A., and Levine, A. J. (1996). Wild-type p53 negatively regulates the expression of a microtubule-associated protein. Genes & Dev. *10*, 2971-80.

Nakano, K., and Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell *7*, 683-94.

Nelson, W., and Kastan, M. (1994). DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol. Cell. Biol. *14*, 1815-23.

Nemunaitis, J., Ganly, I., Khuri, F., Arseneau, J., Kuhn, J., McCarty, T., Landers, S., Maples, P., Romel, L., Randlev, B., Reid, T., Kaye, S., and Kirn, D. (2000). Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B-55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. Cancer Res *60*, 6359-66.

Nicol, C., Harrison, M., Laposa, R., Gimelshtein, I., and Wells, P. (1995). A teratologic suppressor role for p53 in benzo[a]pyrene-treated transgenic p53-deficient mice. Nat. Genet. *10*, 181-7.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053-8.

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., and Taya, Y. (2000). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell *102*, 849-62.

Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature *362*, 857-60.

Pavletich, N. P., Chambers, K. A., and Pabo, C. O. (1993). The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. Genes & Dev. *7*, 2556-64.

Peng, Y., Li, C., Chen, L., Sebti, S., and Chen, J. (2003). Rescue of mutant p53 transcription function by ellipticine. Oncogene *22*, 4478-87.

Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1997). A model for p53-induced apoptosis. Nature *389*, 300-5.

Pricolo, V., Finkelstein, S., Hansen, K., Cole, B., and Bland, K. (1997). Mutated p53 gene is an independent adverse predictor of survival in colon carcinoma. Arch Surg *132*, 371-4; discussion 374-5.

Qian, H., Wang, T., Naumovski, L., Lopez, C. D., and Brachmann, R. K. (2002). Groups of p53 target genes involved in specific p53 downstream effects cluster into different classes of DNA binding sites. Oncogene *21*, 7901-11.

Rippin, T. M., Bykov, V. J., Freund, S. M., Selivanova, G., Wiman, K. G., and Fersht, A. R. (2002). Characterization of the p53-rescue drug CP-31398 in vitro and in living cells. Oncogene *21*, 2119-29.

Rodriguez, M., Ford, R., Goodacre, A., Selvanayagam, P., Cabanillas, F., and Deisseroth, A. (1991). Chromosome 17- and p53 changes in lymphoma. Br J Haematol *79*, 575-82.

Rotman, G., and Shiloh, Y. (1999). ATM: a mediator of multiple responses to genotoxic stress. Oncogene *18*, 6135-44.

Rowan, S, Ludwig, R., Haupt, Y., Bates, S., Lu, X., Oren, M., and Vousden, K. (1996). Specific loss of apoptotic but not cell-cycle arrest function in a human tumor derived p53 mutant. EMBO J. *15*, 827-38.

Rubbi, C. P., and Milner, J. (2003). Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. EMBO J *22*, 6068-77.

Ryan, A. J., Squires, S., Strott, H. L., and T., J. R. (1991). Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistant double strand breaks in replicating DNA. Nucleic Acids Res *19*, 3292-3300.

Sabbatini, P., and McCormick, F. (2002). MDMX inhibits the p300/CBP-mediated acetylation of p53. DNA Cell Biol *21*, 519-25.

Samuels-Lev, Y., O'Connor, D. J., Bergamaschi, D., Trigiante, G., Hsieh, J. K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. (2001). ASPP proteins specifically stimulate the apoptotic function of p53. Mol Cell *8*, 781-94.

Scharer, E., and Iggo, R. (1992). Mammalian p53 can function as a transcription factor in yeast. Nucleic Acids Res. *20*, 1539-45.

Schmidt, T., K, K. r., Karsunky, H., Korsmeyer, S., R, M. l., and T, M. r. y. (1999). The activity of the murine Bax promoter is regulated by Sp1/3 and E-box binding proteins but not by p53. Cell Death Differ *6*, 873-882.

Schmitt, C. A., Fridman, J. S., Yang, M., Baranov, E., Hoffman, R. M., and Lowe, S. W. (2002). Dissecting p53 tumor suppressor functions in vivo. Cancer Cell *1*, 289-98.

Schmitt, C. A., Fridman, J. S., Yang, M., Lee, S., Baranov, E., Hoffman, R. M., and Lowe, S. W. (2002). A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. Cell *109*, 335-46.

Selivanova, G., Iotsova, V., Okan, I., Fritsche, M., Strom, M., Groner, B., Grafstrom, R. C., and Wiman, K. G. (1997). Restoration of the growth suppression function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain. Nat Med *3*, 632-8.

Seoane, J., Le, H. V., and Massague, J. (2002). Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature *419*, 729-34.

Shaulian, E., Schreiber, M., Piu, F., Beeche, M., Wagner, E. F., and Karin, M. (2000). The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. Cell *103*, 897-907.

Shibue, T., Takeda, K., Oda, E., Tanaka, H., Murasawa, H., Takaoka, A., Morishita, Y., Akira, S., Taniguchi, T., and Tanaka, N. (2003). Integral role of Noxa in p53-mediated apoptotic response. Genes Dev *17*, 2233-8.

Shieh, S. Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. (2000). The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes Dev *14*, 289-300.

Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell *91*, 325-34.

Shiohara, M., el-Deiry, W. S., Wada, M., Nakamaki, T., Takeuchi, S., Yang, R., Chen, D. L., Vogelstein, B., and Koeffler, H. P. (1994). Absence of WAF1 mutations in a variety of human malignancies. Blood *84*, 3781-4.

Shiohara, M., Koike, K., Komiyama, A., and Koeffler, H. P. (1997). p21WAF1 mutations and human malignancies. Leuk Lymphoma *26*, 35-41.

Smith, M. L., Ford, J. M., Hollander, M. C., Bortnick, R. A., Amundson, S. A., Seo, Y. R., Deng, C. X., Hanawalt, P. C., and Fornace, A. J., Jr. (2000). p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. Mol Cell Biol *20*, 3705-14.

Stambolic, V., MacPherson, D., Sas, D., Lin, Y., Snow, B., Jang, Y., Benchimol, S., and Mak, T. W. (2001). Regulation of PTEN transcription by p53. Mol Cell *8*, 317-25.

Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29-39.

Suzuki, H., Itoh, F., Toyota, M., Kikuchi, T., Kakiuchi, H., and Imai, K. (2000). Inactivation of the 14-3-3 sigma gene is associated with 5' CpG island hypermethylation in human cancers. Cancer Res *60*, 4353-7.

Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., and Van Dyke, T. (1994). p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell *78*, 703-11.

Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell *92*, 725-34.

Zindy, F., Eischen, C. M., Randle, D. H., Kamijo, T., Cleveland, J. L., Sherr, C. J., and Roussel, M. F. (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev *12*, 2424-33.

Taylor, W. R., Schonthal, A. H., Galante, J., and Stark, G. R. (2001). p130/E2F4 binds to and represses the cdc2 promoter in response to p53. J Biol Chem *276*, 1998-2006.

Thornborrow, E. C., Patel, S., Mastropietro, A. E., Schwartzfarb, E. M., and Manfredi, J. J. (2002). A conserved intronic response element mediates direct p53-dependent transcriptional activation of both the human and murine bax genes. Oncogene *21*, 990-9.

Thut, C. J., Chen, J. L., Klemm, R., and Tjian, R. (1995). p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. Science *267*, 100-4.

Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev *13*, 152-7.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T. K., Hampton, G. M., and Wahl, G. M. (2002). c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol Cell *9*, 1031-44.

Wang, S., Guo, M., Ouyang, H., Li, X., Cordon-Cardo, C., Kurimasa, A., Chen, D. J., Fuks, Z., Ling, C. C., and Li, G. C. (2000). The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest. Proc Natl Acad Sci U S A *97*, 1584-8.

Wang, Y., and Prives, C. (1995). Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. Nature *376*, 88-91.

Wang, Y., Reed, M., Wang, P., Stenger, J. E., Mayr, G., Anderson, M. E., Schwedes, J. F., and Tegtmeyer, P. (1993). p53 domains: identification and characterization of two autonomous DNA-binding regions. Genes & Dev. *7*, 2575-86.

Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., and Liu, E. A. (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science *303*, 844-8.

Venkatachalam, S., Shi, Y. P., Jones, S. N., Vogel, H., Bradley, A., Pinkel, D., and Donehower, L. A. (1998). Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. EMBO J *17*, 4657-67.

Verhaegh, G. W., Parat, M. O., Richard, M. J., and Hainaut, P. (1998). Modulation of p53 protein conformation and DNA-binding activity by intracellular chelation of zinc. Mol. Carcinog. *21*, 205-14.

Verhaegh, G. W., Richard, M. J., and Hainaut, P. (1997). Regulation of p53 by metal ions and by antioxidants: dithiocarbamate down-regulates p53 DNA-binding activity by increasing the intracellular level of copper. Mol. Cell. Biol. *17*, 5699-706.

White, J. D., Rachel, C., Vermeulen, R., Davies, M., and Grounds, M. D. (2002). The role of p53 in vivo during skeletal muscle post-natal development and regeneration: studies in p53 knockout mice. Int J Dev Biol *46*, 577-82.

Wieczorek, A., Waterman, J., Waterman, M., and Halazonetis, T. (1996). Structure-based rescue of common tumor-derived p53 mutants. Nat Med *2*, 1143-6.

Villunger, A., Michalak, E. M., Coultas, L., Mullauer, F., Bock, G., Ausserlechner, M. J., Adams, J. M., and Strasser, A. (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science *302*, 1036-8.

Wu, Z., Earle, J., Saito, S., Anderson, C. W., Appella, E., and Xu, Y. (2002). Mutation of mouse p53 Ser23 and the response to DNA damage. Mol Cell Biol *22*, 2441-9.

Young, K., Lin, S., Sun, L., Lee, E., Modi, M., Hellings, S., Husbands, M., Ozenberger, B., and Franco, R. (1998). Identification of a calcium channel modulator using a high throughput yeast two-hybrid screen. Nat Biotechnol *16*, 946-50.

Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W., and Vogelstein, B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell *7*, 673-82.

KOKKUVÕTE

p53-sõltuva transkriptsiooni regulatsioon

p53 on rakuline valk, mille peamiseks funktsiooniks on ära hoida kahjustatud DNA-ga rakkude paljunemine. p53 ei ole hädavajalik rakkude normaalseks jagunemiseks ega organismi sünnieelseks arenguks, kuid tema puudumisel tekivad hiirtel kiiresti kasvajad. Ka inimeste kasvajates on p53 rohkem kui 50% juhtudest inaktiivne või puudub hoopis.

p53 on transkriptsiooniaktivaator ja oma kasvajaid mahasuruvat funktsiooni täidab ta eelkõige läbi oma märklaudgeenidelt transkriptsiooni aktiveerimise. DNA kahjustus ja teised stressisignaalid toovad kaasa rakus p53 valgu taseme tõusu ja tema aktiivsuse suurenemise. Selle tagajärjel tõuseb p53 märklaudgeenide ekspressioon ja see omakorda viib rakkude paljunemise peatamisele (rakutsükli blokk) või nende elimineerimisele (apoptoos).

Kui metsiktüüpi (wt) ja mutantne (inaktiivne) p53 on ekspresseeritud samas rakus, siis suudab mutantne p53 inhibeerida wt aktiivsust. Peamiseks mehhanismiks peetakse siin seda, et mutantne p53 moodustab wt heterooligomeere (p53 on normaalselt rakus tetrameerina). Selle doktoritöö aluseks olevas esimeses artiklis näitame me, et mutantne p53 suudab p53 transkriptsioonivõimet inhibeerida ka ilma heterooligomeriseerumiseta. Inhibitsioonivõime säilib, kui mutantsel p53 deleteerida kas N- või C- terminus, kuid kaob, kui deleteerida mõlemad. Samas pole mutantne p53 võimeline mõjutama p53-sõltumatut transkriptsiooni. Inhibitsioon ei ole tingitud p53 ekspressiooni või lokalisatsiooni muutustest ning ka p53 võime suruda maha rakkude kasvu on häiritud mutantse p53 poolt oligomeriseerumisest sõltumatult. Nendest tulemustest järeldame, et mutantne p53 seostub mõne p53-spetsiifilise kofaktoriga ja selle tagajärjel kaotab p53 oma võime transkriptsiooni aktiveerida.

Selleks, et tuvastada see (ja ka teised) kofaktorid, lõime me testsüsteemi pärmi *Saccharomyces cerevisiae* rakkudes. Kahjuks ei õnnestunud meil ühtegi kofaktorit identifitseerida.

DNA kahjustuse ulatus võib rakkudes varieeruda suurtes piirides. Teises artiklis näitame me, et p53 valgu kogunemine leiab aset alles siis, kui genotoksilise aine hulk ületab teatud läviväärtust. Seejärel on näha doosisõltuvat p53 taseme tõusu kuni piirväärtuseni, millest edasi p53 valgu tase enam ei muutu. Samas on p53-sõltuv transkriptsioon aktiveeritud juba läviväärtusest madalamate dooside juures. See näitab, et p53 valgu kogunemine ja p53-sõltuva transkriptsiooni aktiveerimine on eraldi reguleeritud ja vastavad signaalirajad on erineva tundlikkusega.

Mingi parameetri muutust rakupopulatsioonis võib saavutada kaht erinevat teed pidi. Esiteks võib toimuda muutus kõikides rakkudes ühtemoodi ja induktori doosist sõltuvalt (graduaalselt). Teiseks võimaluseks on, et muutus toimub individuaalsetes rakkudes lülititaoliselt – on võimalik vaid maksimaalne või minimaalne tase (binaarne regulatsioon). Induktori doos muudab reageerivate rakkude arvu, kuid mitte parameetri muutust ühes rakus. Kolmandas artiklis jälgisime me p53 taseme ja tema transaktivatsioonivõime muutusi vastusena genotoksilisele stressile ühe raku tasemel. p53 valgu tase indutseeritakse graduaalse mudeli alusel, samas võib p53-sõltuv transkriptsioon olla nii graduaalne kui binaarne. Erinevate promootorite

konfiguratsioonide analüüsidest järeldub, et graduaalne regulatsioon vajab spetsiifilist DNA elementi ja selle puudumisel on regulatsioon binaarne. Ka endogeenseid p53 märklaudgeene reguleeritakse mõlema mudeli alusel. Fakt, et esimesel korral reageerinud rakud ei oma eelistust järgmise induktsiooni puhul viitab võimalusele, et valik, kas reageerida või mitte, on tõenäosuslik.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to everyone who helped me during these years. Without you I wouldn't made it.

First of all I wish to thank my Family for their love, care and patience. It took a long time, but its over now.

I am grateful to my first supervisor Rein Sikut for introducing the fascinating world of molecular cell biology to me.

I am thankful to my supervisor Toivo Maimets for exceptional scientific freedom. Only rarely can graduate student pursue its own projects so independently.

I wish to thank all my colleagues, past and present, in Department of Cell Biology as well as in other locations. My special thanks go to Arnold Kristjuhan, Viljar Jaks, Lilian Kadaja and Ilvi Rimm for all these years working together in cell biology lab. I am thankful to Ivar Ilves, Aare Abroi, Jaanus Remme and Tanel Tenson for interesting discussions and Anne Kalling for keeping the cells happy.

I would like to thank Lauri Peil, Toomas Silla and all the others, who have struggled, together with me, through all these years, to keep the Molecular Biology Winter School alive and kicking.

I am grateful to Klas Wiman from Karolinska Institute in Stockholm for nice cooperation, Darius Balciuonas and Hans Ronne from Uppsala University for teaching me the basics of yeast handling.

CURRICULUM VITAE

ARVI JÕERS

Date and place of birth: December 12, 1970, Tallinn, Estonia
Citizenship: Estonian
Family status: married, two children
Work address: Institute of Molecular and Cell Biology, University of Tartu
Riia 23, 51010, Tartu, ESTONIA
Tel: +372 7 375 023
Fax: +372 7 420 286
e-mail: ajoers@ebc.ee
Home address: Kuu 39-60, Tartu, Estonia

Education and professional employment

1978 – 1989	Tallinn Secondary School No 9
1989 – 1993	University of Tartu, Faculty of Biology and Geography, graduated in 1993 as biologist.
1993 – 1996	MSc student in Institute of Molecular and Cell Biology, University of Tartu, MSc degree in cell biology in 1996.
1996 – 2002	PhD student in Institute of Molecular and Cell Biology, University of Tartu.
1997	Visiting PhD student in Dr. Hans Ronne's laboratory at BMC, University of Uppsala, Sweden.
From 2002	Research Scientist in Institute of Molecular and Cell Biology, University of Tartu.

Scientific work

During undergraduate studies I worked on cancer-associated changes in protein glycosylation. As a graduate student my research interests have been molecular mechanisms of action of tumor-suppressor protein p53 and regulation its transactivation function in particular. During the last 3 years my research subject has been the role of p53 in cellular decision-making after genotoxic stress.

ELULOOKIRJELDUS

ARVI JÕERS

Sünniaeg- ja koht: 12. Detsember 1970 Tallinn
Kodakontsus: Eesti kodanik
Perekonnaseis: Abielus, kaks last.
Aadress tööl: Molekulaar- ja Rakubioloogia Instituut, Tartu Ülikool,
Riia 23, 51010, Tartu, Eesti
tel: +372 7 375 023
fax: +372 7 420 286
e-mail: ajoers@ebc.ee
Kodune aadress: Kuu 39-60, Tartu, Eesti

Haridus ja erialane teenistuskäik

1978 – 1989	Tallinna 9. Keskkool
1989 – 1993	Tartu Ülikool, Bioloogia-Geograafiateaduskond, üliõpilane. Diplom bioloogias
1993 – 1996	Magistrant Molekulaar- ja Rakubioloogia Instituudis. Magistrikraad rakubioloogias 1996
1996 – 2002	Doktorant Molekulaar- ja Rakubioloogia Instituudis
1997	Külalisdoktorant Hans Ronne laboris, Uppsala Ülikoolis, Rootsis
alates 2002	Molekulaar- ja Rakubioloogia Instituut, Tartu Ülikool, rakubioloogia teadur

Teadustegevus

Minu uurimistöö on keskendunud tuumor-supressorvalgu p53 molekulaarsete regulatsioonimehhanismide ja rakubioloogilise rolli uurimisele. Erilise tähelepanu all on olnud p53 transaktivatsioonivõime ja selle regulatsioon. Viimastel aastatel olen keskendunud p53 rollile raku saatuse otsustajana peale DNA kahjustust.