THE ACCUMULATION OF MUTANT P53

IN HUMAN CANCER CELLS

PhD Thesis

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Monika Bug

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Affidavit

I hereby declare that this doctoral thesis has been written only by the undersigned and without any assistance from third parties.

Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Göttingen, 30.09.2010

Monika Bug

LIST OF PUBLICATIONS

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TABLE OF CONTENTS

ABSTRACT

The tumor suppressor p53 is mutated in more than 50% of all human solid tumors. This comprises mostly single residue missense point mutations that entail a loss of p53 tumor suppressor function. But at the same time, mutant p53 protein was shown to possess oncogenic activities, i.e. a gain of function, promoting invasion and chemoresistance. Mutant p53 specifically accumulates in advanced tumors, but not in normal tissues, engineered to contain a mutant p53 gene. This means that tumor specific changes evoke the accumulation of mutant p53 during tumor progression. Within this study we observed that mutant p53 accumulates even further, when tumor cells are exposed to some, but not all chemotherapeutic drugs. While the anthracyclines doxorubicin, daunorubicin and epirubicin led to the accumulation of mutant p53, the highly similar compound idarubicin did not. We found the expression of mutant p53 to be regulated at different levels: First, treatment with the topoisomerase II inhibitors daunorubicin, doxorubicin, epirubicin, idarubicin, and etoposide, evokes a DNA damage response that results in the activation of E2F1 and its target gene TAp73. Our data suggest that, upon these genotoxic treatments, E2F1 contributes to the transcriptional activation of mutant p53 pre-mRNA synthesis, both directly and through induction of TAp73. We further show for the first time that the transcription factor E2F1 associates with the promoter DNA of *TP53.* Second, among these chemotherapeutics that induce p53 transcription, we found two members to additionally induce a natural antisense transcript to p53, WRAP53. We further observed that the induction of WRAP53 coincides with impaired p53 mRNA maturation. We therefore hypothesize that the expressed antisense transcript interferes with p53 pre-mRNA stability or its nuclear export. Third, the accumulation that is inflicted on the cells during

carcinogenesis seems to be mostly regulated on the protein level. We performed a high-content siRNA screen, using single-cell based microscopy analysis, and thereby identified the ribosomal S6 kinases to be involved in mutant p53 expression regulation in advanced cancer cells. We believe that our findings should be considered for chemotherapy prescription, since we have shown that some topoisomerase II inhibitors augment mutant p53 expression and thus might favor unwanted tumor progression.

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ABBREVIATIONS

ABBREVIATION	FULL NAME
5'FU	5-fluorouracil
AA/ BAA	Acrylamide/ bisacrylamide
APS	Ammonium persulfate
AraC	Cyctosine arabinose
AraCTP	Cytidine triphosphate arabinose
ARF-BP1	ARF-binding protein 1
ATM	Ataxia-telengiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia-telengiectasia mutated and Rad3-related
Bax	Bcl2-associated X protein
BCLAF	B-cell lymphoma associated factor
BS	Binding site
BSA	Bovine serum albumin
°C	Degree Celsius
CBP	cAMP response element-binding protein binding protein
Cdc	Cell cycle division
Cdk	Cyclin dependent kinase
cDNA	complementary DNA
ChIP	Chromatin immunoprecipitation
Chk	Checkpoint kinase
CK1	Casein kinase 1
COP1	Constitutive photomorphogenesis protein 1
CPT	Camptothecin
Da	Dalton
Dauno	Daunorubicin
dCTP	Deoxycytidine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxynucleoside triphosphate
Dox	Doxorubicin
dTMP	Deoxythimidine monophosphate
dUMP	Deoxyuridine monophosphate
DTT	1,4-Dithiothreitol
DYRK	Dual specificity tyrosine-phosphorylation-regulated kinase
ECL	Enhanced chemoluminescence
Epi	Epirubicin
et al.	et altera
Etop	Etoposide
FCS	Fetal calf serum
g	Gram; Gravitational acceleration
GC rich	Guanine and Cytosine rich
h	Hour
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIPK2	Homeodomain-interacting protein kinase 2
Ida	Idarubicin
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
l	Liter
M	Molar
m	meter
Mdm	Mouse double-minute
min	Minute
MK2	Mitogen-activated protein kinase-activated protein kinase
M-MuLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
µ	Micro
NHEJ	Non-homologous end joining
NP-40	Nonidet P40
P-	Phospho-

PAGE	Polyacrylamide gel elektrophoresis
PBS	Phosphate buffered saline
PCAF	p300/ CBP associated factor
PCR	polymerase chain reaction
PDK	Phosphoinositide-dependent protein kinase
pH	Negative logarithm (base 10) of the molar concentration of dissolved protons
Pirh2	p53-induced protein, RING-H2 domain-containing
PUMA	p53-upregulated modulator of apoptosis
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNA Pol II	RNA polymerase II
RNAse	Ribonuclease
RPL27	Ribocomal protein L26
RPMI	Roswell Park Memorial Institute medium
RPS6K	Ribosomal protein S6 kinase
RREB	Ras-responsive element binding protein
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
sec, s	Second
SDS	Sodium dodecyl sulfate
siRNA	small interfering ribonucleic acid
Taq	*Thermus aquaticus*
TEMED	N,N,N´,N´- Tetramethylethylenediamine
TIP60	TAT-interacting protein, 60-kDa
Tris	tris(hydroxymethyl)aminomethane
TSS	Transcriptional start site
U	Unit of enzyme activity
UV	Ultra violet
V	Volt
w/v	weight per volume
WB	Westernblot = immunoblot
WRAP53	WD repeat-containing antisense to p53
wt	wild type

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Most cells of our body have the potential to divide, an essential process in the renewal of tissues and the scope of our immune system. Some cells proliferate rapidly, others rarely, but proliferation happens in a controlled fashion, with a large set of check points and back-up mechanisms (Pagano and Draetta, 1991). Due to exogenous stimuli like irradiation, exposure to toxins or other forms of stress, key players of this regulation cascade can be mutated and become dysfunctional. If the affected cell thereby gains a certain growth advantage, but is not recognized by the immune system and eliminated, it starts to proliferate in an uncontrolled fashion. Over time, more and more mutations accumulate in these rapidly dividing cells and, depending on the genes that were hit by mutations; this can lead to the development of malignancies.

When a tumor is diagnosed, it is often already in an advanced stage and needs to be treated by chemotherapy. Nowadays a number of chemotherapeutic agents are available; their mechanisms of action are diverse and often not completely understood yet. Depending on the cell type and the mutational spectrum, tumors are treated with different chemotherapeutic agents. Nevertheless, the mechanistic details and the cause of some side effects are widely unknown and a matter of current research.

2.1. CHEMOTHERAPEUTICS

The idea behind most chemotherapeutic drugs is that they trigger a DNA damage response in proliferating cells and subsequently lead to apoptosis (Johnstone *et al.*, 2002). Since tumor cells are normally proliferating faster than most other cells of our body, they are preferentially targeted. Nevertheless,

hematopoetic cells, gastrointestinal mucosal cells and hair are examples of rapidly dividing cells that often get affected by these drugs although their fast proliferation happens in a controlled fashion and is important for their proper function (Tannock, 1986).

Generally, chemotherapeutic drugs can be clustered in three groups according to their mechanism of action: nucleoside analogs, inhibitors of enzymes involved in replication and transcription, and drugs that directly damage the DNA (Pommier and Diasio, 2006).

2.1.1. NUCLEOSIDE ANALOGS

This group of drugs is also called 'antimetabolites' and either inhibits the formation of functional nucleotide triphosphates, or interferes with replication elongation (Daher *et al.*, 1990). All agents that belong to this class prevent efficient DNA synthesis and mostly affect the cells in S phase of the cell cycle. Examples are on the one hand 5-fluorouracil (5'FU), which inhibits the conversion of dUMP to dTMP and causes due to depletion of dTMP defects in DNA synthesis and cell division (Daher *et al.*, 1990). On the other hand, agents like Cytosine arabinose (AraC) affect replication elongation; AraC is recognized by DNA polymerase α as deoxycytosine, but the incorporation of AraCTP in the elongating DNA strand fails due to sterical hindrance resulting in the termination of DNA replication (Chrencik *et al.*, 2003).

2.1.2. ENZYME INHIBITORS

Enzymes with specific functions during replication are the polymerases, topoisomerases and helicases; these also reassemble the most common drug targets of this class. Polymerases are for example targeted by aphidicolin and foscarnet that block dCTP incorporation or pyrophosphate cleavage, respectively (Crumpacker, 1992; Sheaff *et al.*, 1991).

Topoisomerases are enzymes that relax supercoiled DNA by cleavage and religation (D'Arpa and Liu, 1989). The chemotherapeutic drugs camptothecin, etoposide and the anthracyclines daunorubicin, doxorubicin, epirubicin and idarubicin are well known representatives of this class. While camptothecin specifically acts on topoisomerase I, an enzyme that functions through single strand cleavage, and traps the cleavage intermediates (Pommier *et al.*, 2003), the other mentioned drugs are mainly known to act on topoisomerase II (Fortune and Osheroff, 2000). Even though all of these agents in the end lead to double strand breaks that trigger a DNA damage response and induce apoptosis. The topoisomerase II inhibitors additionally can interfere with other metabolic processes of the DNA, like transcription, DNA repair, and chromatin remodeling (Fortune and Osheroff, 2000). In contrast to camptothecin these drugs trap the cleavage intermediate, with the two enzyme subunits of 170 and 180 kDa covalently linked to the DNA. Thereby large protein-DNA adducts are produced that form steric blocks on the template DNA (Fortune and Osheroff, 2000).

The planar structure of anthracyclines additionally allows them to intercalate into DNA, preferentially in GC rich regions. This was shown to stabilize the duplex DNA and to prevent helicases from separating the strands (Bachur *et al.*, 1992).

Inhibitors that act independently of these enzymes, directly involved in replication, but still inhibit cell cycle progression interfere with cyclin-dependent kinases (Cdk) or the checkpoints. The Cdk inhibitors flavopiridol and roscovitine are competitive inhibitors of ATP binding and interfere at various steps in the cell cycle: G1/S transition (restriction point) through Cdk4/6, the activation of replication origins (S-phase) through Cdk2, and the inactivation of these replication origins by Cdk1-cyclin B complexes (De Falco and De Luca, 2010). Additionally, it was shown that these Cdk inhibitors inhibit RNA polymerase II and thereby transcription (Wesierska-Gadek and Krystof, 2009). Nevertheless,

currently most of these kinase inhibitors lack specificity. This also holds true for the checkpoint inhibitor 7-hydroxystaurosporine, a checkpoint kinase 1 (Chk1) inhibitor that was found to additionally inhibit Chk2 and phosphoinositide-dependent protein kinase 1 (PDK1) (Sato *et al.*, 2002; Yu *et al.*, 2002), as well as caffeine, the first drug identified to abrogate a cell cycle checkpoint by inhibiting Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and RAD3-related (ATR), but also a number of additional kinases (Sabisz and Skladanowski, 2008; Sarkaria *et al.*, 1999).

2.1.3. DNA DAMAGING DRUGS

Additionally to radiotherapy there are chemotherapeutic drugs that block the replication fork by inducing DNA template lesions, like DNA adducts, DNA strand breaks, and DNA protein crosslinks. The alkylating agents modify bases within the DNA, either through methylation of Guanine (methylmethanesulfonate), DNA-DNA crosslinks and DNA-protein crosslinks (cisplatin), or interstrand DNA crosslinks (cyclophosphamide) (DeNeve *et al.*, 1990; Hausheer *et al.*, 1989; Mirzayans *et al.*, 1988). In contrast to that, radiomimetic DNA cleaving agents like bleomycin and neocarcinostatin induce single- as well as double-strand breaks of the DNA (Goldberg, 1987; Huang *et al.*, 1981). As for most of the mentioned drugs, the induction of such DNA lesions triggers a DNA damage response, which signals from ATM or ATR down to the effectors p53, E2F1, cell division cycle 25 (CDC25) and others (Darzynkiewicz *et al.*, 2009).

2.2. THE DNA DAMAGE RESPONSE

Originally two different DNA damage pathways were identified. Their activation was observed depending on the kind of damage that was inflicted on the DNA. In response to double strand breaks ATM is recruited to the sites of DNA damage and gets activated, the signal is transduced by Chk2 which in turn

leads to the accumulation and activation of p53, E2F1 and other effectors (Lavin and Khanna, 1999). In contrast to that, ATR is activated by single strand breaks, the signal transduced by Chk1 and finally effectors like p53, Cdc25 and others get activated (Paulsen and Cimprich, 2007). Nowadays, there is a lot more crosstalk between the two pathways known and additional kinases at the levels of ATM, ATR as well as Chk1 and Chk2 were identified.

2.2.1. KINASES IN THE DNA DAMAGE RESPONSE

Not only the role of DNA-dependent protein kinase (DNA-PK), sensing DNA double strand breaks and lesions of non homologous end joining (NHEJ), amends the network of kinases activated in response to DNA damage (Danska and Guidos, 1997; Rathmell *et al.*, 1997), also p38 and its activation of mitogen-activated protein kinase-activated protein kinase 2 (MK2), as well as the recently identified cross talks between the pathways (Reinhardt *et al.*, 2007). The impact of phosphorylations involved in this network is immense and our knowledge about these is most probably far from being complete. The current view on central players within this network and their most prominent targets is summarized in Figure 1.

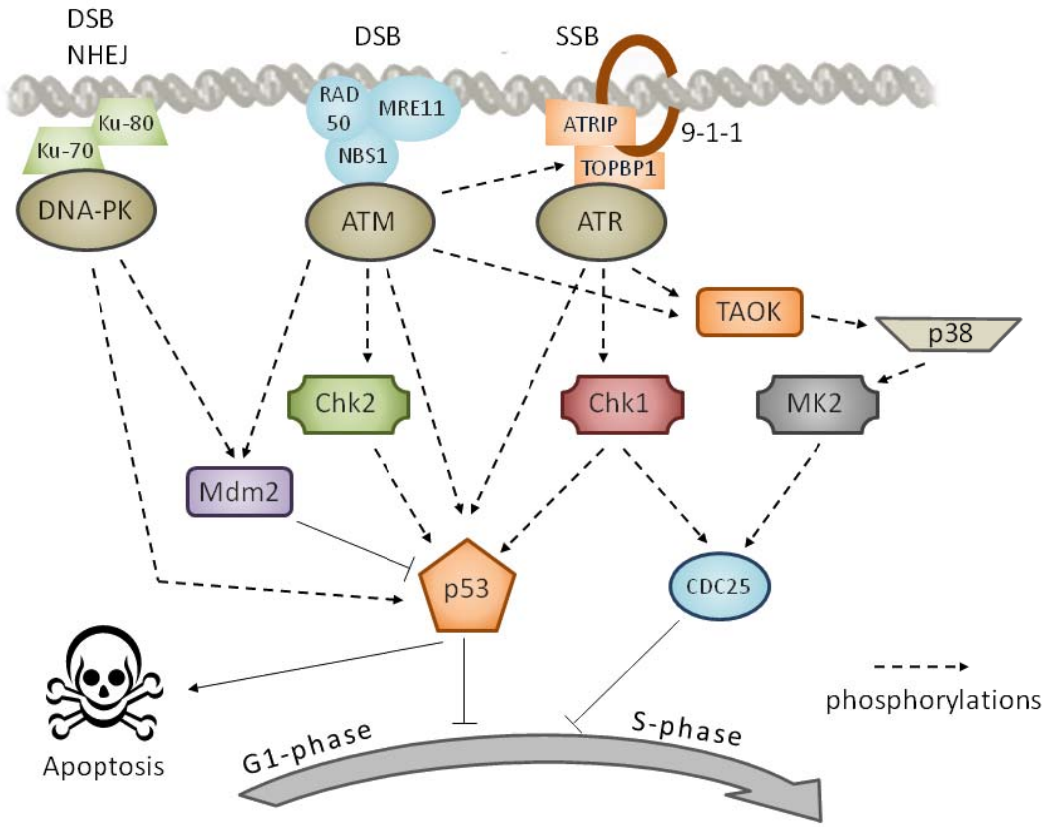


Figure 1: The role of kinases in the DNA damage response pathways.
Due to different stimuli sensor proteins get recruited to the site of DNA damage and trigger a DNA damage response. Double strand breaks (DSB) or lesions of non-homologous end joining (NHEJ) are recognized by the heterodimer Ku70/ 80 and lead to the recruitment and activation of DNA-dependent protein kinase (DNA-PK). This in turn activates p53, on the one hand through direct phosphorylation and on the other hand through phosphorylation of Mouse double-minute 2 (Mdm2). Additionally DSB can be sensed by the MRN complex (Meiotic recombination 11 (MRE11), Rad 50, and Nijmegen breakage syndrome 1 (NBS1)) that activates the transducer kinase Ataxia-telangiectasia mutated (ATM). ATM has a number of different phosphorylation targets that in turn all lead to the activation of p53: Mdm2, checkpoint kinase 2 (Chk2), p53 itself, TAO kinase (TAOK), and also DNA topoisomerase II-binding protein 1 (TOPBP1). The latter itself is part of the DNA single strand break (SSB) sensor complex and involved in the activation of Ataxia-telangiectasia and RAD3-related (ATR). Besides TOPBP1 also the Rad9-Hus1-Rad1 clamp complex (9-1-1) and ATR-interacting protein (ATRIP) contribute to the sensing of DNA single strand breaks and the activation of ATR. Similar to ATM, also ATR has a number of different phosphorylation targets that activate p53 and mark the cell cycle regulatory protein cell division cycle 25 (CDC25) for degradation: p53 itself, checkpoint kinase 1 (Chk1) and TAOK. The latter was found to phosphorylate and activate p38, which in turn phosphorylates and activates mitogen-activated protein kinase-activated protein kinase 2 (MK2), a kinases that was shown to directly phosphorylate CDC25A, leading to its destabilization.

2.2.2. E2F ACTIVITIES IN RESPONSE TO DNA DAMAGE

Since E2F1 was identified in 1987 by Kovesdi *et al.* the number of known E2F family members increased and currently comprises eight genes (E2F1 to 8), which give rise to nine distinct proteins (DeGregori and Johnson, 2006). The transcription factors can be categorized into three groups: E2F1 – 3A are mostly found as activating transcription factors that can get inactivated through their binding to the retinoblastoma protein (Rb). E2F4 and -5 are frequently detected in their inactive state, bound to one of the three pocket proteins (Rb, p107, or p130), but are generally categorized as weak activators. Finally, E2F6 – 8 are classified as transcriptional repressors, which do not interact with any of the pocket proteins (Trimarchi and Lees, 2002). The E2F proteins transactivate several Cdks, as well as cyclins and thereby contribute positively to cell cycle progression and cell proliferation.

Additionally to its cell cycle related functions, E2F1 was found to be an activator of the DNA damage response pathway. It was shown that over-expression of E2F1 leads to increased Chk2 mRNA, as well as protein levels (Rogoff *et al.*, 2004). Over and above, Stevens *et al.* (2003) reported that Chk2 phosphorylates E2F1 and thereby alters the DNA binding specificity of E2F1 from S-phase genes to the pro-apoptotic gene p73. These findings underscore the controversial activities that were implied to E2F1 in the literature. The transcription factor was originally identified as an oncogene, whose hyperactivation leads to uncontrolled cell proliferation, and was later on found to actively suppress tumorigenesis by inducing pro-apoptotic genes in response to DNA damage.

2.2.3. P53 IN THE DNA DAMAGE CASCADE

The tumor suppressor p53, as well as the two E3 ubiquitin ligases Mdm2 and Mdm4 are common phosphorylation targets of Chk1, Chk2, but also the

upstream components of the DNA damage signaling pathways ATM, ATR and DNA-PK (Figure 2) (Meek, 2009).

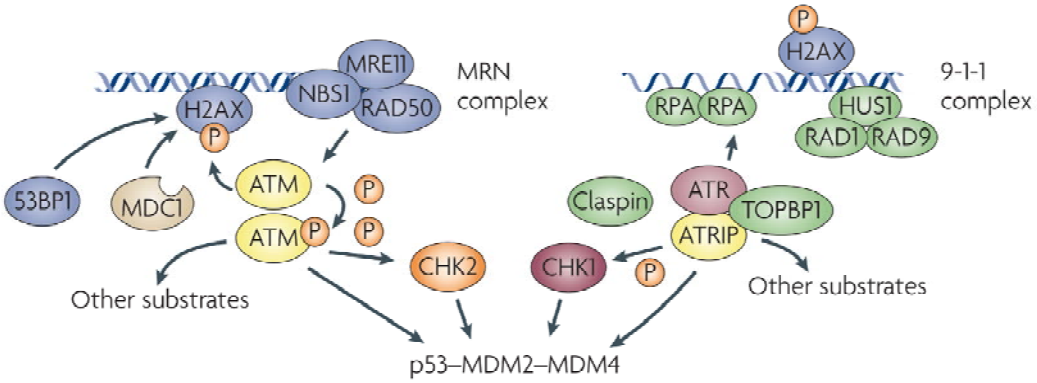


Figure 2: All DNA damage pathways converge at the point of p53 phosphorylation [taken from Meek (2009)].
The activation of Ataxia-telangiectasia mutated (ATM) and checkpoint kinase 2 (Chk2) in response to double strand breaks, sensed by proteins of the MRN complex (Meiotic recombination 11 (MRE11), Rad 50, and Nijmegen breakage syndrome 1 (NBS1)) results in the phosphorylation of p53 and its two antagonists Mouse double-minute 2 (Mdm2) and Mouse double-minute 4 (Mdm4). The same is achieved in response to single strand breaks, which are sensed by the 9-1-1 complex (RAD 9, RAD 1, and HUS 1) and transduced through the activation of the Ataxia-telangiectasia and RAD3-related (ATR) and checkpoint kinase 1 (Chk1) kinases.
H2AX: Histone variant; MDC1: Mediator of DNA damage checkpoint protein 1; 53BP1: p53-binding protein 1; RPA: Replication protein A; TOPBP1: DNA topoisomerase II-binding protein 1; ATRIP: ATR-interacting protein.

While p53 is activated and stabilized through these phosphorylations (Canman *et al.*, 1998), it was shown that its antagonists Mdm2 and Mdm4 get destructed (Maya *et al.*, 2001). Following from the above, p53 is stabilized in two ways in response to DNA damage, since Mdm2 in complex with Mdm4 is known to be the most prominent negative regulator of p53 (Meulmeester *et al.*, 2005; Montes de Oca Luna *et al.*, 1995). These ATM and ATR mediated phosphorylations trigger a cascade of additional posttranslational modifications of p53 that can tailor its response in an appropriate and proportionate manner according to the nature and intensity of the damage (Murray-Zmijewski *et al.*, 2008).

2.3. THE TUMOR SUPPRESSOR P53

Already in the early 90s the human p53 protein was identified to bind to the palindromic DNA sequence Pu-Pu-Pu-C-$^{A}/_{T}$-$^{T}/_{A}$-G-Py-Py-Py and its biological function as transcription factor was proposed (el-Deiry *et al.*, 1992; Kern *et al.*, 1991). The C-terminal oligomerization domain of the protein facilitates its tetramerization, which is essential for DNA binding as well as transcriptional activation of target genes (McLure and Lee, 1998). Nowadays, hundreds of genes regulated by p53 are known that can generally be classified upon their functions in cell cycle arrest, apoptosis, DNA repair, angiogenesis, and senescence (el-Deiry, 1998). The fine tuning of transcriptional activation through p53 mostly happens on the level of posttranslational modifications.

2.3.1. POSTTRANSLATIONAL MODIFICATIONS

The tumor suppressor p53 is known to be modified by all kinds of posttranslational modifications like phosphorylation, acetylation, methylation, neddylation, ubiquitination, and sumoylation. Most sites of modification as well as a number of modifying and demodifying enzymes are known so far (Olsson *et al.*, 2007). Nevertheless, the causes and consequences of the different modification patterns are not completely understood yet and a matter of current research.

PHOSPHORYLATION

Numerous Threonine and Serine residues, mainly within the transactivation domain of p53, have been identified as targets of phosphorylation by kinases like ATM, ATR, DNA-PK, Chk1, Chk2, CK1, JNK, HIPK2 and DYRK2 (Bode and Dong, 2004). These modifications often lead to the stabilization of the protein and hence to its activation in response to genotoxic and other forms of stress. Data from in vitro or over-expression studies indicate that phosphorylation at Serine 15 stimulates p53-dependent transactivation, growth arrest and apoptosis in response to DNA damage, whereas it is still under

debate whether phosphorylation of this site affects Mdm2 binding (Dumaz and Meek, 1999). Two groups established mouse models that express a mutant version of p53 where Serine 18 (corresponding to Serine 15 in humans) is replaced by Alanine and can therefore not be phosphorylated any longer. Thymocytes of these mice displayed a reduced induction of DNA damage mediated apoptosis, indicating that this phosphorylation in vivo contributes to the specific activation of target genes (Chao *et al.*, 2003; Sluss *et al.*, 2004).

ACETYLATION

The histone acetyltransferase (HAT) heterodimers CBP/p300 were found to acetylate p53 at Lysines 370, 372, 373, 381, and 382 (Gu and Roeder, 1997). In contrast, Lys320 and Lys305 in the nuclear localization domain of p53 are acetylated by PCAF and p300 respectively (Liu *et al.*, 1999). Some studies reported an enhancement of sequence-specific DNA-binding activity of acetylated p53, as well as more potent transcriptional activation of target genes (Barlev *et al.*, 2001; Sakaguchi *et al.*, 1998). Along that line, it was shown by two independent groups that acetylation of Lys120 of p53, by the MYST acetyltransferases MOF and TIP60, leads to the preferential induction of pro-apoptotic target genes such as PUMA and Bax, whereas the expression of other target genes like p21 and Mdm2 remains unaffected (Sykes *et al.*, 2006; Tang *et al.*, 2006). As the lysine residues within the C-terminal domain of p53 are also targets for ubiquitination, it was proposed that acetylation of these residues may promote the stabilization of p53 by interfering with proteasomal degradation (Brooks and Gu, 2003; Ito *et al.*, 2002).

To elucidate the impact of these acetylations in vivo, different mouse models were generated harboring up to 7 Lysine to Arginine mutations. Unfortunately these studies were not conclusive, since the phenotypes of these mice were very mild. The fact that various posttranslational modifications are conjugated to the same set of Lysines implies that the biological consequences, caused by

these, cancel each other out and therefore burrow the actual activities (Olsson *et al.*, 2007).

UBIQUITINATION

In contrast to the above it appeared to be very conclusive when mouse models were used to unravel the biological consequences of p53 ubiquitination. Montes de Oca Luna *et al.* (1995) generated a knock-out mouse line for Mdm2, the most prominent E3 ubiquitin ligase of p53. This resulted in embryonic lethality of the mice, a strong phenotype that was rescued by the additional knock-out of p53. These observations indicate that the lack of Mdm2-mediated p53 degradation leads to massive apoptosis and therefore to embryonic lethality of the mice. The E3 ubiquitin ligases COP-1, Pirh2, and ARF-BP1 were as well described to ubiquitinate p53 and to induce its proteasomal degradation (Chen *et al.*, 2005a; Dornan *et al.*, 2004; Leng *et al.*, 2003). Nevertheless, the above mentioned Mdm2 knock-out study suggests that in unstressed cells no additional E3 ubiquitin ligase is able to prevent the accumulation of p53 and its induction of apoptosis.

2.3.2. REGULATION OF P53 EXPRESSION

The expression levels of p53 are mainly regulated on the protein level. Mdm2, the above described essential p53 ubiquitin ligase, is itself one of the p53 target genes and thereby forms an autoregulatory feedback loop with the tumor suppressor (Freedman *et al.*, 1999). Mdm2 binds to the N-terminus of p53 and ubiquitinates it, either at C-terminal Lysines, or at Lysines within the DNA binding domain, this subsequently leads to the nuclear export or proteasomal degradation of the protein (Li *et al.*, 2003). Even though it seems to be an energetically unfavorable mechanism, the constant transcription, translation and proteasomal degradation of p53 allows the cell to rapidly react to various stress conditions, like DNA damage, oncogene activation, hypoxia and other inducers of the p53 network (Figure 3).

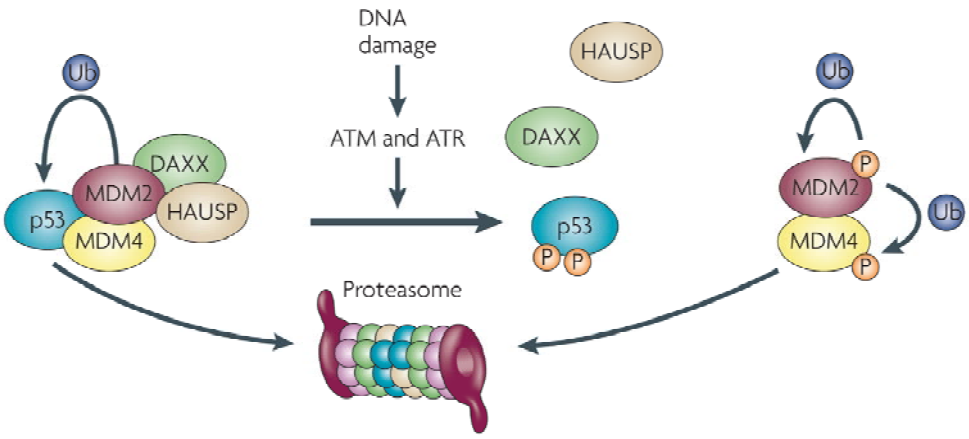


Figure 3: The fate of p53: between proteasomal degradation and DNA damage induced phosphorylation
[taken from Meek (2009)].
In unstressed cells the expression of p53 is kept at low levels. The E3 ubiquitin ligases Mouse double-minute 2 (Mdm2) and Mouse double-minute 4 (Mdm4), as well as the deubiquitinating enzyme Ubiquitin-specific protease (HAUSP) regulate its nuclear export and proteasomal degradation. In response to DNA damage, signaling cascades via Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and RAD3-related (ATR) lead to the phosphorylation of p53 and the E3 ligases Mdm2 and Mdm4. While p53 gets stabilized through these modifications, its antagonists get ubiquitinated (Ub) and subjected to proteasomal degradation.

Other kinds of p53 regulation received less attention. But, meanwhile it became evident that there are a few regulators that induce p53 expression through transcriptional activation. First, HOXA5 was found as a positive regulator of p53 transcription in response to DNA damage with the additional observation that its loss of mRNA expression in tumor samples is positively correlated with a loss of p53 mRNA expression (Raman *et al.*, 2000). Liu *et al.* (2007) reported that upon exposure to genotoxic stress, PKCdelta gets activated and interacts with the death-promoting transcription factor Btf (alias BCLAF) to co-occupy promoter elements within *TP53*. They furthermore reported that siRNA mediated knock-down of Btf suppresses p53-mediated apoptosis in response to DNA damage. Wang and el-Deiry (2006) found that p53 itself and, its structurally und functionally related family member, TAp73 are capable of regulating the expression of p53 on the mRNA level. They described three potential p53/ TAp73 responsive elements in the promoter region of p53, further identifying one of them to be essential using luciferase assays. Recently also

Ras-responsive element binding protein 1 (RREB1) was identified as potential transcriptional activator of p53 expression in response to DNA damage (Liu *et al.*, 2009).

In contrast to the forecited transcription factors, Mahmoudi *et al.* (2009) discovered an additional mechanism of p53 mRNA expression regulation. The natural antisense transcript to p53 (WRAP53) was found to mediate p53 mRNA stability in response to DNA damage. It was identified as a predicted gene within the *TP53* genomic locus on chromosome 17, encoded on the opposite strand of the tumor suppressor. The biological role of WRAP53 protein is completely unknown, whereas the specific over expression of certain transcripts was shown to increase p53 mRNA expression.

2.3.3. THE INTERPLAY OF P53/ P73 AND E2F1

In response to DNA damage p53 and E2F1 both get stabilized through phosphorylation by the same set of kinases: ATM, Chk1, and Chk2. The phosphorylation of E2F1 through Chk1 and Chk2 then leads to the induction of pro-apoptotic target genes like TAp73 (Stevens *et al.*, 2003). This is proposed to be a backup mechanism, when p53 is defective, since TAp73, a paralog of p53, is known to transactivate the same pro-apoptotic target genes as p53 (McKeon, 2004).

But, there is also direct cross-talk between the two transcription-factors p53 and E2F1 reported. On the one hand, deregulated E2F was found to directly transactivate the expression of $p14^{ARF}$, which inhibits Mdm2 and thereby leads to the stabilization and activation of p53 (Bates *et al.*, 1998). While in the absence of $p14^{ARF}$, E2F1 was found to stimulate p53 phosphorylation. Within the same study, it was claimed that this, most probably ATM or ATR dependent, posttranslational modification of p53 is crucial for E2F1-mediated apoptosis (Rogoff *et al.*, 2002).

2.3.4. MUTATIONS OF P53

In 1979 p53 was identified as a protein accumulated in the majority of the analyzed tumors and therefore characterized as tumor antigen (Crawford *et al.*, 1981; DeLeo *et al.*, 1979; Rotter *et al.*, 1980). Almost 10 years later Finlay *et al.* (1988) among others discovered that for all the work that was performed meanwhile a mutant p53 clone was used and that p53 in fact acts as a tumor suppressor. The mutations found in p53 do not reflect the classical spectrum known from other tumor suppressors, where frame shifts or large deletions mainly cause the loss of tumor suppressor activity. On the contrary, point mutations of single amino acids, as they are found in p53, are characteristic for oncogenes. Nevertheless, point mutations in oncogenes normally affect a small number of codons, encoding residues involved in their enzymatic activity, whereas the mutational spectrum of p53 ranges throughout the whole DNA binding domain of the protein, with a number of hotspot mutations that occur more frequently than others (Soussi and Lozano, 2005) (Figure 4).

A comprehensive list of published studies where p53 mutations have been analyzed by gene sequencing is provided on the IARC *TP53* database (http://www.iarc.fr/p53/). Evaluation of these data revealed that in about 70% of the reported studies the presence of a *TP53* mutation is significantly associated with bad prognosis, whereas only 5% of the studies reported a significantly good prognosis upon *TP53* mutation (Olivier *et al.*, 2005).

These observations indicate that cancer-associated mutant p53 isoforms are more than just relics of wt p53 inactivation and possess distinctive roles in tumor cells. Firstly, this can be achieved through dominant-negative effects over co-expressed wild type p53 proteins, forming mixed tetramers that are incapable of DNA binding and transactivation. Secondly, the generated mutant p53 protein might possess activities of its own, which could actively contribute to tumor progression.

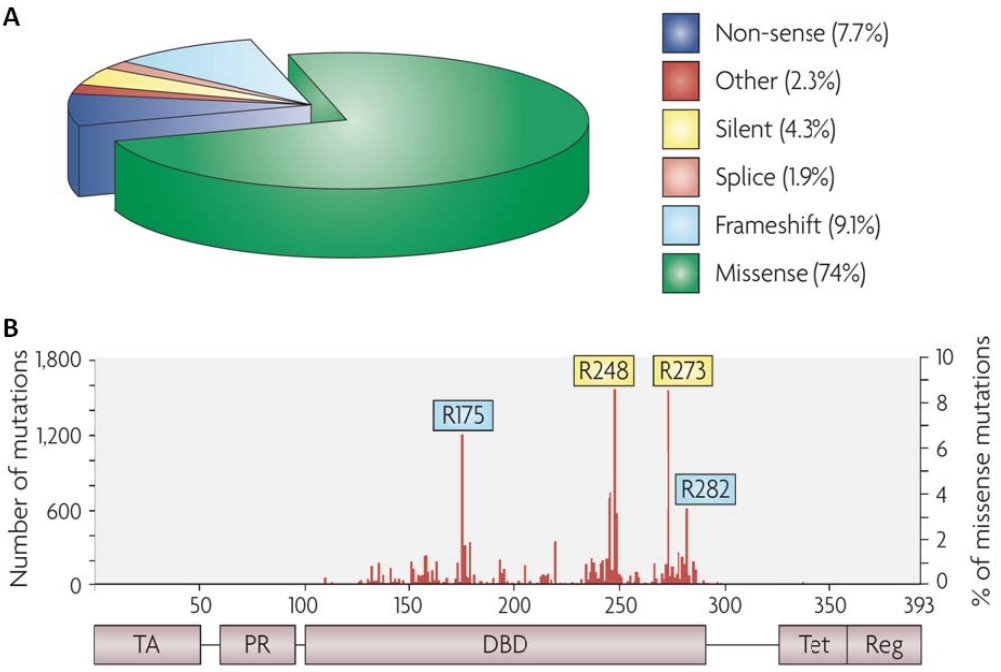


Figure 4: The tumor suppressor p53 is most frequently inactivated through a missense mutation within the DNA binding domain
[adopted from Brosh and Rotter (2009)].
A: Pie chart representing the different tumor-derived mutation types reported in the IARC *TP53* Mutation Database. **B:** Almost every amino acid of the central DNA binding domain of p53 is hit by a point mutation leading to p53 loss of function. Four amino acids at the positions 175, 248, 273, and 282 are more frequently hit and represent the so called hotspot mutations of p53.
TA: transactivation domain; PR: proline-rich domain; DBD: DNA binding domain; Tet: tetramerization domain; Reg: carboxy-terminal regulatory domain. Data derived from the IARC *TP53* Mutation Database version R13 (November 2008).

2.3.5. P53 GAIN OF FUNCTION

Mutant p53 gain of function (GOF) was described first by Dittmer *et al.* (1993). They showed that ectopic mutant p53 expression can transform p53-null cells, leading to increased colony formation in culture and raised numbers of tumors in mice. Meanwhile three hallmarks of transformation were described to promote mutant p53 gain of function:

GENOMIC INSTABILITY

Mutant p53 expression was shown to disrupt normal spindle checkpoint control leading to polyploid cells (Gualberto *et al.*, 1998). Additionally, mice with over-expressed mutant p53 were described to exhibit a high degree of genomic

instability with aberrant centrosome amplification, as well as chromosome translocations (Caulin *et al.*, 2007).

ANTIAPOPTOTIC SIGNALING

Mutant p53 can suppress c-myc induced apoptosis in leukemic cells and thereby allows the cell to benefit from the pro-proliferative effects of the oncogene, without inducing apoptosis at the same time (Lotem and Sachs, 1995). Additionally, mutant p53 expression decreases the induction of apoptosis in response to chemotherapeutics, as well as other kinds of DNA damage, thereby conferring chemoresistance on the tumor cells (Blandino *et al.*, 1999; Li *et al.*, 1998).

CELL MIGRATION AND INVASION

In vitro studies by Adorno *et al.* (2009) and Wang *et al.* (2009) indicated that mutant p53 can augment cell migration and invasion. Nevertheless, it is believed that this process is highly cell-context dependent and in many cases additional signals like oncogenic Ras or TGF-β are needed to unleash this gain of function activity. To estimate the biological relevance of these observations, data from different mouse models were used. Both, mutant p53 over-expressing cells intravenously inoculated into syngeneic mice and knock-in studies, where the endogenous wt p53 was replaced by its mutant variant, revealed that mutant p53 expression leads to the development of more aggressive, metastatic tumors. This supports the concept that mutant p53 gain of function actively contributes to tumor progression (Heinlein *et al.*, 2008; Pohl *et al.*, 1988).

The mechanistic understanding of the role of mutant p53 in tumor cells is still not complete, but the available reports offer some insights. Microarray analysis yielded a large list of genes regulated in their expression by mutant p53. Nevertheless, it was also shown that most mutant p53 variants cannot directly bind to the p53 responsive elements, since either the amino acids involved in direct DNA binding are mutated or the gained mutations lead to overall changes

in the conformation of the protein. Therefore, the effects of mutant p53 on the transcriptional regulation of other genes have to occur indirectly (Figure 5) (Oren and Rotter, 2010). First, mutant p53 was found in complex with its two family members p63 and p73, thereby inhibiting their transcription factor activities (Gaiddon *et al.*, 2001; Strano *et al.*, 2002). Second, mutant p53 was shown to bind to a number of other transcription factors, either leading to the repression of their activity, or recruiting transcriptional activators that facilitate the transcription of the downstream genes (Di Agostino *et al.*, 2006; Stambolsky *et al.*, 2010; Weisz *et al.*, 2007). Last but not least, it was shown that mutant p53 can bind specific DNA elements, such as matrix attachment regions, in a conformation dependent manner. This is proposed to block the binding of other transcription factors to adjacent binding sites, resulting in transcriptional inhibition (Gohler *et al.*, 2005).

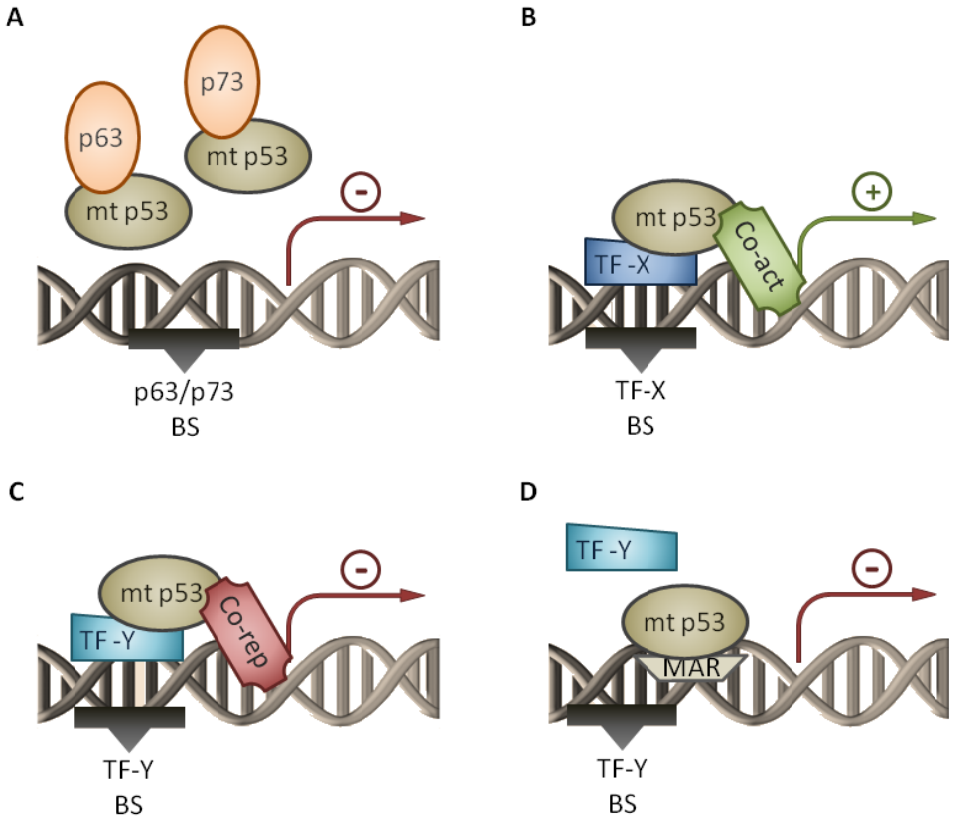


Figure 5: Transcriptional regulation by mutant p53 independently of its DNA binding activity.
A: Transcriptional inhibition through direct interaction and inactivation of its family members p63 and p73. **B:** Transcriptional activation of target genes through interaction with other sequence specific transcription factors (TF-X) and the recruitment of co-activators. **C:** Transcriptional repression of target genes through interaction with other sequence specific transcription factors (TF-Y) and the recruitment of co-repressors. **D:** Association with matrix-attachment regions (MAR) that partially overlap with other transcription factor binding sites and thereby prevent the binding and transactivation of the down-stream genes.
[Adopted from Oren and Rotter (2010)]

2.3.6. MUTANT P53 ACCUMULATION

Mutant p53 protein often accumulates in tumor cells and reaches steady-state levels that greatly exceed those of wt p53. Additionally, it was reported that mutant p53 is not intrinsically stable. This was on the one hand observed in primary cells derived from Li-Fraumeni syndrome patients, who carry germ line p53 mutations (Yin *et al.*, 1992), and on the other hand in the knock-in studies performed by Lang *et al.* (2004) and Olive *et al.* (2004), where mutant p53 protein was shown to only accumulate in tumors, but not in the surrounding tissue.

2.4. THE AIM OF THIS WORK:

THE MECHANISMS OF MUTANT P53 ACCUMULATION

During the last 30 years of p53 research, it was repeatedly shown that the tumor suppressor p53 strongly accumulates in response to chemotherapeutic treatment, going along with posttranslational modifications of the protein at various sites. A similar or even stronger accumulation of p53 is observed in tumor cells that express a mutant variant of the protein. Missense mutations of the protein were shown to not only abrogate its tumor suppressive activities, but also to actively promote oncogenic functions, ranging from genomic instability, over antiapoptotic signaling to increased metastasis and proliferation.

The question that arises from the above is whether the mechanisms leading to p53 accumulation in response to chemotherapeutic treatment, act synergistically with the generally observed augmentation of p53 expression in cancer cells harboring a p53 point mutation. Within this study we observed that such a synergism can lead to the further accumulation of mutant p53 in cancer cells upon treatment with some but not all chemotherapeutic drugs. Therefore we investigated the mechanistic details of mutant p53 accumulation on the one hand gained due to cellular transformation and on the other hand through chemotherapeutic treatment. We found that mutant p53 expression is regulated differently during these two processes causing its accumulation. To achieve the benefits of chemotherapeutic treatment and at the same time circumvent the undesired side effects of mutant p53 accumulation, it would be advantageous to use the obtained information for the development of new therapeutics that could be used in combination with classical chemotherapeutics.

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. TECHNICAL DEVICES

EQUIPMENT	COMPANY
Agitator, magnetic, heated (MR 3001)	Heidolph
Bioruptor (UCD-200TM-EX)	Diagenode
Blotting-chamber (EasyPhor Wet-Blotter)	BioZym
Centrifuge, mini (GMC-060 LMS)	Eppendorf
Centrifuge (5415R)	Eppendorf
Centrifuge (Megafuge 1.0 R)	Heraeus Instruments
ChemoCam Imager (ECL detection)	Intas
Countess	Invitrogen
Electrophoresis-System, for SDS-PAGE	Amersham Biosciences
Foil swelding machine	KRUPS
Freezer -20°C	Liebherr
Freezer -80°C	Heraeus Instruments
Heating block (HTB-1-131 HLC)	Haep Labor Consult
Thermomixer comfort	Eppendorf
Ice-machine (B100)	Ziegra
Incubator for cell cultures (Hera Cell 150)	Heraeus Instruments
Laminar flow cabinet (Hera Safe)	Heraeus Instruments
Light microscope (Axovert 40C)	Zeiss
Liquid Nitrogen Tank (LS 4800)	Taylor-Wharton
Microscope, fluorescent (AxioImager.Z1)	Zeiss
Microscope, automated (Pathway 855)	Becton, Dickinson and Company
PCR machine Thermocycler (T personal)	Biometra
pH-Meter (WTW-720)	WTW, Weilheim, DE
Pipet, electric (Portable-XP)	Drummond
Pipets 2.5, 20, 200, 1000 µl	Eppendorf
Power supply unit (Powerpack P25T)	Biometra

Power supply unit (Power-Pac Basic)	Biorad
Real-time PCR machine (Chromo4™)	Bio-Rad Laboratories
Real-time PCR machine (CFX96; C1000)	Bio-Rad Laboratories
Refrigerator 4°C	Liebherr
Rotator (PTR 300)	Grant Bio
Scales (Acculab ALC-6100.1, LE623S)	Sartorius
Shaker (DRS-12)	neo Lab
Shaker (Promax 2020)	Heidolph
Shaker (Rocky)	Schütt Labortechnik
Spectrophotometer NanoDrop (ND-1000)	PeqLab
UV-transilluminator (Intas UV system)	Intas
Vacuum pump	IBS Integra Biosciences
Vortex (Genie 2)	Scientific Industries
Water bath (TW 20)	Julabo Labortechnik

3.1.2. CONSUMABLE MATERIALS

CONSUMABLE	**COMPANY**
6- and 12- well cell culture plates (Cellstar)	Greiner-bio-one
96 well imaging plates (black)	BD-Falcon
96 well PCR plate (duo plate, skirted)	Sarstedt
Adhesive aluminum foil	Sarstedt
Cell scraper (16mm, 25mm)	Sarstedt
Centrifuge tubes (15ml, 50ml)	Sarstedt
Cryo Tube Vials (1.8ml)	Nunc
Gloves (Latex Safe Skin PFE)	Kimberly Clark
Micro tubes (0.5ml, 1.5ml, 2ml)	Sarstedt
Nitrocellulose, poresize: 0.2µM (Protran BA83)	Omnilab
Pasteur pipets, glass (230mm)	VWR international
Parafilm	Pechiney
Pipet tips with or without filter (20 µl, 200 µl, 1000 µl)	Sarstedt
Sealing tape (optically clear for 96 well PCR Duo plates)	Sarstedt
Syringe (1 ml)	BD Plastipak
Syringe needles (0,6 x 25mm)	BD Microlance
Tissue culture dish (CELLSTAR 60x15 mm)	Greiner-bio-one

Tissue culture dish (CELLSTAR 100x20 mm)	Greiner-bio-one
Tissue culture dish (CELLSTAR 145x20 mm)	Greiner-bio-one
Whatman paper (GB002)	Schleicher & Schuell

3.1.3. CHEMICALS

NAME	COMPANY
10 x Taq buffer with KCl (B38)	Fermentas
2-mercaptoethanol	Roth
2-propanol	Roth
25 mM $MgCl_2$ (R0971)	Fermentas
β-glycerol phosphate disodium salt pentahydrate (β-glycerophosphate)	Fluka
Ammonium persulfate (APS)	Roth
Bromphenol blue	Sigma-Aldrich
BSA	Roth
Calcium chloride ($CaCl_2$)	Roth
Camptothecin	Sigma-Aldrich
Chelex 100	Bio-Rad
Chloroform	Roth
Ciprobay 200	Bayer
Cycloheximide	Sigma-Aldrich
DAPI dilactate	Sigma-Aldrich
Di-sodiumhydrophosphate dihydrate (Na_2HPO_4 x H_2O)	Roth
Dithiothreitol (DTT)	Roth
dNTPs, 25 µM each (U1420)	Promega
Dulbecco's Modified Eagle's Medium (31600-091)	GIBCO/ Invitrogen
EDTA	Roth
Ethanol, >99.9%	Merck
Ethanol denatured, 99.8%	Roth
Ethidium bromide	Roth
Fetal Calf Serum (FCS)	GIBCO / Invitrogen
Formaldehyde, 37%	Roth
Glycerol	Roth
Glycine	Roth

GlycoBlue (AM9516)	Ambion
HEPES	Roth
Hydrochloric acid (HCl)	Roth
Immobilon western chemiluminescent HRP substrate	Millipore
Iodacetamide	AppliChem
L-glutamine	GIBCO / Invitrogen
Lipofectamine 2000	Invitrogen
Magnesium chloride ($MgCl_2$)	Roth
McCoy's Medium 5A	GIBCO / Invitrogen
Methanol	Roth
Milk powder, non fat	Roth
NEBuffer for M-MuLV reverse transcriptase (B0253)	NEB
N-ethylmaleimide	Sigma
N,N,N´,N´-Tetramethylendiamin (TEMED)	Roth
NP-40	USB
PBS tablets (18912-014)	GIBCO/ Invitrogen
Penicillin/Streptomycin	GIBCO / Invitrogen
pH-Solution 10,01	Roth
pH-Solution 4,01	Roth
pH-Solution 7,01	Roth
Ponceau S	Roth
Proteinase K (EO 0491)	Fermentas
Protein A sepharose CL-4B (17-0780-01)	GE Healthcare
Rotiphorese Gel 30 (30% acrylamide bisacrylamide solution; ratio 37.5:1)	Roth
Potassium chloride (KCl)	Roth
Potassium dihydrogen phosphate (KH_2PO_4)	Roth
RNase Inhibitor (M0307)	NEB
RPMI Medium 1640	GIBCO / Invitrogen
Sepharose CL-4B (17-0150-01)	Amersham
Sodium acetate	Roth
Sodium deoxycholate	AppliChem
Sodium dodecyl sulfate (SDS)	Roth
Sodium chloride (NaCl)	AppliChem
Sodium hydrogen carbonate ($NaHCO_3$)	Roth

Sodium hydroxide (NaOH)	Roth
Sonicated salmon sperm DNA (201190-81)	Stratagene
SuperSignal west femto maximum Sensitivity (34095)	Pierce
Tetracycline	Sigma
Trasylol (aprotinin 500.000 KIE)	Bayer
Trichostatin A	Sigma-Aldrich
Tris	Roth
Triton X-100	AppliChem
Trizol (15596-018)	Invitrogen
Trypanblue	Invitrogen
Trypsin-EDTA	GIBCO / Invitrogen

3.1.4. ENZYMES

NAME	COMPANY
M-MuLV Reverse Transcriptase (M0253)	NEB
Taq DNA polymerase	PrimeTec

3.1.5. CHEMOTHERAPEUTICS

NAME	COMPANY
5-Fluorouracil	SIGMA
Camptothecin	SIGMA
Daunorubicin	SIGMA
Doxorubicin	Santa Cruz
Epirubicin	SIGMA
Etoposide	SIGMA
Idarubicin	SIGMA

3.1.6. BUFFERS

BLOCKING SOLUTION	
PBS	
FCS	10%

CHIP BUFFER	
Tris-HCl pH 8.0	50 mM

NaCl	150 mM
EDTA pH 8.0	5 mM
NP40	0.5% (v/v)
Triton X-100	1% (v/v)

CHIP++ BUFFER

ChIP buffer	
Aprotenin/ Leupeptin	1 µg/ ml each
Pepstatin A	1 µg/ ml
Pefablock	1 mM

LAEMMLI BUFFER 6X

Tris-HCl pH 6.8	0.35 mM
Glycerol	30% (v/v)
SDS	10% (w/v)
DTT	9.3% (w/v)
Bromphenol blue	0.012% (w/v)

PBST

PBS	
Tween-20	0.1%(v/v)

RIPA-BUFFER

Tris-HCl pH 7.5	20 mM
NaCl	150 mM
Sodium deoxycholate	1% (w/v)
SDS	0.1% (w/v)
Triton-X 100	1% (v/v)
EDTA	10 mM
Trasylol	5 % (v/v) (equals 100,000 KIE)
pH was adjusted to 7.5 with 1M NaOH	

SDS RUNNING BUFFER (FOR SDS-PAGE)

Tris	25 mM
Glycine	192 mM
SDS	0.1% (w/v)

WESTERN SALTS

Tris	25 mM
Glycine	192 mM
SDS	0.02% (w/v)
Methanol	15%
pH was adjusted to 8.3 with HCl	

3.1.7. OLIGONUCLEOTIDES

siRNAs

NAME	ID	SEQUENCE (5' → 3')
N.C. 1		UNKNOWN
N.C. 2		UNKNOWN
BCLAF1	s18874	CAUUGAUCGCCGUAGAAAAtt UUUUCUACGGCGAUCAAUGtc
BCLAF1_2	s18875	CGCGAUUACAGAAAUAAUAtt UAUUAUUUCUGUAAUCGCGac
CBP	s3495	GAAUCUUUCCCAUAUCGAAtt UUCGAUAUGGGAAAGAUUCag
CBP_2	s3496	GGAUAUUGCUGUGGACGCAtt UGCGUCCACAGCAAUAUCCaa
HOXA5	s6765	GACUACCAGUUGCAUAAUUtt AAUUAUGCAACUGGUAGUCcg
HOXA5_2	s6766	CCAGUUGCAUAAUUAUGGAtt UCCAUAAUUAUGCAACUGGta
p300	s4696	CCACUACUGGAAUUCGGAAtt UUCCGAAUUCCAGUAGUGGat
p300_2	s4697	GCCUGGUUAUAUAACCGGAtt UCCGGUUAUAUAACCAGGCat
p53	s605	GUAAUCUACUGGGACGGAAtt UUCCGUCCCAGUAGAUUACca
PCAF	s16895	GGUACUACGUGUCUAAGAAtt UUCUUAGACACGUAGUACCta
PCAF_2	s16896	GGAGUUCGACAGAUUCCUAtt UAGGAAUCUGUCGAACUCCat
RPL26	s12203	GAAAUAUGUUAUCUACAUUtt AAUGUAGAUAACAUAUUUCtt
RPS6KA1	s12273	CACUGAUUCUGAAGGCGAAtt UUCGCCUUCAGAAUCAGUGtc
RPS6KA1_2	s12275	CCAUUGACUGGAAUAAGCUAtt UAGCUUAUUCCAGUCAAUGgt
RPS6KB1	s12283	GGUUUUUCAAGUACGAAAAtt UUUUCGUACUUGAAAAACCtt
RPS6KB1_2	s12284	GGACUAUGCAAAGAAUCUAtt UAGAUUCUUUGCAUAGUCCaa
RPS6KB2	s12286	CCCUUUUUCCGGCACAUGAtt UCAUGUGCCGGAAAAAGGGat

RPS6KB2_2	s12285	ACAUCAAACUGACCGACUUtt AAGUCGGUCAGUUUGAUGUgg
RPS6KL1	s38111	GGUACUUUGUGAGCGAGGAtt UCCUCGCUCACAAAGUACCtg
RPS6KL1_2	s38110	CGAUGUUAGUGAGGACUAUtt AUAGUCCUCACUAACAUCGcg
RREB1	s12354	CCAUCUCCUCUGAAACGUAtt UACGUUUCAGAGGAGAUGGag
RREB1_2	s12356	GGAGUUUGUUUGCAAGUAUtt AUACUUGCAAACAAACUCCtt
TIP60	s20630	GCAAGCUGCUGAUCGAGUUtt AACUCGAUCAGCAGCUUGCcg
TIP60_2	s20631	GGACGGAAGCGAAAAUCGAtt UCGAUUUUCGCUUCCGUCCtg
TP73	s14319	GCAAUAAUCUCUCGCAGUAtt UACUGCGAGAGAUUAUUGCct
TP73_2	s14320	CCACCAUCCUGUACAACUUtt AAGUUGUACAGGAUGGUGGtg
WRAP53_1	s30251	CCUCUGCUUUCAUCCCGAUtt AUCGGGAUGAAAGCAGAGGtg
WRAP53_2	s30252	GAAGCAAACGGGAGCCUUUtt AAAGGCUCCCGUUUGCUUCtt

PCR

NAME	**SEQUENCE (5' → 3')**
36B4_for	GATTGGCTACCCAACTGTTG
36B4_rev	CAGGGGCAGCAGCCACAAA
p53_for	ATGGAGAGAGCCGCAGTCAGATC
p53_rev	GGGAGCAGCCTCTGGCATTCTG
p53-Int1_for	GCCGAGACGGGCCATTCGTG
p53-Int1_rev	TCTCACCGCTCACCTGCCCA
WRAP53-Exon1a_for	CGGAGCCCAGCAGCTACC
WRAP53-Exon1a_rev	TTGTGCCAGGAGCCTCGCA
WRAP53-Exon2_rev	GTCCTGGTCTGAAGGACAGC
WRAP53-Exon7_for	GACTGCGAGGTCCGAGCCACATTTG
WRAP53-Exon8_rev	GAGCCATCATCCCAGGCATACAGAC
E2F1_for	CGGTGTCGTCGACCTGAACT
E2F1_rev	AGGACGTTGGTGATGTCATAGATG
TAp73_Exon1_for	GGGCTGCGACGGCTGCAG
TAp73_Exon3_rev	GATGTAGTCATGCCCTCCAGG

NOXA_for	GGACTGTTCGTGTTCAGCTCGC
NOXA_rev	GCCGGAAGTTCAGTTTGTCTCC

ChIP

NAME	SEQUENCE (5' → 3')
ChIP_p53-E2F1 BS-I_for	TGCACCCTCCTCCCCAACTCC
ChIP_ p53-E2F1 BS-I_rev	GCTCCCTGGACGGTGGCTCT
ChIP_p53-E2F1 BS-II_for	CCCGGGAGGAGAGGCGAACA
ChIP_ p53-E2F1 BS-II_rev	TGGGTCGCCCGCGAAATCTG
ChIP_p53+19.5kb_for	GCCACGGCTGGCACAAGGTT
ChIP_p53+19.5kb_rev	GCTGCCCCCACTTTCCTGGG
ChIP_p107-E2F1 BS_for	AGGCAGACGGTGGATGACAACAC
ChIP_p107-E2F1 BS_rev	TCAGCGTGGGGCTTGTCCTCGAA
ChIP_TP73-E2F1 BS_for	GAGCGCCGGGAGGAGACCTT
ChIP_TP73-E2F1 BS_rev	GCGGGCGTTAGCGCCTTTTT

3.1.8. ANTIBODIES

PRIMARY ANTIBODIES

NAME	DILUTION	APPLICATION	SOURCE	COMPANY
E2F1 KH95 and KH129	1:500 each	WB	mouse monoclonal	Santa Cruz Biotechnology
E2F1 KH20 and KH95	1µg	ChIP	mouse monoclonal	Upstate
p53 D0-1 HPR-conjugated	1:8000	WB	mouse monoclonal	Santa Cruz Biotechnology
p53 FL-393	1:500	IF	rabbit polyclonal	Santa Cruz Biotechnology
p53 (pSer15)	1:1000	WB	mouse monoclonal	Cell Signaling
p53 (acLys382)	1:1000	WB	rabbit polyclonal	Cell Signaling
RNA pol II	1µg	ChIP	rabbit polyclonal	Santa Cruz
β-actin	1:10000	WB	mouse monoclonal	Abcam

SECONDARY ANTIBODIES

NAME	DILUTION	APPLICATION	COMPANY
Donkey α-mouse IgG	1:10000	WB	Jackson ImmunoResearch

(H+L) HPR-conjugated			
Donkey α-rabbit IgG (H+L) HPR-conjugated	1:10000	WB	Jackson ImmunoResearch
Alexa Fluor 488 anti-rabbit	1:500	IF	Molecular Probes, Invitrogen
Alexa Fluor 594 anti-rabbit	1:500	IF	Molecular Probes, Invitrogen

3.1.9. EUKARYOTIC CELL LINES

NAME	SOURCE	P53 STATUS
5637	human bladder carcinoma	R280T
A431	human squamous cell carcinoma	R273H
HCT116	human colon carcinoma; p21 wt or $p21^{-/-}$	wt
U251	human glioma cells	R273H
U2OS	human osteosarcoma	wt

3.1.10. CELL CULTURE WORKING SOLUTIONS

DULBECCO'S MODIFIED EAGLE'S MEDIUM (DMEM -)

Dulbecco's Modified Eagle's Medium	10g
NaHCO3	3.7 g/L
HEPES	5.96 g/L
H_2O	ad 1L

The medium was filtered and stored at +4°C

DULBECCO'S MODIFIED EAGLE'S MEDIUM WITH SUPPLEMENTS (DMEM + FCS)

DMEM -	450 ml
FCS	10%
Penicillin/Streptomycin	50 U/mL
Tetracycline	2 µg/mL
L-glutamine	200 µM
Ciprobay 200	10 µg/mL

DMEM+FCS was and warmed up to +37°C directly before use

RPMI MEDIUM 1640 WITH SUPPLEMENTS (RPMI + FCS)

RPMI -	450 ml
FCS	10%
Penicillin/Streptomycin	50 U/mL
Tetracycline	2 µg/mL

L-glutamine	200 µM
Ciprobay 200	10 µg/mL

DMEM+FCS was and warmed up to +37°C directly before use

MCCOY'S MEDIUM 5A WITH SUPPLEMENTS (MCCOY'S + FCS)

McCoy's 5A -	450 ml
FCS	10%
Penicillin/Streptomycin	50 U/mL
Tetracycline	2 µg/mL
L-glutamine	200 µM

DMEM+FCS was and warmed up to +37°C directly before use

PBS BUFFER

PBS tablets	
H2O	500 ml

PBS for cell culture was autoclaved and stored at +4°C

CELL FREEZING SOLUTION

DMSO	10 %
FCS	90 %

3.2. METHODS

3.2.1. CELL BIOLOGY

MAINTENANCE OF CELL CULTURES

All cell lines were cultivated at 37°C in a humidified incubator with 5% CO_2 either in RPMI medium 1640 (A431, U251, 5637) or in Dulbecco's Modified Eagle's medium (DMEM) (U2OS), or McCoy's medium (HCT116 wt, HCT116 $p21^{-/-}$), all supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 µg/ml streptomycin, 200 µM Glutamine, 2 µg/ml tetracycline, and 10 µg/ml Ciprobay 200 (not for the HCT116 cells). Sub cultivation was performed every 3-4 days, as soon as the cells reached 70-80% of confluence. For passaging, the medium was removed; the cells were rinsed once with PBS and incubated at 37°C for a few minutes with 0.05% Trypsin-EDTA solution, to induce detachment from the culture dish. Upon neutralization of the Trypsin with fresh medium the cells were carefully resuspended and diluted 1:8 - 1:10 in fresh

medium. For experiments, the cell number was determined using trypanblue staining of living cells that was subsequently quantified using the Countess system. The required amount of cells was seeded into the corresponding culture dishes/ well plates. For long term storage, the cells were frozen in liquid nitrogen.

CELL FREEZING PROCEDURE

The cells from a 10 cm culture dish at 70-80% confluency were frozen in 1 cryovial. After trypsinization and dilution with fresh medium+FCS as described above, the cell suspension was centrifuged 10 min at 800 rpm. The supernatant was aspirated; the cell pellet was resuspended in 1ml cold cell freezing solution (10% DMSO in FCS) and transferred into pre-cooled cryovials. The vials were stored in -80°C for 2 days and then kept in a liquid nitrogen tank for long term storage.

To take frozen cells in culture, the vials were quickly thawed by hand and immediately transferred into a 15cm culture dish with prewarmed medium. After one day of incubation at 37°C, the medium was changed to remove the residual DMSO.

REVERSE SIRNA TRANSFECTION

Pre-designed or validated siRNAs from Applied Biosystems were used for all siRNA transfection experiments. Both the siRNAs and the transfection reagent Lipofectamine 2000 were diluted in cell culture medium without supplements and incubated for 5 minutes. The solutions were combined in an empty well-plate and incubated for additional 20 minutes to allow the siRNA-lipid-micelles to form. The cells were counted and the appropriate number of cells was added to the transfection mix and diluted with medium+FCS to the final volume of the corresponding well plate.

Different amounts of oligonucleotides, transfection reagent, cells and medium were used depending on the well sizes:

	siRNA + MEDIUM	LIPOF. 2000 + MEDIUM	CELL NUMBER	TOTAL VOLUME
6-well	30 pmol	2.7 µl	$2.5 - 3 \cdot 10^5$	2 ml
12-well	15 pmol	1.35 µl	$1.5 - 1.8 \cdot 10^5$	1 ml
96-well	1 pmol	0.25 µl	8000	100 µl

After 48 hours the cells were fixed for immunofluorescence staining or harvested for immunoblot analysis or RNA isolation.

LONG-TERM SIRNA TRANSFECTION:

The cells were reverse transfected as described above. 48 hours later the samples were trypsinized in the well plate and 25 - 33% of the cells were used for a second reverse transfection following the same protocol as above. After an additional incubation for 72 hours the cells were harvested for RNA isolation or immunoblot analysis.

CHEMOTHERAPEUTIC TREATMENT

Most treatments were performed for 24 hours; therefore the cells were either seeded about 12 hours before treatment or siRNA transfected 24 hours before treatment. The medium was removed from the cells and fresh medium containing the chemotherapeutic drug at the desired final concentration was added to the cells. The mock sample was treated with the same volume of dissolvent only.

	STOCK CONC.	FINAL CONC.	DISSOLVENT
5-Fluorouracil	0.3 M	500 µM	DMSO
Camptothecin	2.87 mM	2.87 µM	DMSO
Daunorubicin	4 mM	500 nM	H_2O
Doxorubicin	4 mM	500 nM	H_2O
Epirubicin	4 mM	500 nM	H_2O
Etoposide	20 mM	100 µM	DMSO
Idarubicin	4 mM	500 nM	H_2O

3.2.2. MOLECULAR BIOLOGY

TOTAL RNA ISOLATION

For the preparation of total RNA 0.5 - $1 \cdot 10^6$ cells per sample are needed (corresponds to one 6-well). The medium was aspirated and 800 µl Trizol (Invitrogen) was added to the cell layer for lysis. After 5 minutes at room temperature the lysates were transferred into microtubes and supplemented with 180 µl chloroform. The mixture was vigorously shaken and further incubated for 3 min at room temperature, followed by a centrifugation step (4°C, 16000 g, 20 min). The upper aqueous phase, containing RNA, was carefully transferred into a new microtube and supplemented with the same amount of isopropyl alcohol. The samples were mixed vigorously by hand and incubated for 3 min at room temperature and for 2 - 24 hours at -20°C. RNA was precipitated by centrifugation (4°C, 16000 g, 20 min), the pellet was washed with 70% ethanol, centrifuged (4°C, 9000 g, 10 min), air dried for 10 minutes at 37°C and resuspended in 30 µl nuclease free water.

QUANTIFICATION OF RNA

The RNA concentration was measured, using a NanoDrop spectrophotometer (PeqLab). The absorbance at 260 nm was used to determine the concentration, whereas the ratios 260:230 and 260:280 were used as indicators for the purity of the isolated RNA. The ratios 260:230 around 1.9-2.0 and 260:280 in the range of 2.0-2.1 were considered as ‘pure’ RNA. In case these values were appreciably lower, RNA was additionally purified, as described in the following section.

PURIFICATION OF RNA

The RNA sample (30µl) was mixed with 20µl H_2O, 1µl 125 mM EDTA, 1µl 3M sodium acetate and 70µl 100% ethanol. The samples were vortexed and incubated for 5 min at room temperature. After shock-freezing in liquid N2 the samples were centrifuged at 4°C for 20 min at 16000 g. The pellet was washed with 70 µl 70% ethanol and centrifuged (4°C, 10 min, 9000 g). The Supernatant

was aspirated and the pellet was air dried for 10 minutes at 37°C. The precipitated RNA was resuspended in 30µl nuclease free water and the concentration and purity of the RNA was determined, again using the NanoDrop spectrophotometer as above.

REVERSE TRANSCRIPTION

Reverse transcription was performed using moloney murine leukemia virus (M-MuLV)-derived reverse transcriptase (NEB). The following stock solutions were prepared and aliquots were kept at -20°C:

- Combined primer stock: 15µM random nonamers (N9) and 50µM oligo dT23VN
- Deoxyribonucleoside triphosphates (dNTPs): 2.5mM of each (dCTP, dATP, dTTP, dGTP) (Promega)

1µg of total RNA was used for the reverse transcription and diluted with nuclease free water to a final volume of 10µl. 2µl of the combined primer stock and 4µl dNTP mix were added to the diluted RNA. The samples were incubated for 5 minutes at 70°C. Meanwhile a master mix of 2µl NEBuffer for M-MuLV reverse transcriptase (NEB), 0.25µl RNase inhibitor (10U, NEB), 0.125µl M-MuLV reverse transcriptase (25U, NEB), and 1.625µl nuclease free water per sample was prepared. The transcriptase master mix was added to the RNA samples and incubated for 1 hour at 42°C. The enzyme was inactivated at 95°C for 5 min and the cDNA was diluted with 30µl nuclease free water. It was either directly used for real-time PCR or stored at -20°C. To control for genomic DNA contamination each reaction was also performed as noRT control, omitting the reverse transcriptase in the master mix.

REAL-TIME PCR

Real-time PCR (qPCR) was used to obtain semi-quantitative measurements of gene expression or to quantify the recovered sheared DNA from chromatin

immunoprecipitation experiments. The following master mix was prepared on ice and aliquoted in microtubes:

	FINAL CONC.	FILTERED
Tris-HCl pH 8.8	75 mM	✓
$(NH_4)_2SO_4$	20mM	✓
Tween-20	0.01 %	✓
$MgCl_2$	3 mM	✓
Triton X-100	0,25%	✓
Trehalose	300 mM	✓
Sybr Green	1:80000	
dNTPs	0.2 mM	
Taq-Polymerase	20U/ ml	

The tubes were shock-frozen in liquid N2 and kept at -20°C for up to 3 month.

For the PCR reaction 14µl master mix were mixed with 0.075µl of each primer (stock concentration: 100µM) and 9.85µl H_2O. This was prepared as a master mix and aliquoted into a 96-well PCR plate. 1µl cDNA/ sheared DNA was added into the corresponding wells. The plate was sealed, centrifuged (30sec, 600rpm) and the following PCR program was used for the specific gene amplifications:

STEP	TEMPERATURE	TIME	FLUORESCENT READ STEP	
1 - DNA melting	95°C	3 min		
2 - DNA melting	95°C	15 sec		39x
3 - primer annealing and elongation	60°C	1 min	X	
4 - melting curve	60°C - 95°C		every 0.5°C	

For the semi-quantitative analysis of target mRNA expression the C_t-values of the genes of interest as well as the reference gene 36B4 were obtained. The ΔΔCt-method was used to determine the relative expression of the analyzed target genes:

$$\Delta\Delta C_t = \frac{C_t \text{ (36B4, untreated)} - C_t \text{ (target gene, untreated)}}{C_t \text{ (36B4, treated)} - C_t \text{ (target gene, treated)}}$$

(Livak and Schmittgen, 2001)

For ChIP analysis a serial dilution of the input DNA was used to determine the relative amounts of target DNA in the input samples, as well as the IP samples. The recovered DNA is diagramed relative to the input DNA.

$$\% \text{ of input DNA} = \frac{\text{rel. amount of target DNA (IP sample)}}{\text{rel. amount of target DNA (input sample)}}$$

3.2.3. BIOCHEMISTRY AND IMMUNOLOGICAL METHODS

IMMUNOBLOT ANALYSIS

CELL HARVESTING AND LYSIS

Adherent cells were grown, treated, or transfected in a 12-well plate for immunoblot analysis. For harvesting they were scraped in the growth medium, transferred to a microtube and centrifuged (5 min, 1000 rpm). The cell pellet was resuspended and the cells were lysed in 60µl of RIPA/ 6x Laemmli buffer (1:1 mixture). The samples were incubated for 5 min at 95°C for protein denaturation. The samples were centrifuged (1 min, 13000 rpm) and stored at -20°C or directly used for SDS-PAGE.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was developed 1967 by (Shapiro *et al.*, 1967) to determine the molecular weight of proteins. The detergent SDS coats the denatured proteins and translates their molecular weight into a negative charge, therefore a sample buffer developed by (Laemmli, 1970) is widely used. An electric field is applied to the gel and the negatively charged proteins migrate towards the anode. Within the stacking gel the pores are large and therefore the proteins form a concentrated stack between the leading chloride ions and the trailing ion Glycine. As soon as the sample migrates into the resolving gel, which obtains a pH that is 2 units higher than that of the stacking gel and pores that are

restrictively small for the proteins, the sample starts to resolve according to the molecular weight of the proteins. Depending on the molecular weight of the protein of interest different percentages of acrylamide/ bisacrylamide (AA/ BAA) are used within the resolving gel. All SDS-PAGE experiments within this study were performed using 10% AA/ BAA.

CHEMICAL	STACKING GEL (5%)	RESOLVING GEL (10%)
Acrylamide/ bisacrylamide	850 µl	4.15 ml
Tris-HCl pH 6.8	625 µl	-
1.5 M Tris-HCL pH 8.8	-	3.15 ml
H_2O	3.4 ml	5 ml
10% SDS	50 µl	125 µl
10% APS	50 µl	75 µl
TEMED	10 µl	7.5 µl

The resolving gel was casted between two glass plates, separated by spacers (1mm thick) and covered by a layer of 2-propanol to prevent air contact for polymerization. The solidified gel was rinsed with water to remove any residual 2-propanol and the stacking gel was casted on top of the resolving gel. A comb, either with 10 or 15 teeth was inserted into the stacking gel before polymerization in order to form separated slots for sample loading.

After gel polymerization, 10 to 20 µl of cell lysate were loaded into the pockets of the stacking gel. Electrophoresis was performed at 15mA per gel until the samples migrated into the resolving gel, then it was increased to 20 mA per gel.

WESTERN BLOT

For immunodetection of the proteins they were transferred onto a nitrocellulose membrane (pore size: 0.2µM) after the separation through SDS-PAGE using the tankblot technique (Bittner *et al.*, 1980). A stack of sponges, filter papers, the gel, the membrane, filter papers, and sponges was prepared, all soaked in transfer buffer. This was then placed within the vertical blotting chamber, filled up with transfer buffer and again an electric field was applied. After blotting for 1 hour at 100V all proteins were bound to the nitrocellulose membrane. The

quality of the transfer was controlled through the reversible protein staining with Ponceau S solution.

IMMUNOSTAINING

For specific protein visualization after western blotting, membranes were subjected to immunostaining. First, membranes were blocked with a 5% non-fat milk solution in PBST (milk) for 1 hour followed by the incubation with primary antibody, diluted in milk for 2 hours at room temperature or overnight at 4°C. Subsequently, membranes were washed twice according to the following protocol: 3 times in PBST followed by 15 min in milk. To visualize the specifically bound primary antibodies the membranes were incubated with HPR-conjugated secondary antibodies for one hour. Washing was repeated the same way as described above. All washing and incubation steps were fulfilled with gentle shaking at room temperature, if not specified otherwise. For protein detection enhanced chemiluminescence solutions (ECL) were used and the signal was measured using the ChemoCam Imager (Intas). For quantification the LabImage 1D software (Intas) was used.

CHROMATIN IMMUNOPRECIPITATION

$1 \cdot 10^6$ U251 cells were seeded per 10 cm culture dish and treated 18 hours later with 500nM doxorubicin. 24 hours after treatment protein-DNA crosslinking was performed using 1.42% (v/v) formaldehyde in PBS for 15 min and stopped by the addition of Glycine to a final concentration of 138 mM for 5 min. After washing with PBS twice, the cells were scraped in 1ml ChIP++ buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 0,5% NP-40, 1% Triton X-100, Leupeptin (1µg/ml), Aprotenin (1µg/ml), Pepstatin A (1µg/ml), Pefabloc (1mM)), transferred into a microtube and centrifuged at 1000 rpm for 5 min. The Pellets were washed once with 1 ml ChIP++ buffer and resuspended in 300 µl of the same buffer. The lysates were sonicated in an icewater bath sonicator (Bioruptor) to shear the chromatin to a length of 500 – 1000 base pairs (3 times 10 minutes using 10 sec on/ off cycles at maximum power). The lysates were

diluted in ChIP++ buffer before pre-clearing for 1 hour at 4°C with 100µl sepharose, washed 3 times in ChIP buffer and finally resupended in ChIP++ buffer to achieve a 50% slurry. The samples were centrifuged at 12000 rpm, 10 min, at 4°C, and the supernatants were transferred to new microtubes. The pre-cleared chromatin was diluted with ChIP++ buffer according to the number of immunoprecipitations that were performed. 1 µg of antibody per 50 µl of lysate was used for the immunoprecipitation (IP), additionally 50µl of the precleared DNA were used as input control. The IP samples were further diluted with ChIP++ buffer up to 500 µl and incubated overnight at 4°C with rotation. The input samples (50 µl) were mixed with 1µl glycogen (Glycoblue) and 100µl 100% ethanol and placed at - 20°C overnight for DNA precipitation. Protein A sepharose (GE Healthcare) beads were incubated over night in a 15ml tube filled up with ChIP buffer to allow the beads to swell. At the same time 0.5g BSA and 100µl sheared salmon sperm DNA were added to block the beads and avoid unspecific precipitation. Blocked protein A sepharose was washed three times with ChIP buffer (centrifuged at 2000 rpm, 2 min, 4°C) and finally resuspended in ChIP++ buffer to get a 50% sepharose slurry. 30µl of this slurry were added to each immunoprecipitation reaction and samples were incubated for 2 hours at 4°C with rotation. Meanwhile the input samples were centrifuged (13000 rpm, 20 min, 4°C), and the DNA pellets were washed once with 500 µl of 70% ethanol before they were air dried for 10 min at 37°C. The immune-sepharose complexes were washed 8 times with 1 ml cold ChIP buffer, centrifuged for 2 min at 2000 rpm and 4°C. 100 µl 10% (w/v) Chelex 100 slurry was added to the washed beads and to the input DNA pellet. After brief vortexing the samples were heated to 95°C for 10 min. 30µg Proteinase K was added to each sample and incubated at 55°C for 30 min with shaking at 1000 rpm. For the inactivation of Proteinase K the samples were heated to 95°C for 10 min. All beads were precipitated by centrifugation (12000 rpm, 1min, 4°C) and the supernatants were carefully transferred into new tubes. For

quantification of the precipitated/ recovered DNA 1µl of the supernatant was used for qPCR analysis.

IMMUNOFLUORESCENCE

Cells were grown in 96-well imaging plates (BD Falcon). Prior to immunofluorescence staining the cells were fixed using 3.7% formaldehyde in PBS for 20 minutes. After fixation the cells were washed with PBS containing 50mM Glycine in order to inactivate residual free formaldehyde that could otherwise unspecifically cross-link the primary antibodies to proteins of the cells. Permeabilization was achieved through 10 minutes incubation with PBS containing 0.5% Triton X-100. All buffers that were used from this step on contained 0.2% Triton X-100 in order to keep the cells in a permeabilized state. Incubation for 10 minutes in blocking solution (10% FCS in PBS + 0.2% TX100) was performed to block all unspecific binding sites in the cells before they were incubated for 1 hour with the primary antibodies (for dilutions see 3.1.8). The remaining primary antibodies were washed away with blocking solution 3 times for 5 minutes. The secondary antibodies coupled to the fluorophores Alexa488 or Alexa546 were incubated in a 1:500 dilution in blocking solution for 45 minutes in the dark. A nuclear stain (Hoechst 33342 or Doxorubicin) was additionally used during this incubation. We observed that the previous treatment of the cells with red fluorescent chemotherapeutic drugs (Daunorubicin, Doxorubicin, Epirubicin, or Idarubicin) has an impact on the fluorescent signal of Hoechst 33342; therefore we used in these cases a high dose of doxorubicin (10µM) for nuclear stain. The free secondary antibodies were washed away with blocking solution for 5 minutes, PBS +0.2% TX100 for 5 minutes and with PBS for additional 5 minutes; all incubations were performed in the dark. Finally the cells were kept in 100µl PBS and the plate was sealed with aluminum foil.

The fluorescent pictures were taken, using the BD Pathways system. In each well at least 9 pictures were taken using a 10x or 20x magnification. On the

basis of the nuclear stain the single nuclei within each well were defined and the average fluorescent intensity of the immunodetected proteins in each of these nuclei was measured. The results are either presented as average intensity per well, or the single nuclei intensities in each well are plotted in histograms.

3.2.4. THE SCREEN

THE LIBRARY

For the kinase screen a siRNA library (Silencer Human Kinase siRNA Library V3) was obtained from Applied Biosystems containing 3 different siRNAs against each of the 719 kinases included in the library. The siRNAs were obtained lyophilized in 96 well plates, containing 8 empty wells that were used for internal controls. The 3 siRNAs targeting the same gene were always localized on different plates. Before transfection all siRNAs were dissolved in nuclease free water at a final concentration of 50µM and dilution plates containing 5µM of the siRNAs were prepared. All pipetting steps were performed by the Biomek 2000 (Beckmann Coulter).

TRANSFECTION

For the siRNA transfections in a 96 well format the Biomek 2000 was used. The robot was programmed according to the siRNA transfection protocol as it was described in section 3.2.1 (REVERSE SIRNA TRANSFECTION), but using 4.5 times more siRNA. The reason for this is that the library consists of Silencer siRNAs that are less efficient compared to the Silencer Select siRNAs that were used throughout the other experiments.

IMMUNOFLUORESCENCE STAINING AND ANALYSIS

48 hours after reverse siRNA transfection of the cells they were fixed and stained for immunofluorescence analysis as described in 3.2.3 (IMMUNOFLUORESCENCE). The polyclonal p53 antibody (FL-393) was used for the immunostaining at a dilution of 1:500 in combination with an anti-rabbit

secondary antibody coupled to the fluorophore Alexa488 at a dilution of 1:500. Hoechst 33342 was used as nuclear stain to identify the regions of interest during the analysis. In each well 12 pictures were taken at a 10x magnification covering different positions. For the analysis around 10000 nuclei per well were used.

DATA MINING

To identify the HITs statistical methods were applied to the average expression per well data. Therefore the fluorescent signals were normalized to the average signal of the plate, this compensates for differences within the staining procedure or the microscopy. To finally evaluate the impact of the single knock-downs on the expression of p53 Z-scores were determined:

$$z\text{-}score = \frac{x - \mu}{\sigma}$$

x = average intensity in the well; μ = average intensity of all wells;
σ = standard deviation of the intensities of all wells

The relative p53 expression intensities of the individual siRNAs as well as the sum of the three siRNAs targeting the same kinase were used for the z-score analysis. On the basis of these results the kinases that revealed the strongest down-regulation of mutant p53 expression were further analyzed on the basis of the following three criteria:

1) How many of the siRNAs revealed this down-regulation?
2) Do we see a peak-shift in the histograms of the p53 staining intensities, when comparing the three siRNAs to the negative control siRNAs?
3) Visual inspection of the microscopic raw data. Do we observe morphological changes or increased cytoplasmic staining upon knock-down?

RESULTS

Most chemotherapeutic agents induce a DNA damage response in the cells subsequently leading to apoptosis. This comprises the activation and stabilization of the tumor suppressor p53, mainly through posttranslational modifications (Murray-Zmijewski *et al.*, 2008). It is estimated that 50% of all human tumors carry a p53 mutation, accompanied by a strong accumulation of the mutant p53 protein. Since most of these mutations are substitutions of single amino acids, we expect that at least some of the enzymes that were found to be responsible for the modification of wild type p53 also affect the mutant variants of the protein in response to DNA damage. Therefore, we first investigated whether the evoked DNA damage response upon chemotherapeutic treatment influences the modification of mutant p53 and whether this goes along with a further stabilization of the protein.

4.1. THE ACCUMULATION OF MUTANT P53 UPON DOXORUBICIN TREATMENT

4.1.1. THE EXPRESSION LEVELS OF MUTANT P53 PROTEIN ARE ELEVATED IN RESPONSE TO DOXORUBICIN

U251 cells are derived from a glioma and harbor the hotspot p53 point mutation R273H. As most tumor cell lines, that express a mutated form of p53, these cells accumulate high levels of the protein. Nevertheless, we observed by immunoblotting that the expression levels of p53 in these cells get elevated even further, when treated with the chemotherapeutic drug doxorubicin (Figure 6A).

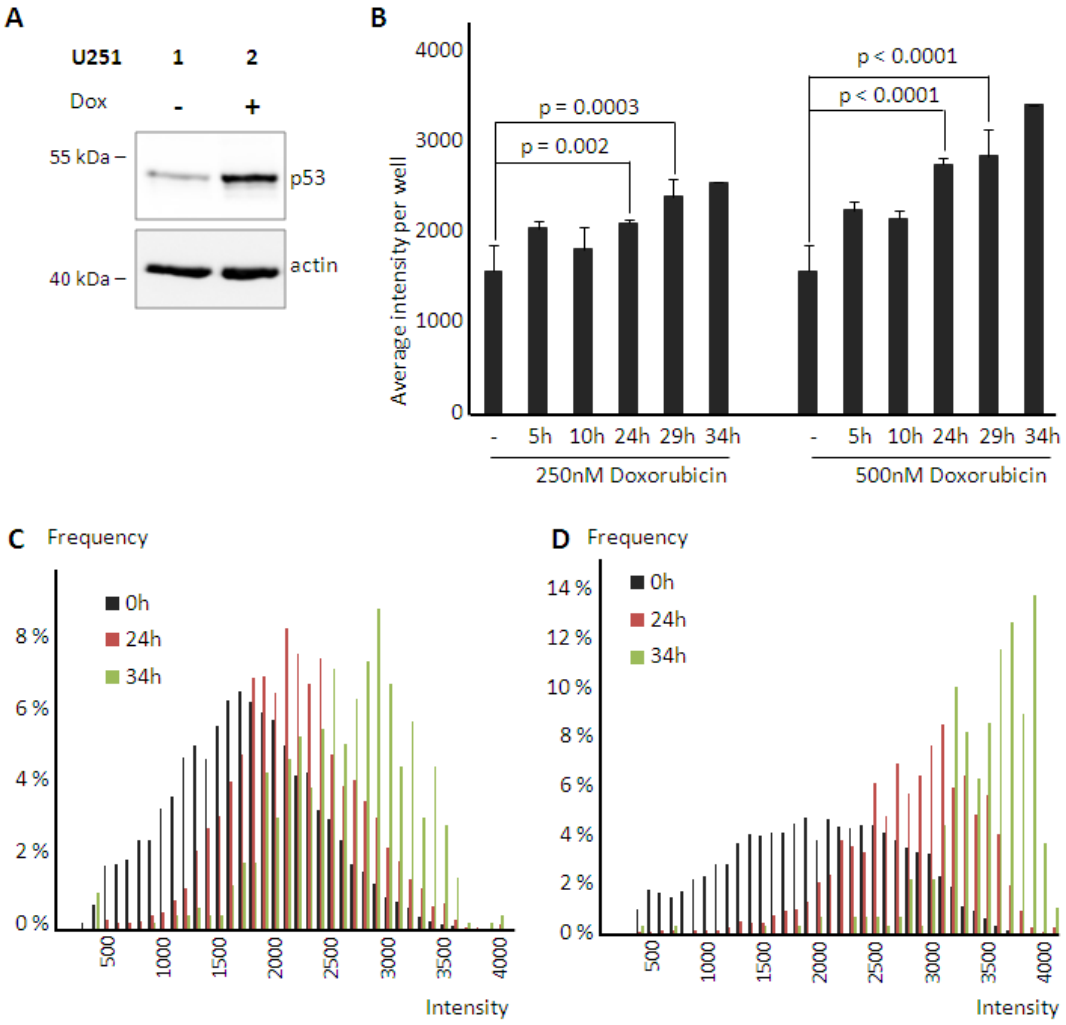


Figure 6: The accumulation of mutant p53 in response to doxorubicin treatment.
U251 cells (p53 R273H) were treated with 500nM doxorubicin for 24h. **A:** Total cell lysates were subjected to immunoblot analysis using antibodies against p53. Actin staining was used as loading control. **B-D:** Cells were fixed and stained for immunofluorescence analysis using antibodies against p53. Single nuclei were identified using Hoechst 33342 staining. The average p53-staining intensity was determined per nucleus. The experiment was performed in triplicate **B:** Means and standard deviations of the average p53-intensities per well are depicted. A Student's t-test (α=0.05) was performed for statistical analysis. **C, D:** Histograms of the p53-intensities per nucleus were generated for three different time-points at 250nM (**C**) and 500nM (**D**) final concentration of the drug.

This result was confirmed by quantitative immunofluorescence microscopy analyzing the p53 expression levels upon doxorubicin treatment in a time dependent manner at two different concentrations. The average expression of p53 was measured for each nucleus. In Figure 6B the mean intensities per well are diagramed, as they were determined in triplicate. The levels increased significantly after 29 hours of treatment using 250nM doxorubicin, as well as

after 24 hours of treatment with a final drug concentration of 500nM. The representation of the data in histograms (Figure 6C and D) shows that with both concentrations the majority of the cells accumulate their mutant p53 protein over time.

The mechanisms by which mutant p53 is generally stabilized in tumor cells are poorly understood. About the further accumulation of the protein upon chemotherapeutic treatment even less is known.

4.1.2. POSTTRANSLATIONAL MODIFICATIONS OF MUTANT P53 ARE INDUCED IN RESPONSE TO DOXORUBICIN TREATMENT, EVEN THOUGH THEY DO NOT DIRECTLY INFLUENCE ITS STABILITY

Kurz *et al.* (2004) showed that doxorubicin acts through the activation of the transducer kinase ATM. Additionally, it is known that ATM phosphorylates p53 at Serine 15, which in turn leads to the acetylation of Lysine 382 of p53 (Dumaz and Meek, 1999; Sakaguchi *et al.*, 1998). This suggests itself that also mutant p53 might get posttranslationally modified at these sites in response to doxorubicin treatment. We therefore analyzed the response of U251 cells to chemotherapeutic drug exposure by immunoblotting using antibodies against Serine 15 phosphorylated and Lysine 382 acetylated p53. We did not detect any modified p53 in untreated cells, but after incubation with doxorubicin for 24h the levels were dramatically increased (Figure 7A).

This result was confirmed using immunofluorescence analysis, quantifying the expression of Serine 15 phosphorylated p53 in individual cells treated with 500nM doxorubicin for 24 hours. The histogram of the obtained data clearly shows a peak shift towards higher intensities upon treatment (Figure 7B).

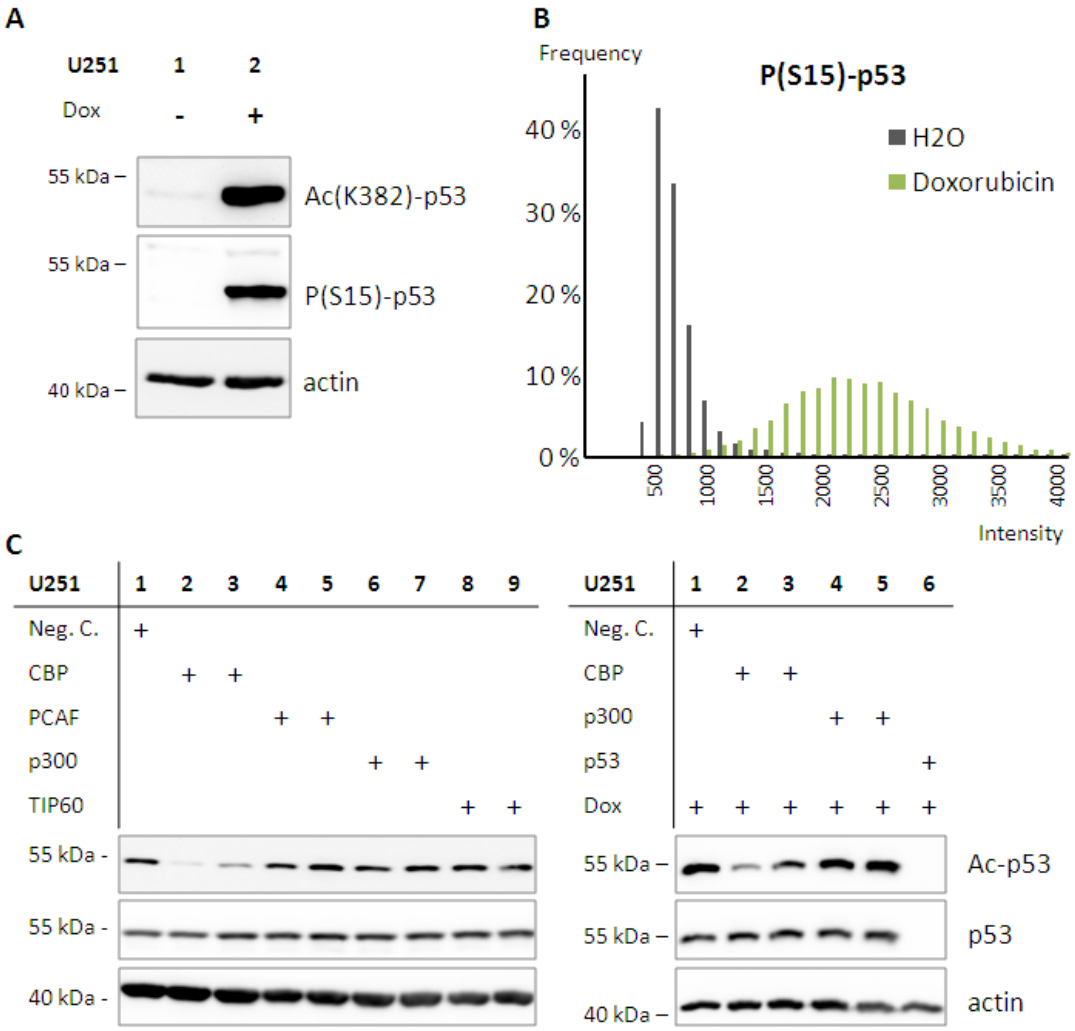


Figure 7: Mutant p53 gets posttranslationally modified upon doxorubicin treatment.
A: U251 cells were treated with 500nM doxorubicin for 24h. Total cell lysates were subjected to immunoblot analysis using antibodies against acetyl(Lys382)-p53 or phospho(Ser15)-p53. Actin staining was used as loading control. **B:** U251 cells were treated for 24 hours with 500nM doxorubicin. The cells were fixed and stained for immunofluorescence analysis using antibodies against phospho(Ser15)-p53. Single nuclei were identified using 10µM doxorubicin staining. The average p53-staining intensity per nucleus is diagramed in a histogram. **C:** U251 cells were reverse transfected with two different siRNAs per gene for 48 hours. As indicated, the samples in the right panel were additionally treated with 500nM doxorubicin for the last 24 hours. Whole cell lysates were subjected to immunoblot analysis using antibodies against acetyl(Lys382)-p53 and total p53. Actin staining served as loading control.

The impact of these modifications is unknown, but we cannot exclude that, as for wt p53, the protein gets stabilized through these modifications. To explore their functional significance with respect to the stabilization of the protein, we used siRNA mediated knock-down of known p53 acetyltransferases, as Ito *et al.* (2002) described their role in the regulation of wild-type p53 stability. 48

hours post transfection of U251 cells with siRNAs, targeting the 4 histone acetyl transferases CREB binding protein (CBP), p300/CBP-associated factor (PCAF), p300, and Lysine acetyl transferase 5 (KAT5 alias TIP60) total cell lysates were subjected to immunoblot analysis. Surprisingly, only the knock-down of CBP led to a clear reduction of Lys382-acetylated p53 in these cells. Nevertheless, this did not have any impact on the expression levels of total p53 protein (Figure 7C, left panel).

Since we have shown that the levels of Lys382-acetylated p53 are increased dramatically in response to doxorubicin (Figure 7A), we additionally investigated whether the knockdown of CBP and p300 impairs the further accumulation of mutant p53 protein levels upon doxorubicin treatment. But, we again did not detect any changes in the expression levels of total p53 protein (Figure 7, right panel). Therefore we conclude that the posttranslational modification of Lysine 382 of mutant p53 is not the primary regulator of its stability, neither in the default state of the cells, nor in response to chemotherapeutic treatment.

4.1.3. U251 CELLS DISPLAY AUGMENTED MRNA LEVELS OF P53 IN RESPONSE TO DOXORUBICIN TREATMENT

Apart from protein stability, many proteins are regulated in their expression on the transcriptional level. Even though there are only a few reports claiming that p53 gets differentially expressed due to transcriptional activation, it appears to be logic that if the general accumulation of mutant p53 is associated with increased half life of the protein the response to chemotherapeutic treatment happens to be regulated by other means. Therefore, we next aimed to test whether doxorubicin mediated accumulation of mutant p53 is caused by transcriptional activation of the gene. To investigate this, we isolated total RNA from U251 cells treated for 24 hours with 500nM doxorubicin. Strikingly,

quantitative real-time PCR analysis revealed that there was about 5 times more p53 mRNA upon doxorubicin treatment (Figure 8).

The activation of E2F1 through ATM, ATR and the checkpoint kinases Chk1 and -2 upon DNA damage leads to its stabilization and preferential transactivation of apoptotic target genes like TAp73 and NOXA (Hershko and Ginsberg, 2004). In our experiments the up-regulation of TAp73 mRNA is always used as a positive control for proper induction of the DNA damage response. It should be noted that the transcription of p53 mRNA in response to doxorubicin is surged as strong, as the well known E2F1 target gene TAp73 (Figure 8).

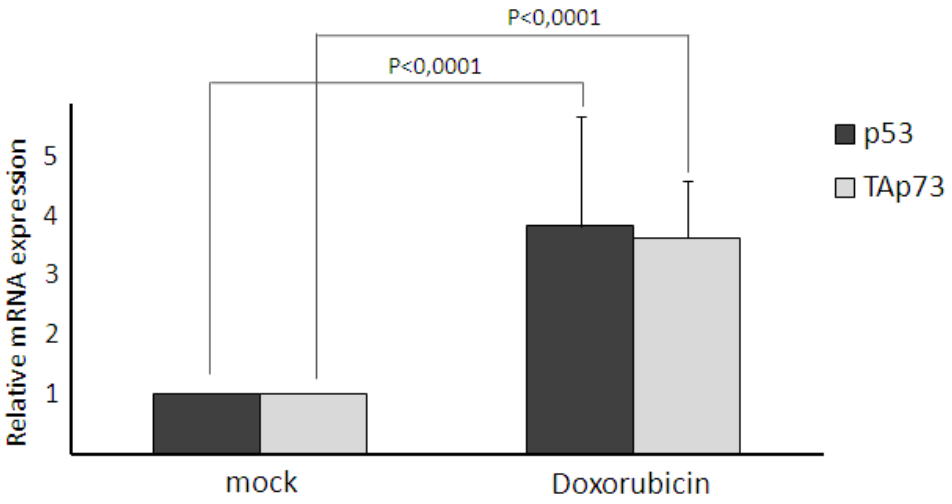


Figure 8: Doxorubicin induces the transcription of p53.
U251 cells were treated with 500nM doxorubicin for 24 hours. Total RNA was isolated and reverse transcribed into cDNA. Relative mRNA expression levels were determined using 36B4 as control gene. The mean and standard deviation of 8 independent replicates were plotted. For statistical analysis a Student's t-test was performed (α=0.05).

4.2. THE MECHANISMS OF P53 TRANSCRIPTIONAL REGULATION

It was previously reported that the two transcription factors HOXA5 and RREB1 contribute to the transcriptional regulation of p53. We confirmed that in our system HOXA5, as well as RREB1 contribute to the up-regulation of p53 mRNA in response to doxorubicin, as it was shown by Raman *et al.* (2000) and Liu *et al.* (2009) respectively (data not shown).

4.2.1. THE TRANSCRIPTION FACTORS E2F1 AND TAP73 ARE NECESSARY FOR THE INDUCTION OF P53 IN RESPONSE TO DOXORUBICIN

In addition to the above, Ren *et al.* (2002) published a ChIP-on-chip study where the promoter of p53 was found occupied by the transcription factor E2F4 four fold over average. Nevertheless, E2F4 is thought to be primarily involved in the repression of E2F-responsive genes (Dyson, 1998), whereas its homolog E2F1, which is known to be stabilized and activated in response to doxorubicin, is a potent transcriptional activator of its target genes (Dyson, 1998).

As a first step to determine whether E2F1 regulates the expression of p53, the messenger RNA levels of p53 were analyzed in response to doxorubicin treatment upon the previous knock-down of E2F1. The up-regulation of TAp73 transcription in this experiment occurred mainly through the activation of E2F1, as the increased transcription of TAp73 is abolished completely after knock-down of E2F1 with two different siRNAs (Figure 9, light grey bars). At the same time we observed that the knock-down of E2F1 diminishes the accumulation of p53 mRNA (Figure 9, dark grey bars) upon doxorubicin to a large extent even though the effect is not as strong, as it was observed for TAp73. The knock-down efficiencies for both siRNAs were very high as determined by qRT-PCR (Figure 9, upper chart) and immunoblot analysis (Figure 10C).

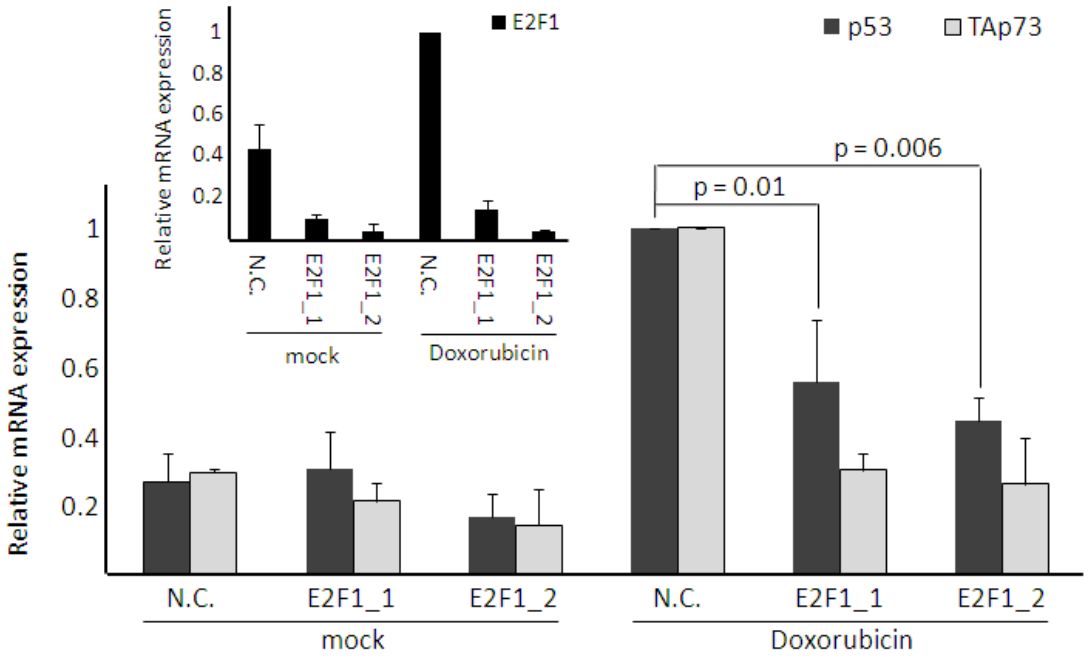


Figure 9: The knock-down of E2F1 alleviates the effects of doxorubicin on p53 transcription
U251 cells were transfected with siRNAs targeting E2F1 for 24 hours, before they were treated with 500nM doxorubicin for additional 24 hours. Total RNAs were reverse transcribed and quantified, relative to 36B4, by real-time PCR. The means and standard deviations of four biological replicates are depicted. For statistical analysis a Student's t-test was performed (α=0.05).

To further support the theory that the augmented transcription of p53 upon doxorubicin treatment is dependent on the transcription factor E2F1, we performed quantitative immunofluorescence microscopy, as well as immunoblotting. In both cases, we observed that also on the protein level the induction of p53 through doxorubicin treatment gets diminished by the knock-down of E2F1 (Figure 10).

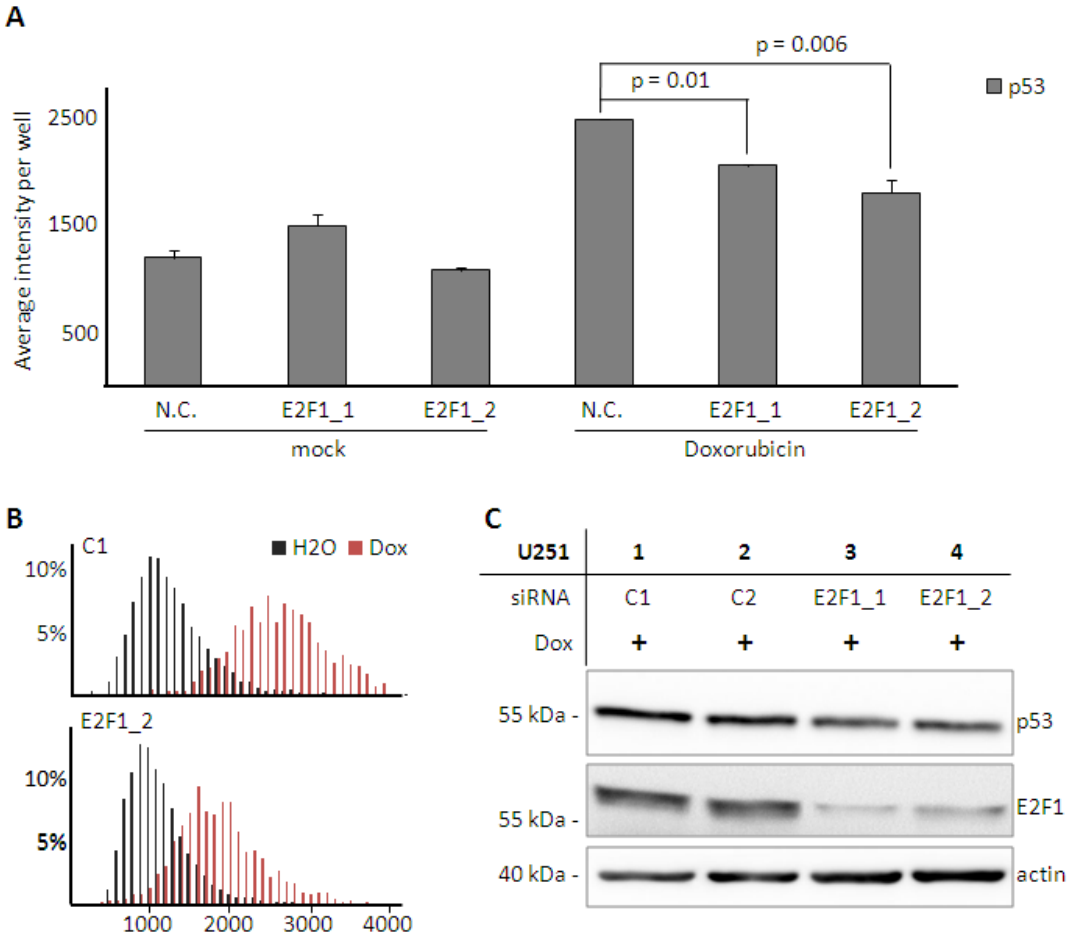


Figure 10: E2F1 contributes to the enhanced protein levels of mutant p53 upon doxorubicin.
U251 cells were transfected with two different siRNAs targeting E2F1 for 24 hours, and then they were treated for additional 24 hours with 500nM doxorubicin. **A, B:** The cells were fixed and stained for immunofluorescence microscopy. Single nuclei were identified using 10µM doxorubicin. The p53 staining was quantified per nucleus. **A:** The average intensity per well was determined in triplicate, means and standard deviations are depicted. For statistical analysis a Student's t-test was performed (α=0.05). **B:** Representation of the data in histograms. **C:** The cells were harvested for immunoblot analysis and stained for p53 as well as E2F1. Actin was used as a loading control.

Bearing in mind the observations of Wang and el-Deiry (2006) that p53 and TAp73 are capable of directly regulating the transcription of p53, the observed may in part be due to the E2F1 dependent induction of TAp73.

To investigate whether E2F1 is only indirectly acting on p53 through the co-regulation of its target gene TAp73 we used siRNA mediated knock-down of the p53 paralog. And indeed, TAp73 is as well necessary for the induction of p53 mRNA (Figure 11), even though it should be noted that the knock-down of

E2F1 had a stronger effect on the expression of p53 mRNA compared to the knock-down of p73, whereas the expression levels of TAp73 were unequivocally lower in the latter case. Therefore, we believe that E2F1 is acting on the transcriptional regulation of p53 not exclusively through TAp73.

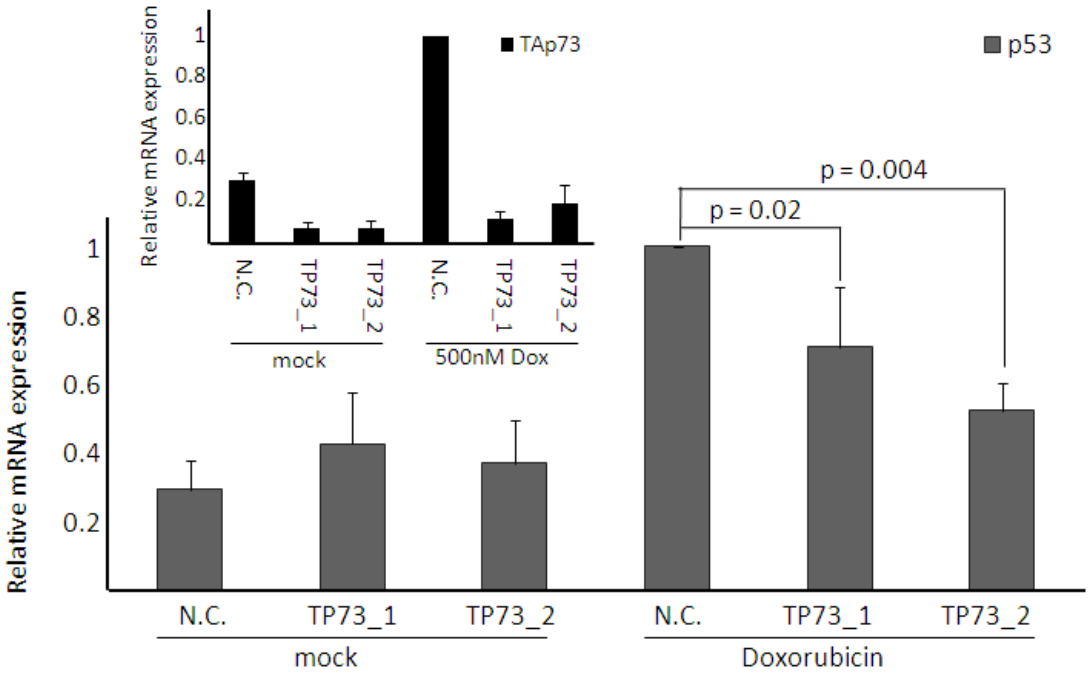


Figure 11: The induction of p53 transcription is partially dependent on p73.
U251 cells were treated with 500nM doxorubicin for 24 hours. The isolated RNA was reverse transcribed and the expression of p53 and TAp73 was quantified relative to 36B4 using real-time PCR. The experiment was performed in four biological replicates. The means and standard deviations were plotted. For statistical analysis a Student's t-test was performed (α=0.05).

4.3. E2F1 REGULATES P53 DIRECTLY AS A TRANSCRIPTION FACTOR

The finding that E2F1 knock-down has a stronger effect on the doxorubicin induced augmentation of p53 transcription, compared to p73, pinpoints to the additional involvement of E2F1 in the regulation of p53. To identify potential E2F1 binding sites and their biological relevance we first used bioinformatical tools to *in silico* predict potential binding sites that were then confirmed by chromatin immunoprecipitation analysis (ChIP).

4.3.1. *IN SILICO* IDENTIFICATION OF POTENTIAL E2F1 BINDING SITES WITHIN THE P53 PROMOTER REGION

E2F1 is an important transcription factor regulating the expression of various genes in response to its activation through DNA damage or other stimuli. The DNA sequence TTTSSCGC is described to be the canonical binding site motif for E2F1; nevertheless, some ChIP-on-chip studies revealed that a large proportion of E2F binding occurs at sites where this recognition sequence cannot be found (Bieda *et al.*, 2006). In collaboration with Martin Haubrock (Department of Bioinformatics, University of Göttingen) all these ChIP-on-chip data sets were used to generate a scoring matrix, helping to identify potential E2F1 binding sites (Table 1).

Table 1: Scoring matrix for the identification of potential E2F1 binding sites
Sequence information from known E2F1 binding site motifs were integrated to generate a scoring matrix for the identification of potential new E2F1 binding sites. The resulting consensus motif is displayed in the left column. N: any (A, C, G, or T); K: ketone (G or T); S: strong bonds (C or G)

Nucleotide / Consensus	A	C	G	T
N	1	4	3	5
K	0	1	5	7
T	2	0	0	11
S	0	7	6	0
S	0	5	8	0
C	0	10	3	0
G	0	3	10	0
C	0	8	4	1

This matrix was then applied to the genomic sequence around the transcriptional start site (TSS) of p53 in order to identify potential binding sites. In Figure 12A the determined scores are plotted against the genomic region. Two sites within the analyzed sequence revealed a score greater than 0.9 and

were therefore considered as potential E2F1 binding sites. First, E2F1 BS-I, at position 7590195-7592195 (hg19 chromosome 17) with the sequence ACTGGCGC revealed a score of 0.911, and second, E2F1 BS-II, at position 7590195-7592195 (hg19 chromosome 17) with the sequence TTTCGCGG resulted in a score of 0.954. Analysis of the evolutionary conservation of the predicted binding sites showed that E2F1 BS-I close to the TSS is highly conserved, whereas E2F1 BS-II even though displaying a higher score lacks this conservation (Figure 12B).

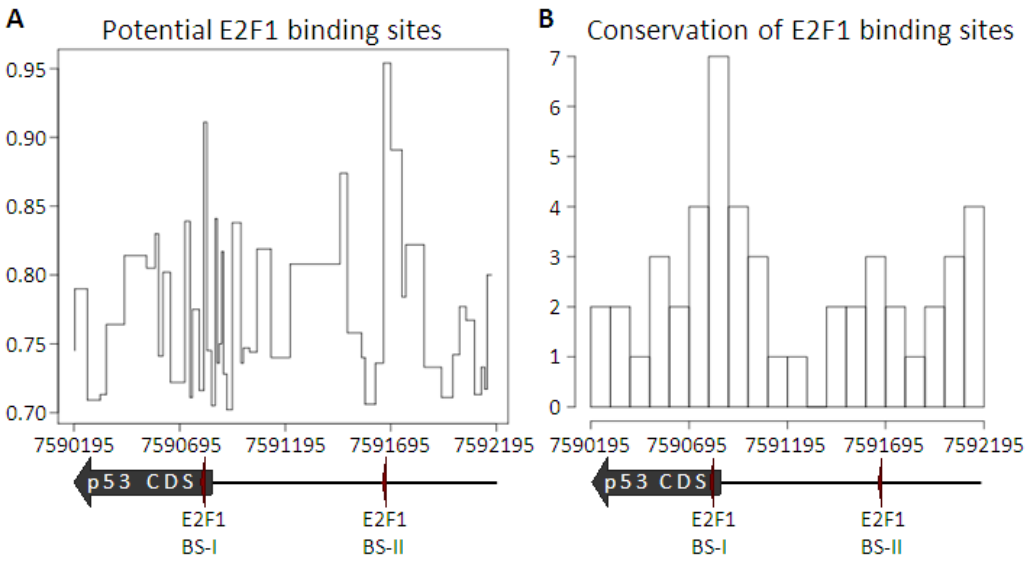


Figure 12: Bioinformatical analysis of the p53 promoter uncovers two E2F1 binding sites.
A scoring matrix (Table 1) to discover potential E2F1 binding sites was applied to the DNA sequence of the p53 promoter region (-1500 to +500 around the TSS). **A:** The calculated scores are plotted against the genomic region. **B:** The level of conservation within the analyzed genomic region is depicted. **A, B:** A schematic representation of the p53 gene locus, with the two binding motifs displaying the highest score and sequence conservation, is shown underneath both plots.

4.3.2. THE TRANSCRIPTION FACTOR E2F1 BINDS TO ONE OF THE POTENTIAL E2F1 BINDING SITES WITHIN THE P53 PROMOTER REGION

Within 2000 base pairs around the TSS of p53 two potential E2F1 binding sites were identified using bioinformatical tools. In order to confirm the biological relevance of these binding sites ChIP analysis was performed. E2F1 protein,

cross linked to its DNA binding elements, was precipitated using antibodies, generated against the transcription factor. ChIP-grade IgG antibodies were used for negative control precipitation. The recovered DNA was amplified by quantitative real-time PCR using primers spanning the two potential E2F1 binding sites on the p53 promoter. Additionally, primers spanning the well known E2F1 binding sites on the p107 and TAp73 gene loci were used as positive controls. Primers amplifying a region 19.5kb downstream of the p53 TSS, a region where no binding of E2F1 is expected, served as negative control. The data show that E2F1 is bound to the predicted E2F1 BS-I roughly 40bp downstream of the TSS, but not to the less conserved second potential binding site (Figure 13, upper panel).

As expected, E2F1 also associated with the promoters of its target genes p107 and TAp73. In contrast, the recovery of a distant fragment of the p53 genomic locus (p53 +19.5kb) was at the background level (similar to precipitation with non-specific IgG) (Figure 13, lower panel).

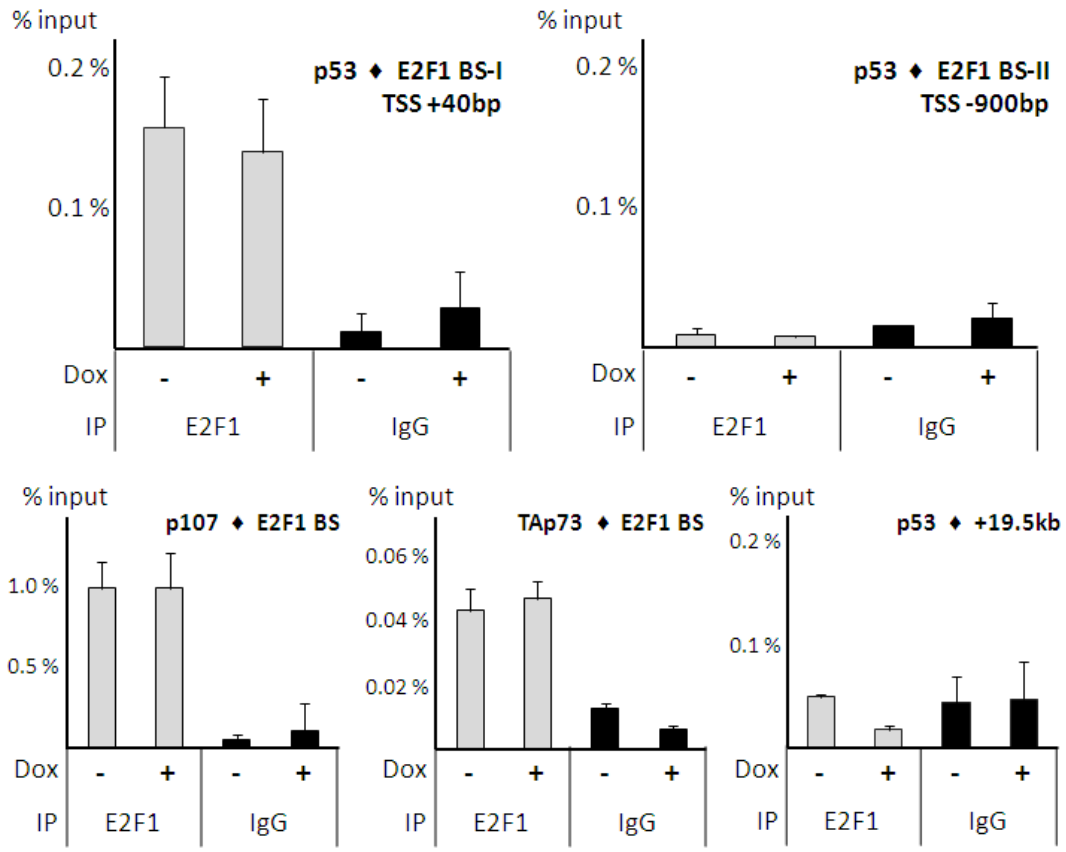


Figure 13: E2F1 is bound to one of the predicted binding sites under physiological conditions.
U251 cells were treated with 500nM doxorubicin for 24 hours. Cross linked DNA – protein complexes were precipitated using antibodies against E2F1 or IgG, as negative control. The recovered DNA was quantified by real-time PCR using primers flanking the two potential E2F1 binding sites, as well as two positive control sequences (p107 and TAp73) and a negative control region (p53 +19.5kb). The means and standard deviations of three replicates are depicted as percentile of the input DNA.

4.4. IS THE INCREASED TRANSCRIPTION OF P53 A GENERAL RESULT OF CHEMOTHERAPEUTIC TREATMENT?

4.4.1. THE INFLUENCE OF DIFFERENT CHEMOTHERAPEUTIC DRUGS ON THE TRANSCRIPTIONAL REGULATION OF P53 EXPRESSION

Treatment of U251 cells with the topoisomerase I inhibitor camptothecin (CPT), the topoisomerase II inhibitor etoposide, as well as the nucleoside analog 5-fluorouracil revealed that the induction of p53 transcription is specific to doxorubicin, rather than a general result of the evoked DNA damage response.

The treatment of the cells with CPT resulted in a very strong induction of NOXA, another target gene of E2F1, whereas the induction of TAp73 and p53 was not detected under these circumstances (Figure 14A). Also 5-fluorouracil (5'FU), as nucleoside analog, caused similar to CPT an induction of NOXA, but neither TAp73 nor p53 were elevated in their expression (Figure 14A).

Additionally, we tested etoposide, a chemotherapeutic drug more closely related to doxorubicin, since it also acts through the inhibition of topoisomerase II. In contrast to CPT and 5'FU this drug led to the induction of TAp73, as it was previously observed for doxorubicin. But, to our surprise, the levels of p53 messenger RNA remained unaffected (Figure 14A). Other than the mentioned induction of TAp73 transcription, we also observed that etoposide treatment mediates an increase in Serine 15 phosphorylation of p53 in U251 cells (Figure 14B). This could be explained by the evoked DNA damage response and the accompanied activation of ATM. Nevertheless, we observed in the same experiment that the total levels of p53 protein remain unchanged in response to etoposide treatment (Figure 14C).

In conclusion, these data show that the induction of TAp73 in the DNA damage response seems not to be sufficient to augment the transcription of p53, although we demonstrated before (4.2.1) that it is necessary.

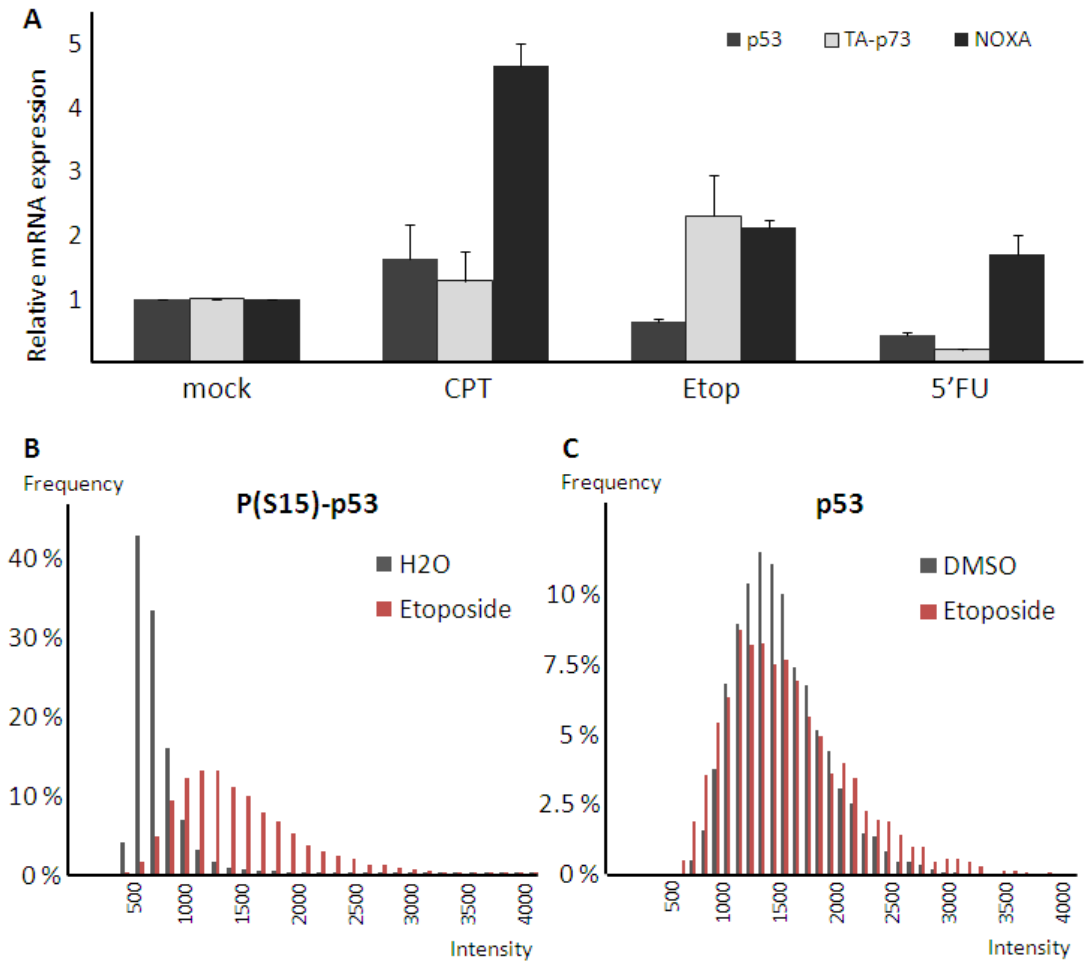


Figure 14: The induction of p53 is not simply caused by the inhibition of topoisomerase. **A:** U251 cells were treated with CPT (2,87µM), etoposide (100µM), or 5'FU (500µM) for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. The means and standard deviations of three biological replicates are depicted. **B, C:** U251 cells were treated with 100µM etoposide for 24 hours each. Upon fixation, the cells were stained for immunofluorescence analysis using antibodies against phospho(S15)-p53 (**B**) or total p53 (**C**). Single nuclei were identified using 10µM doxorubicin. The average fluorescence intensity per nucleus was determined. The results are represented in histograms.

4.4.2. THE IMPACT OF DIFFERENT ANTHRACYCLINES ON THE TRANSCRIPTIONAL REGULATION OF P53

The mechanism by which doxorubicin acts in the cell is controversially discussed in the literature, some reports claim that its ability to intercalate into DNA leads to decreased transcription through inhibition of helicase activity (Bachur *et al.*, 1992) or through DNA cross linking (Swift *et al.*, 2006), others claim that doxorubicin mainly functions by stalling of topoisomerase II on the

DNA and the stabilization of a reaction intermediate in which the DNA strands are cut and covalently linked to the enzyme (Tewey *et al.*, 1984a; Tewey *et al.*, 1984b). Additionally, there are mechanisms of free radical formation, DNA alkylation, direct membrane effects, and direct induction of apoptosis discussed (Gewirtz, 1999). The observation that etoposide, in contrast to doxorubicin, lacks the ability to induce p53 transcription, argues against the theory that the general inhibition of topoisomerase II activity is sufficient to induce the transcription of p53. In addition to doxorubicin, there are other anthracyclines currently used in the clinics, namely daunorubicin, epirubicin, and idarubicin. These drugs are described to have slightly different sequence specificities, but are all believed to interfere with DNA transcription through intercalation as well as topoisomerase II inhibition (Minotti *et al.*, 2004).

These 4 structurally related drugs share a common tetracyclic ring system containing an anthraquinone chromophore with a daunosamine moiety attached to the A-ring (C7), but they differ in their substitutions to this basic structure (Figure 15A, differences compared to doxorubicin are highlighted with red circles). Interestingly, analysis of their ability to induce the transcription of p53 revealed that dauno-, doxo-, and epirubicin induced p53 in a comparable manner. Whereas idarubicin, differing from daunorubicin only in a methoxy-group at C4 (D-ring), has no effect on p53 transcription, even though it induces TAp73 the same way as the other three compounds (Figure 15B).

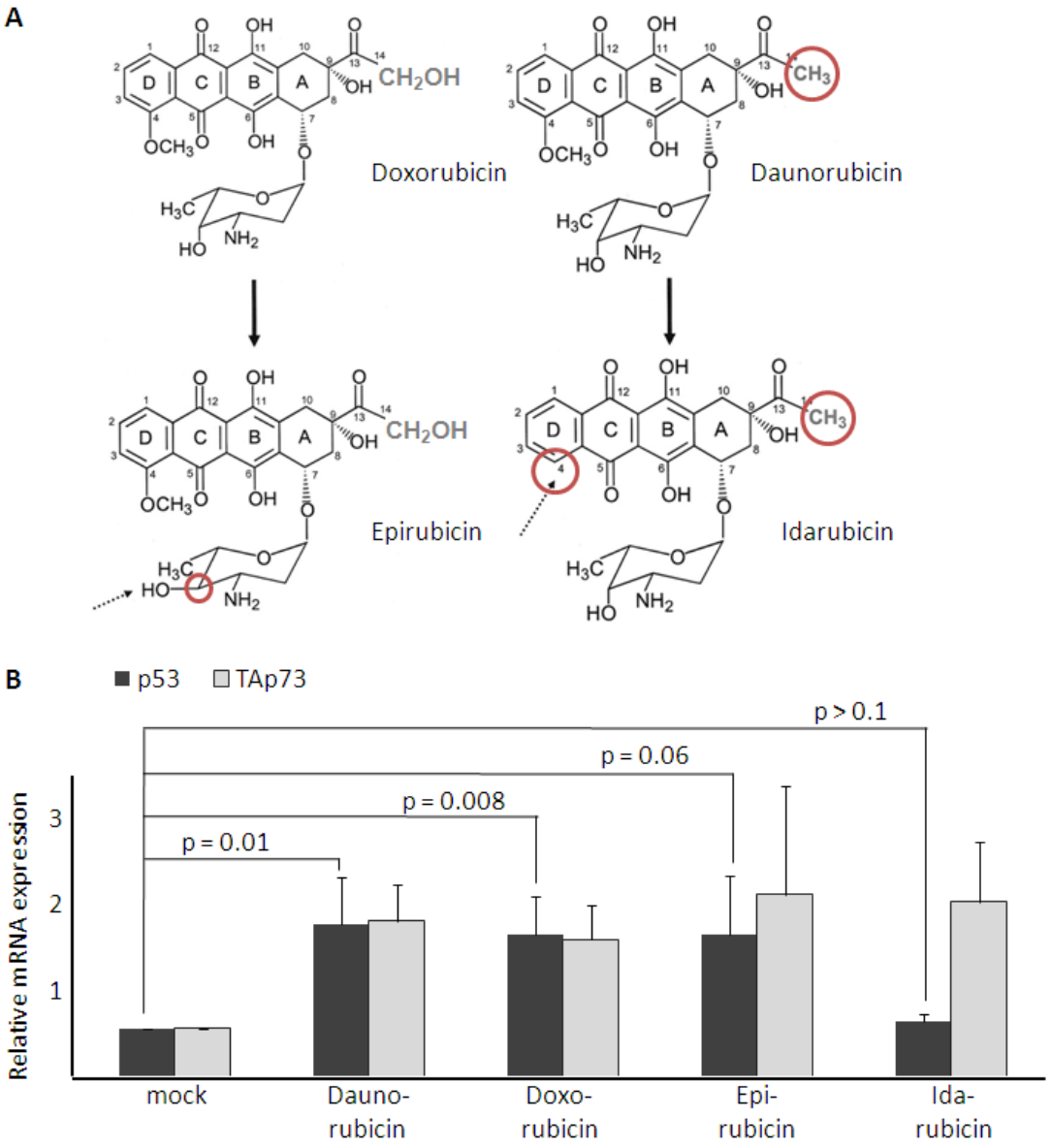


Figure 15: Three out of four anthracyclines activate the transcription of p53.
A: Chemical structure of the four anthracyclines doxorubicin, daunorubicin, epirubicin and idarubicin [Adopted from Minotti *et al.* (2004)]. Differences with respect to doxorubicin are highlighted with red circles. **B:** U251 cells were treated with 500nM dauno-, doxo-, epi-, or idarubicin for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. The experiment was performed in triplicate. The means and standard deviations of the three experiments are diagramed. For statistical analysis a Student's t-test was performed (α=0.05).

4.4.3. ANTHRACYCLINE MEDIATED ACTIVATION OF P53 TRANSCRIPTION

Quantification of the p53 pre-mRNA expression revealed that upon doxorubicin treatment not only the levels of mature p53 mRNA are elevated, which could as well be explained by increased mRNA stability, but also the direct product of

transcription, the pre-mRNA. This argues in favor of the hypothesis that the two transcription factors TAp73 and E2F1 facilitate the active transcription of the p53 gene (Figure 16).

Interestingly, we found that idarubicin, the anthracycline that is structurally related to doxorubicin, but not capable of augmenting the levels of p53 mRNA, induced the expression of p53 pre-mRNA the same way as the other anthracyclines (Figure 16; data not shown).

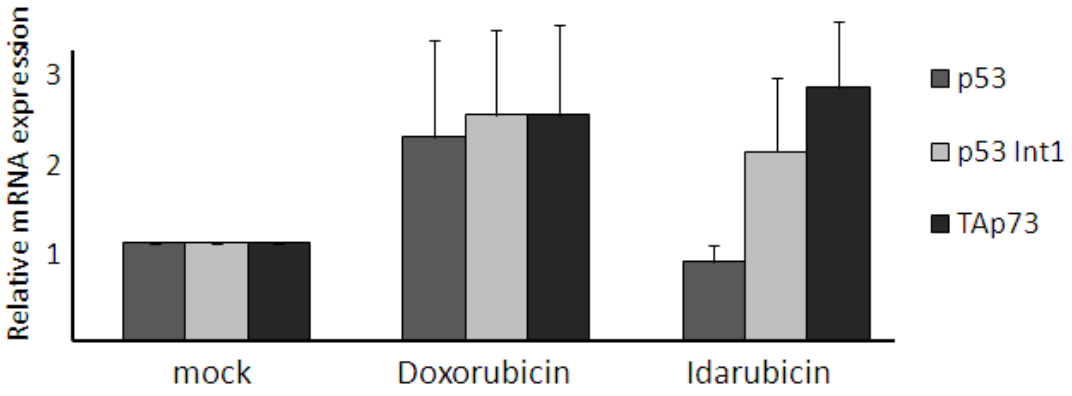


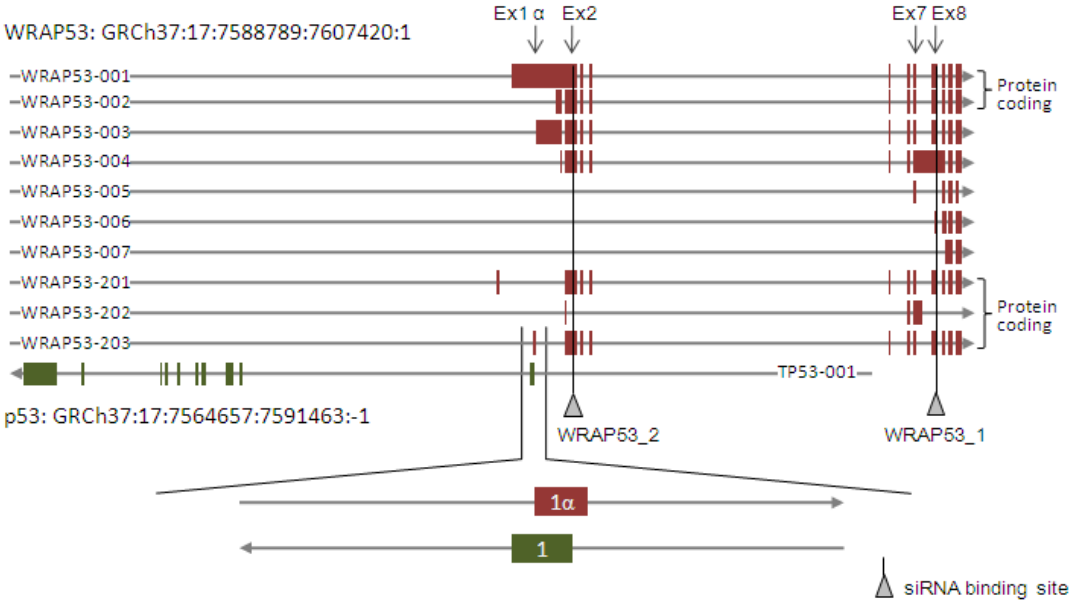
Figure 16: p53 pre-messenger RNA is elevated in response to all anthracyclines.
U251 cells were treated with 500nM doxorubicin or idarubicin for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. Means and standard deviations of three biological replicates are depicted.

4.5. WRAP53 AS REGULATOR OF P53 MRNA EXPRESSION

The recent finding of Mahmoudi *et al.* (2009) that the pre-mRNA stability of p53 is regulated through a natural antisense transcript prompted us to investigate whether the expression of the described WRAP53 (WD repeat containing, antisense to p53) gene contributes to the regulation of p53 in response to anthracyclines.

WRAP53 was identified as a gene located immediately upstream of *TP53* on the opposite strand. Mahmoudi *et al.* (2009) found at least 17 variants of this gene, generated through alternative splicing, as well as three different TSS. In Figure 17 the 10 transcripts listed in the Ensembl database were aligned with the p53 gene. Two of the transcripts (WRAP53-001 and WRAP53-203) contain

exon 1α, which is overlapping with a large portion of exon 1 of *TP53*. So far the function of WRAP53 protein is unknown; whereas it is claimed by Mahmoudi *et al.* (2009) that exon 1α of the WRAP53 mRNA contributes to the induction of p53 in response to DNA damage.



Transcript \ Primer	WRAP53 1α	WRAP53 1α-2	WRAP53 7-8
WRAP53-001	X	—	X
WRAP53-002	—	—	X
WRAP53-003	—	—	X
WRAP53-201	—	—	X
WRAP53-203	X	X	X

Figure 17: Schematic representation of the *TP53* genomic locus on chromosome 17
The 10 transcripts of WRAP53 listed in the Ensembl database are aligned with *TP53*. Both genes are encoded within the same genomic locus on opposite strands of the DNA. Exon 1α of WRAP53 and exon 1 of p53 overlap with the majority of their sequence, as depicted in the zoom-in. A table of the transcripts amplified using three different primer pairs is depicted in the lower part.

To explore the functional significance of WRAP53 in the context of anthracycline induced transcription of p53, we analyzed its mRNA expression using three different primer pairs: WRAP53 7-8 is used to amplify the majority of the transcripts independent of the TSS; WRAP53 1α is used to amplify both transcripts containing exon 1α; and WRAP53 1α-2 is used to specifically amplify WRAP53-203 (an overview of the transcripts targeted by the primer pairs is listed in Figure 17, lower part).

In response to doxorubicin we observed a slight up-regulation of WRAP53 1α. In contrast to that, our analysis yielded a massive induction of the WRAP53 transcripts containing exon 1α in response to idarubicin (Figure 18A), the anthracycline that was shown before to induce the pre-mRNA levels of p53 (4.4.3), but keeps the levels of mature mRNA low (4.4.2). An even stronger induction of WRAP53 1α was observed in response to etoposide treatment (Figure 18B), the topoisomerase II inhibitor that as well induced the DNA damage response through TAp73, but failed to augment the expression levels of p53 (4.4.1).

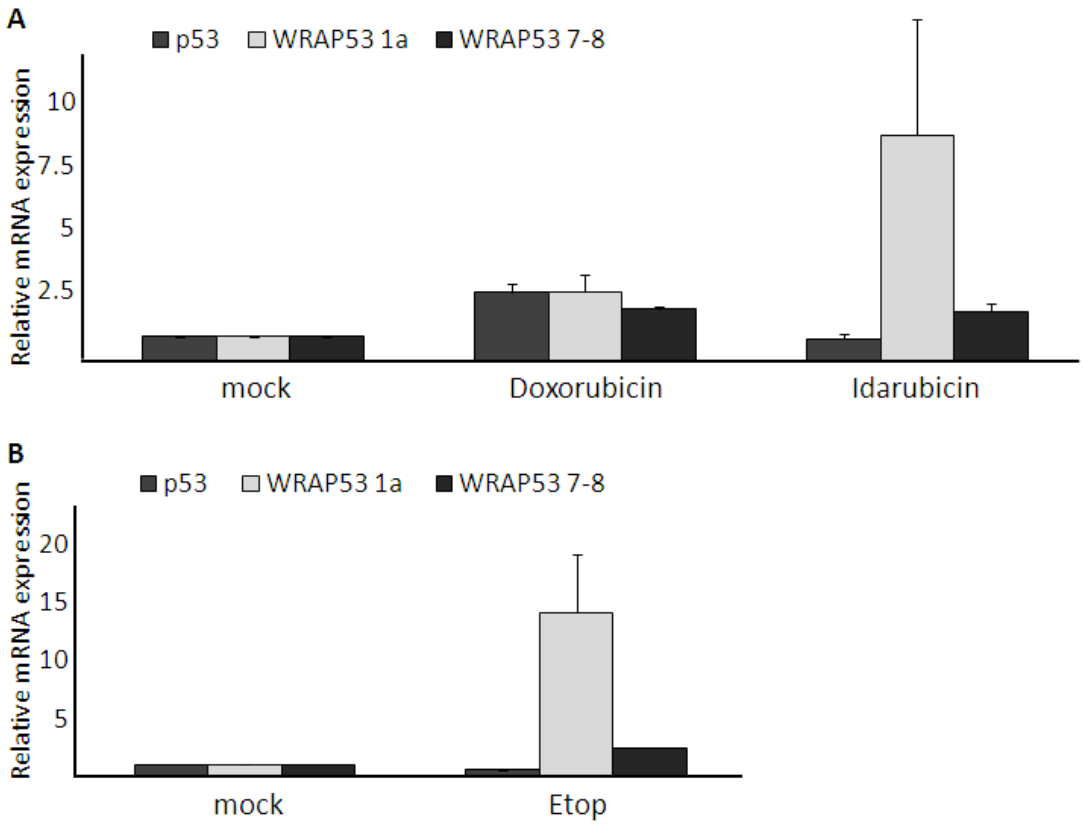


Figure 18: The natural antisense transcript of p53 is expressed antagonistic to p53 itself. U251 cells were treated with 500nM doxorubicin or idarubicin (**A**) or 100µM etoposide (**B**) for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. The depicted diagram represents the means and standard deviations of four biological replicates (**A**), experiment **B** was performed in triplicate.

The total levels of WRAP53 are slightly induced in response to any kind of DNA damage that was subjected to the cells, but remained the same for idarubicin

and etoposide, when compared to doxorubicin treatment (Figure 18A, B; black bars). These data show a clear correlation between the induction of WRAP53 transcripts containing exon 1α and the diminished response of p53 mRNA expression to E2F1 activation. Nevertheless, it should be mentioned that these data stand in contrast to the study published by Mahmoudi *et al.* (2009), where WRAP53 was shown to stabilize p53 mRNA in response to DNA damage.

Quantification of the p53 mRNA levels, upon siRNA mediated knock-down of WRAP53 (the siRNA target sites are depicted in Figure 17) and subsequent treatment with idarubicin was used to elicit the role of WRAP53 in the regulation of p53. To our surprise, the levels of p53 mRNA remained low upon knock-down of the antisense transcript WRAP53 and subsequent idarubicin treatment (Figure 19, white bars). Nevertheless, when we checked for the knock-down efficiency of the used siRNAs, we observed a discrepancy depending on the primer pair that was used for the analysis. Quantification of WRAP53 mRNA using primers to amplify either all transcripts, or specifically WRAP53-203 led to a reduction upon siRNA transfection of about 90%. Whereas, the usage of primers amplifying all transcripts containing exon 1α revealed that almost 60% of mRNA escaped the knock-down (Figure 19). A possible explanation for this discrepancy is that a WRAP53 transcript exists that contains exon 1α, but lacks exon 2 and 8, where the used siRNAs bind. Alternatively, it could be suggested that due to RNA masking or inhibited nuclear export, this WRAP53 transcript escapes the siRNA mediated knock-down.

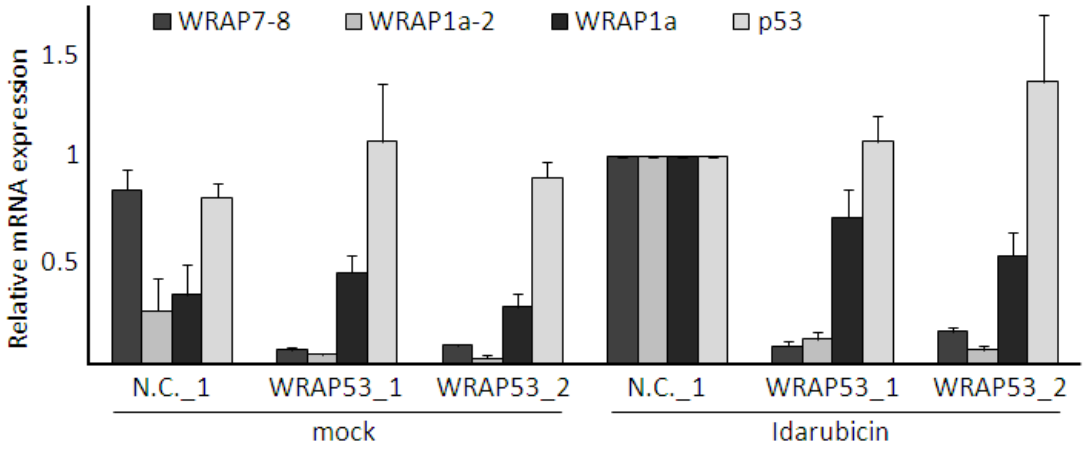


Figure 19: Not all transcripts of WRAP53 are targeted by the used siRNAs.
U251 cells were long-term transfected with two different siRNAs targeting WRAP53. Total RNA was isolated and reverse transcribed. Quantitative real-time PCR was used to determine the expression of the different WRAP transcripts as well as p53 mRNA. 36B4 was used as reference gene. Means standard deviations of three biological replicates are diagramed.

4.6. IS THE OBSERVED TRANSCRIPTIONAL REGULATION OF P53 THROUGH TOPOISOMERASE II INHIBITORS LIMITED TO U251 CELLS?

An important feature of tumor derived cell lines is their individual collection of mutations, rendering their physiological balance between different pathways. Thus, the behavior of cell lines might be different when they get exposed to certain stimuli. To substantiate that the presented mechanisms of p53 transcriptional regulation have general validity, we analyzed the effect of doxorubicin treatment using additional cell lines, also harboring different p53 mutations, as well as wild type p53.

A431 cells, derived from an epidermoid carcinoma (p53 R273H) and the bladder carcinoma cell line 5637 (p53 R280T) displayed an up-regulation of p53 mRNA levels in response to doxorubicin. In both cases the transcription of p53 is induced even stronger than that of TAp73, the gene that served throughout the study as a positive control for the triggered DNA damage response (Figure 20A). In contrast to these results we observed that the osteosarcoma cell line U2OS, that was used as a representative of wt p53

expressing cells, responded to doxorubicin treatment with an invariant expression of p53 mRNA (Figure 20A). Similar data were obtained for the colon carcinoma derived cell line HCT116 that as well expresses wt p53 (data not shown).

According to the general knowledge of the pathways within the p53 network, we hypothesized that a negative feedback loop from p53, via the cyclin dependent kinase inhibitor p21 and the Retinoblastoma protein, to E2F1 explains the decreased response of p53 mRNA expression to doxorubicin treatment. Of note, due to p53 loss of function, this feedback loop is permanently silenced in cells expressing mutant p53. To investigate the impact of the mentioned feedback loop in U2OS cells, we used siRNA mediated knock-down of p21, to intercept the pathway. The treatment of U2OS cells with doxorubicin after silencing of p21 resulted in the same slight up-regulation of p53 mRNA levels, as it was observed upon control siRNA transfection (Figure 20B). This indicates that the mentioned negative feedback loop, from transcriptionally active p53 via p21 to E2F1, is not causing the diminished response of p53 mRNA expression to doxorubicin treatment.

The result, we obtained in U2OS cells upon doxorubicin treatment, reminded us of, what we have seen in U251 cells with idarubicin before, the levels of TAp73 increase, but there is almost no change in the p53 expression. This prompted us to investigate, whether the expression of the p53 natural antisense transcript WRAP53 might again be involved in the regulation of p53. Strikingly, this theory was approved, treatment of U2OS cells with doxorubicin leads as well to the up-regulation of WRAP53 1α (Figure 20C) and thereby resembles another example of inverse correlation between the expression of opposing transcripts from the *TP53* locus.

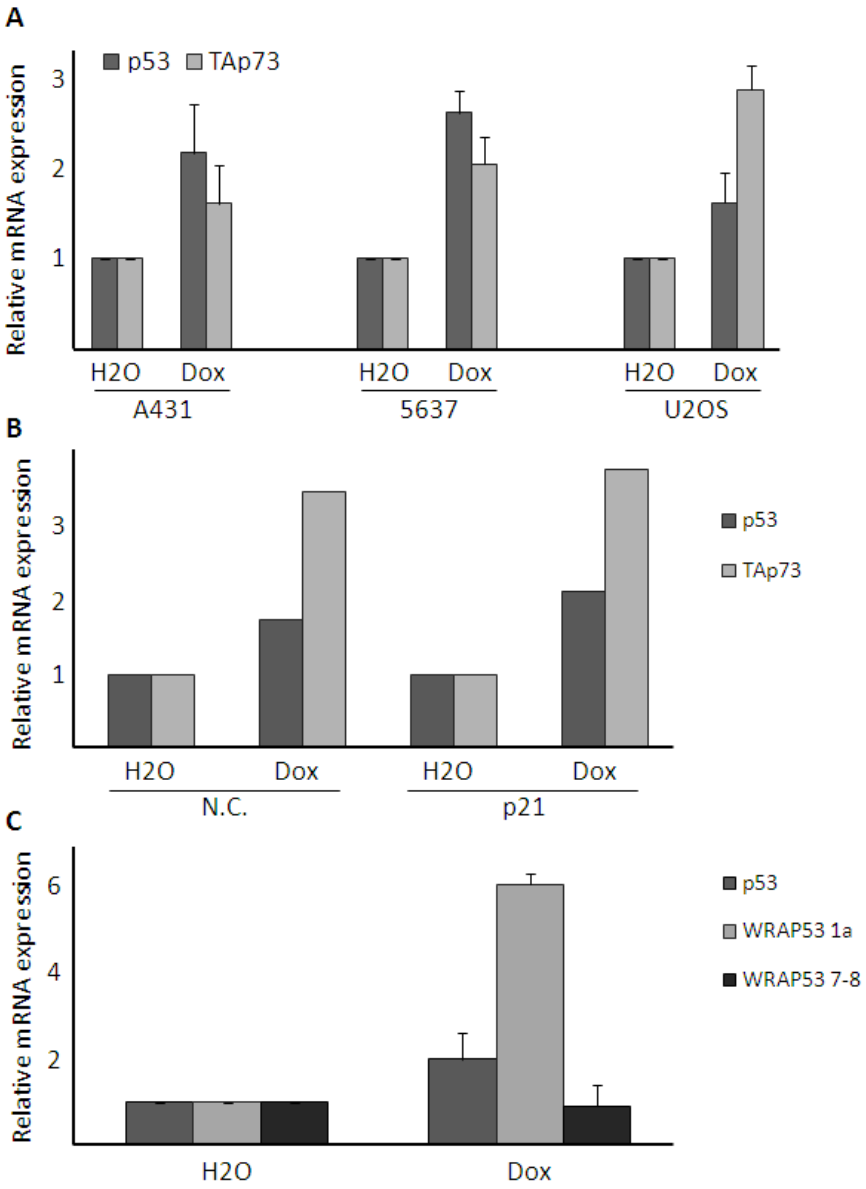


Figure 20: The discovered mechanisms of p53 regulation also apply to other cell lines
A: A431, 5637, and U2OS cells were treated with 500nM doxorubicin for 24 hours. **B:** U2OS cells were transfected with siRNAs as indicated for 48 hours. The last 24 hours the samples were additionally treated with 500nM doxorubicin. **C:** U2OS cells were treated with 500nM doxorubicin for 24 hours. **A-C:** Total RNA was isolated and subjected to reverse transcription. The levels of p53, TAp73, and WRAP53 were quantified relative to 36B4 using real-time PCR. Means and standard deviations of the experiments are diagramed.

Having elucidated important parts of mutant p53 expression regulation in response to different chemotherapeutics, the question remains whether these pathways also contribute to the general regulation of mutant p53 accumulation, as it is observed during tumor progression. The finding that E2F1, as well as TAp73 knock-down keeps the expression of p53 mRNA and protein levels more

or less constant in the absence of doxorubicin (4.2.1) pinpoints to the requirement of additional mechanisms regulating the expression of mutant p53.

4.7. A KINASE SCREEN DISCLOSES FURTHER CANDIDATES INVOLVED IN THE EXPRESSION REGULATION OF MUTANT P53 IN TUMOR DERIVED CELLS

Already 30 years ago, p53 was identified as a protein frequently accumulated in tumor cells and served as a diagnostic marker (Crawford *et al.*, 1981; DeLeo *et al.*, 1979; Rotter *et al.*, 1980). Until now, it is not clear why mutant p53 accumulates so strongly, whenever it is mutated. For many years, it was believed that the loss of p53 function goes along with low levels of Mdm2, the E3 ubiquitin ligase that is on the one hand a direct target gene of p53, but on the other hand its most important inducer of proteasomal degradation. This was a perfect explanation for the increased half-life of mutant p53 until Lang *et al.* (2004) and Olive *et al.* (2004) generated transgenic mouse models harboring various p53 hotspot mutations. Using these mouse models, they demonstrated that mutant p53 is specifically accumulated in the cells of advanced tumors, but remained at low levels in the surrounding tissue. These observations prompted us to investigate which pathways, imbalanced through tumor specific mutations, contribute to the expression regulation of p53.

The mutational spectrum in cancer cells mostly affects the pathways regulating cell cycle progression as well as DNA damage response, in order to keep a cell proliferating and alive. These pathways involve a series of constitutive phosphorylation events as to multiply the signal. Kinases, the enzymes that perform all these phosphorylations, are therefore central players and common targets of deregulation in the progression of tumor formation.

Investigating, whether these imbalanced pathways entail the accumulation of mutant p53, we performed a high content siRNA screen in 5637 cells. The so far known mutational spectrum of this bladder carcinoma derived cell line is

limited to the p53 mutation R280T and a nonsense mutation within the Rb gene. In addition to that, we found the cells to be siRNA transfected with a very high efficiency and perfectly shaped for single-cell based immunofluorescence analysis. The *Silencer* Human Kinase siRNA Library V3 targets 719 human kinases and kinase subunits with three individual siRNAs per gene, including validated siRNAs for more than half of the targets.

After 48 hours of siRNA transfection the cells were fixed and stained for single cell based immunofluorescence analysis. Hoechst 33342, as a nuclear stain, was used to define the individual nuclei of the cells. The average p53 staining in each of these nuclei was measured and used to calculate the mean expression of p53 upon each of the individual siRNA transfections. For the final hit determination Z-scores were calculated (Figure 21).

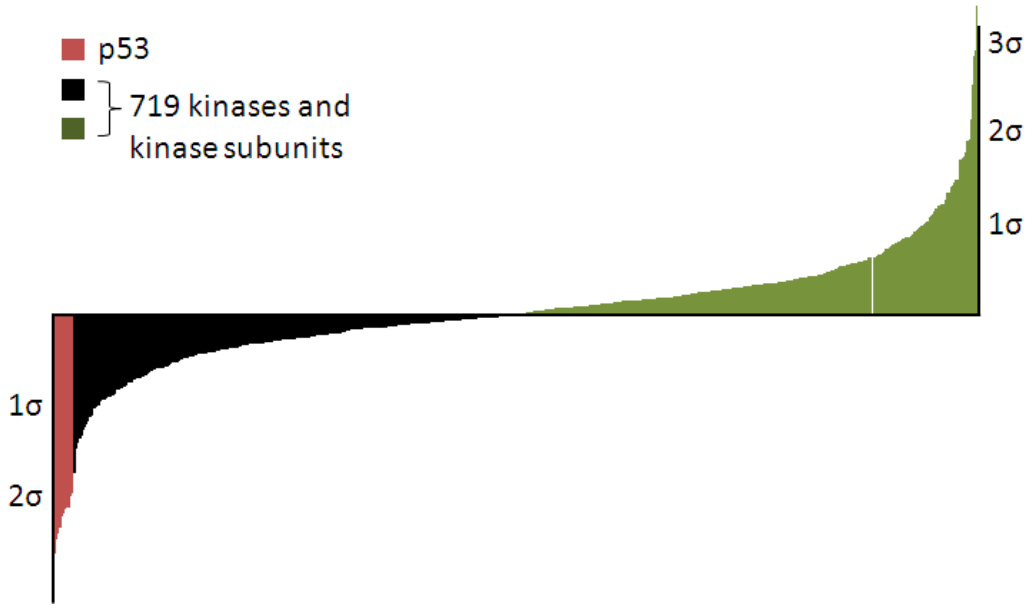


Figure 21: The influence of 719 human kinases on the expression of mutant p53 protein. 5637 cells were transfected with siRNAs targeting 719 human kinases and kinase subunits. Each target was silenced by three different siRNAs in individual wells. 48 hours after transfection the cells were fixed and stained using antibodies against p53. Hoechst 33342 was used to identify the individual nuclei within which the average expression levels of p53 were determined. Z-scores were assigned to each of the targeted kinases as a measure of p53 induction/ repression. For detailed description of the analysis please see 3.2.4. On each plate two wells were transfected with siRNAs targeting p53 itself as positive control for the down-regulation of mutant p53; the results of these controls are depicted in red.

The knock-down of 18 kinases revealed a Z-score lower than -1.1. These targets were considered as potential hits and analyzed in more detail. For 4 of these kinases it turned out that only one of the siRNAs had a striking effect, whereas the other two did not influence the expression of p53 at all. The chances that the effects of these kinases are caused by an off-target effect are very high and the targets were excluded from further analysis.

We assigned the remaining 14 hits to the pathways or cellular processes, where they were previously found to be involved in (Table 2).

Table 2: Hits from the screen.
The knock-down of the listed genes displayed a down-regulation of p53 in 5637 cells. Three siRNAs per gene were used, in column 3-5 the individual scores are listed and in column 6 the score of the average signal determined for the three siRNAs. The last column assigns the identified kinase to the pathway in which it is known to act.
PI5K: Phosphatidylinositol 5-kinase; PI3K: Phosphatidylinositol 3-kinase; mTOR: mammalian target of Rapamycin; PKC: Protein kinase C

	Kinase	Score A	Score B	Score C	Ø Score	Pathway
1	PIP5K1A	-1,32	-1,65	-1,75	-1,58	PI5K
2	PIK3C2G	-1,52	-1,58	-1,56	-1,55	PI3K → mTOR
3	RPS6KL1	-1,48	-1,56	-1,53	-1,52	PI3K → mTOR
4	PIM2	-1,28	-1,55	-1,64	-1,49	Cell proliferation, Meiosis
5	PIM3	-1,28	-1,08	-1,91	-1,43	Cell proliferation, Meiosis
6	PIP5K1C	-1,08	-1,49	-1,56	-1,38	PI5K
7	PGK2	-1,65	-0,65	-1,78	-1,36	Glycolysis, testis-specific
8	PINK1	-1,84	-1,76	-0,47	-1,35	Mitochondrial stress response
9	PIK3R3	-1,40	-1,14	-1,39	-1,31	PI3K → mTOR
10	PIK3CB	-1,20	-1,34	-1,25	-1,26	PI3K → mTOR
11	PIP5K2B	-0,73	-1,04	-2,02	-1,26	PI5K
12	PIP5K1B	-1,28	-0,99	-1,46	-1,24	PI5K
13	PKN2	-0,75	-1,34	-1,47	-1,19	PKC related, function unknown
14	RPS6KB2	-0,84	-0,84	-1,81	-1,16	PI3K → mTOR

Besides the two kinases PGK2 (Phosphoglycerate kinase 2) and PKN2 (Protein kinase N2) that are either very tissue specific or functionally unknown, three groups of kinases remained, as potential targets to be followed up on. First, the

PIM kinases, they are as well largely unknown in their function, but it became evident that two out of three paralogs appeared within the hit list. Second, the PI5K, they are represented by four members in the hit list, nevertheless, so far they are rather known to regulate cellular polarity and membrane trafficking, processes, in which the regulation of p53 expression would not be expected to happen. Third, the PI3K → mTOR pathway, it is represented by three members of the PI3K family and two members downstream of mTOR. Additionally, it should be mentioned that PINK1 (PTEN induced kinase 1) gets activated by members of the PI3K → mTOR pathway, even though its described function is so far restricted to the mitochondrial stress response in Parkinsons disease. Only very recently, it was observed by Morimoto *et al.* (2010) that the up-regulation of PINK1 expression in amyotrophic lateral sclerosis patients is positively correlated with the phosphorylation and stabilization of wt p53, suggesting that there is a link between the two pathways.

Taking all these information together, we decided to follow up on RPS6KL1 and –B2. These are two kinases that act quite far downstream in a pathway that is represented by 5 kinases within the hit list. In addition to this, there is one report claiming that some members of the large protein family of RPS6 kinases directly phosphorylate wild type p53, which was shown using an *in vitro* kinase assay (Cho *et al.*, 2005).

The S6 kinases were named according to their primary function of phosphorylating the ribosomal protein S6. The protein family consists of 10 members that can be grouped in three functionally more related subfamilies. First the RSKs (Ribosomal S6 kinases) consisting of RPS6KA1, RPS6KA2, RPS6KA3, and RPS6KA6, second the MSKs (Mitogen- and stress-activated kinases) with RPS6KA4 and -5, and third the S6Ks (S6 kinases) namely RPS6KB1 and RPS6KB2. Additionally, RPS6KC1 and RPS6KL1, as structurally related, but functionally mostly unknown kinases, belong to this family. Similar as described earlier for the E2Fs, also the RPS6 kinases fulfill

partially opposing activities and it can therefore not be expected that all 10 members of the protein family have an impact on the mutant p53 expression regulation.

4.7.1. VALIDATION OF THE IDENTIFIED HITS: RPS6KB2 AND RPS6KL1

RPS6KL1 and RPS6KB2 were identified in the performed kinase screen as potential regulators of mutant p53 expression in the tumor cell line 5637 by quantitative immunofluorescence microscopy. This is a very sensitive method that was on the one hand enabling us to detect kinases that have slight effects on the expression of mutant p53, but on the other hand also requires optimization until the effects can get validated by less sensitive methods like immunoblotting.

First, we tried to find another cell line, still harboring a p53 mutation, but expressing higher levels of the identified kinases, in order to observe whether S6 kinases have an even stronger impact on p53 levels in such a system. Nakamura *et al.* (2008) published the two glioma cell lines U251 and U373 to express detectable levels of the kinases RPS6KB1 and -2. Since RPS6KL1 is largely unknown and the available antibodies fail to specifically detect the protein, we could not optimize the cell line with respect to the expression level and activity of this kinase, which originally showed a stronger effect on the regulation of p53. In order to confirm that the knock-down of RPS6KB2, as well as RPS6KL1, contributes to the expression regulation of p53 in U251 cells we performed immunofluorescence analysis. The experiment was carried out the same way, as in the screen, but using U251 cells. This revealed that U251 cells are a good model system to analyze the impact of S6 kinases on the expression regulation of mutant p53. The impact of RPS6KL1 on mutant p53 levels was as well confirmed under these circumstances (Figure 22).

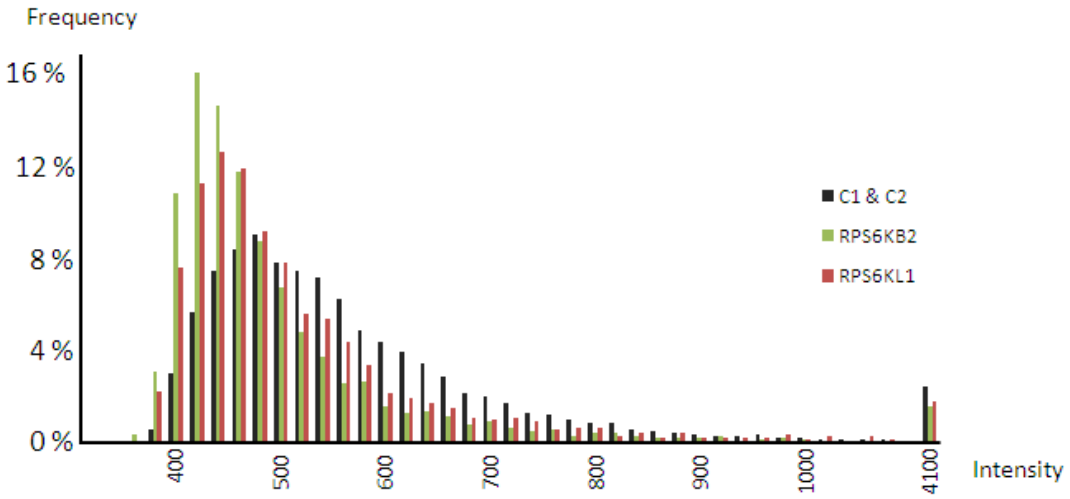


Figure 22: The knock-down of RPS6KB2 and RPS6KL1 decreases the expression levels of mutant p53 protein in U251 cells.
U251 cells were transfected with siRNAs against RPS6KB2 and RPS6KL1 for 48 hours. The fixed cells were stained for immunofluorescence analysis using antibodies recognizing total p53. Hoechst 33342 was used to identify the individual nuclei. The average expression of p53 per nucleus was determined; the data are diagramed in a histogram.

Taking into account that mutant p53 proteins in tumor cells have a much longer half-life than wild type p53, we further optimized the assay with respect to the duration of the knock-down. Using a double siRNA transfection protocol (3.2.1 LONG-TERM SIRNA TRANSFECTION), we confirmed the role of RPS6KB2 and RPS6KL1, as well as RPS6KB1, in the regulation of mutant p53 expression (Figure 23). The knock-down was performed for 5 days before the cells were harvested for immunoblot analysis. The structurally related kinase RPS6KA1 was used as an additional negative control, since it was found in the screen to keep the expression levels of p53 constant compared to non-targeting scrambled siRNAs.

The knockdown efficiency was monitored indirectly, by staining for the expression of Serine 235/236 phosphorylated S6, a well known target of the analyzed kinases RPS6KB1 and -2, due to the lack of specific antibodies recognizing the S6 kinases. This way, we observed that for RPS6KB1, as well as RPS6KB2 the first siRNAs mediated a stronger knock-down, compared to the second. This reflects the same pattern, as it is observed for the expression

of mutant p53. The knock-down of RPS6KL1 as well diminished the expression of mutant p53 to a large extent. Unfortunately, we could not monitor the knock-down efficiency of the protein, since there are neither specific antibodies available, nor a well characterized substrate that could be used for this analysis (Figure 23).

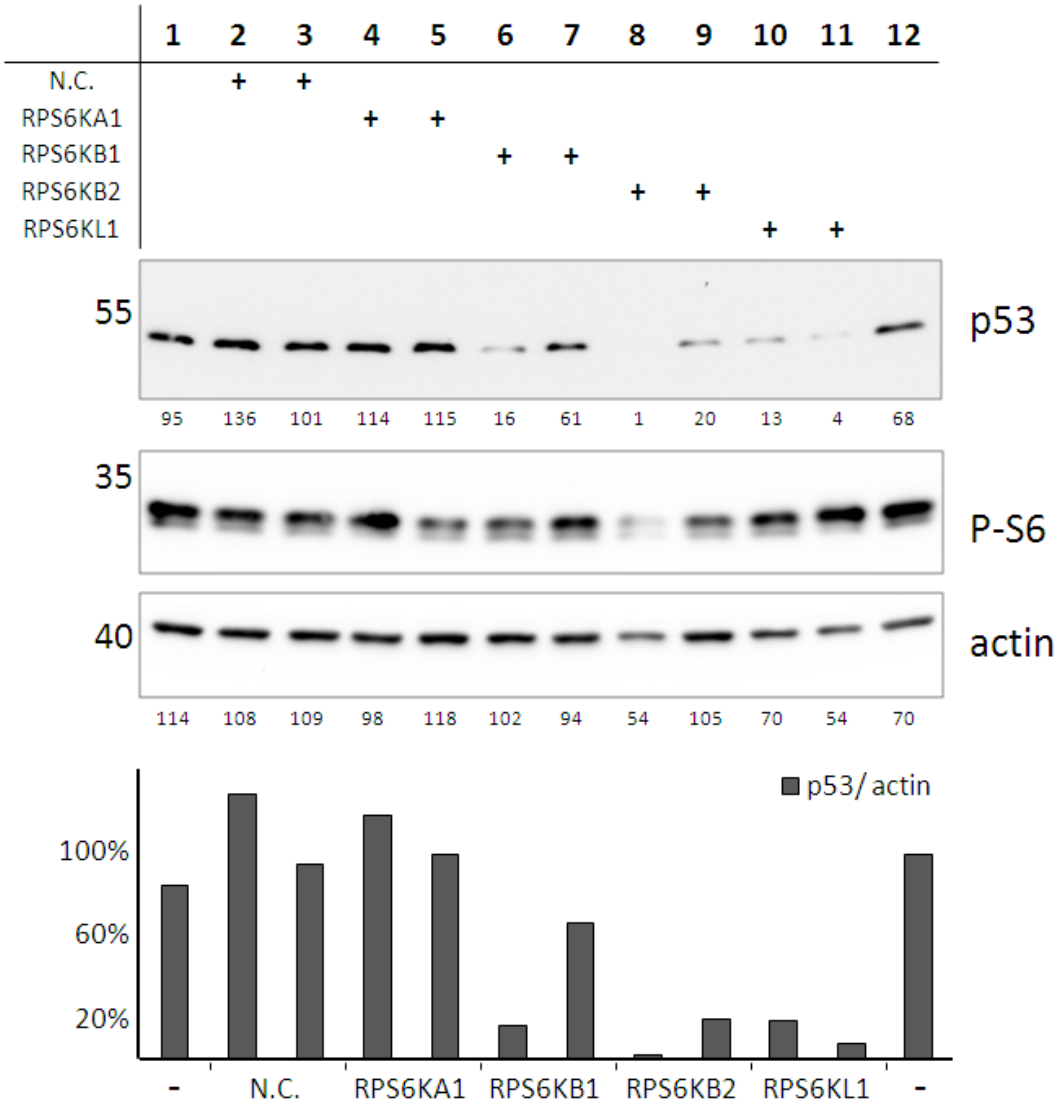


Figure 23: The S6 kinases RPS6KB2 and -B1, as well as RPS6KL1 contribute to the regulation of mutant p53 expression in tumor cells
U251 cells were double-transfected with siRNAs targeting four members of the RPS6 kinase family. Five days after the first transfection the cells were harvested and whole cell lysates were subjected to immunoblot analysis. The expression of p53, P(Ser 235/236)-S6, and actin was detected. The blots were quantified using LabImage 1D (lower part).

To test whether the knockdown of the identified kinases affects mutant p53 expression on the protein level, and not, as previously observed, on the transcriptional level, we isolated total RNA from cells upon siRNA mediated knockdown of the S6 kinases. This experiment clearly revealed that all three RPS6 kinases, that were shown to have an impact on mutant p53 expression, do not change its mRNA levels (Figure 24). This strongly argues, in line with previous observations, that the accumulation of mutant p53 during tumor progression happens on the protein level.

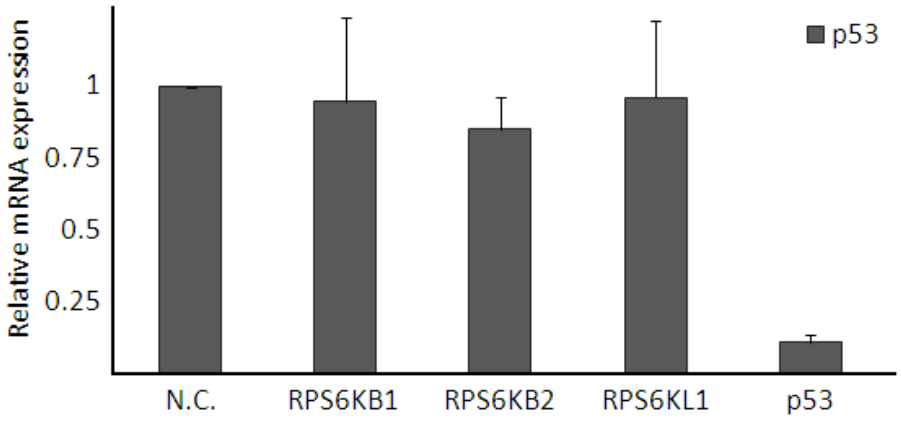


Figure 24: S6 kinases do not regulate the expression of p53 on the transcriptional level. U251 cells were siRNA transfected for 48 hours. Total RNA was isolated and quantified relative to 36B4 using real-time PCR. Means and standard deviations of four biological replicates were diagrammed.

Other than the depletion of S6 kinases, we also investigated, whether modulation of their activity would result in decreased expression of mutant p53. As described by Nobukuni *et al.* (2005) and Hidayat *et al.* (2003), inactivation of the kinases can be achieved by the withdrawal of FCS and the two amino acids Arginine and Lysine and can be reverted by 30min incubation with fresh medium containing FCS (Figure 25; P-S6 staining). The levels of mutant p53 decreased as well upon starvation and recovered after 30min of incubation in full medium supplemented with 10% FCS (Figure 25).

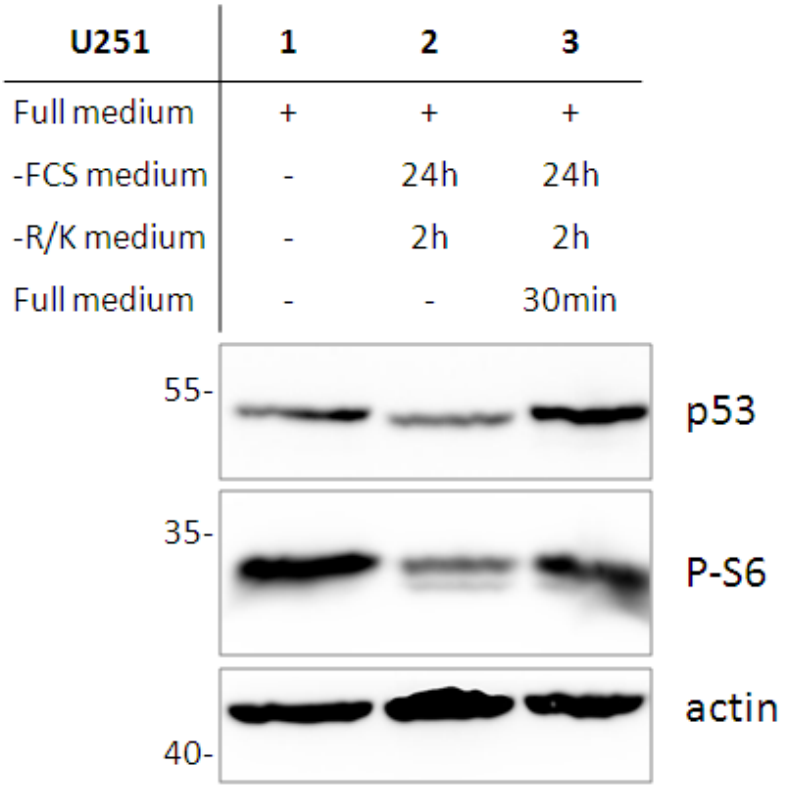


Figure 25: Serum starvation abolishes S6 kinase activity and leads to the down-regulation of mutant p53 expression.
U251 cells were seeded in 12 wells. After the cells attached the medium was changed, and the cells were kept in serum deprived medium. 24 hours later the medium was again changed to Arg/ Lys free medium without FCS, to further starve the cells. After 2 hours the cells were supplemented with fresh medium containing 10% FCS for 30 minutes. The cells were harvested and whole cell lysates were subjected to immunoblot analysis, using antibodies against p53 and P(Ser235/ 236)-S6. Actin staining was used as loading control.

This experiment argues that the protein level of p53 is regulated through the activity of S6 kinases and not simply through their abundance. Nevertheless, we don't know yet, whether this is a direct activity of the S6 kinases, as it was claimed by Cho *et al.* (2005) or, whether it still might be indirectly affected by other intermediates.

DISCUSSION

It is characteristic for most tumor cells that they proliferate rapidly in an uncontrolled fashion. The mechanistic principle behind most chemotherapeutic agents takes advantage of this feature, by preferentially inducing apoptosis in rapidly dividing cells. In most cases this is achieved through the induction of a DNA damage response, going along with the stabilization and activation of the tumor suppressor p53. This, depending on the severity and the nature of the damage, leads to cell cycle arrest or apoptosis. Sequence analysis of thousands of tumor samples revealed that p53 is mutated in more than 50% of all human solid tumors. In contrast to other tumor suppressors, p53 is mainly inactivated through single point mutations within the central DNA binding region of the protein. This mutation leads not only to the loss of tumor suppressor activity, but at the time confers oncogenic properties to the expressed gene product. According to different studies, this comprises increased chemoresistance, as well as a higher frequency of metastasis formation. It has previously been shown that knock-in mice, harboring one of the p53 hotspot mutations, differ from p53 null mice with respect to the frequency of metastasis formation (Lang *et al.*, 2004; Olive *et al.*, 2004). Histochemical analysis of the tumors revealed that mutant p53 was specifically over-expressed in high grade tumors, whereas its expression was at the background level in the surrounding tissue (Terzian *et al.*, 2008). The molecular signals that cause this accumulation are not known so far, nevertheless, studies of various groups indicate that Mdm2, as well as other E3 ubiquitin ligases like Cop1, ARF-BP1, and CHIP (C terminus of Hsc70-interacting protein) become inactivated due to the deregulation of tumor specific pathways (Lukashchuk and Vousden, 2007).

Within this study we identified three different levels of mutant p53 expression regulation. Due to different stimuli, either DNA damage responsive transcription factors were identified as central players of mutant p53 expression regulation, or the natural antisense transcript of p53, recently identified by Mahmoudi *et al.* (2009), or kinases involved in the PI3 kinase → mTOR pathway.

5.1. TRANSCRIPTIONAL REGULATION OF MUTANT P53 EXPRESSION IN RESPONSE TO CHEMOTHERAPEUTIC TREATMENT

In response to genotoxic stress, conferred by chemotherapeutic agents, like the three anthracyclines daunorubicin, doxorubicin, and epirubicin, the expression of mutant p53 in tumor cells is augmented due to increased transcription (Figure 15). Within the performed experiments, we have shown that the transcription factors HOXA5, RREB1, TP73, and E2F1 are all necessary for the observed up-regulation in response to treatment with the named anthracyclines, while they have no impact on the basal transcriptional expression regulation of mutant p53 in the absence of DNA damage (Figure 9, Figure 11, data not shown). The transcription factor E2F1 was further shown, to directly bind to a, so far not described binding site within the p53 promoter (Figure 13). Interestingly, ChIP analysis revealed no differences in E2F1 binding to the promoter of p53 upon doxorubicin treatment. Nevertheless, for the well known DNA damage responsive E2F1 target gene TAp73, we as well observed invariant E2F1 binding regardless of doxorubicin treatment. These data indicate that the E2F1 that we recover in our ChIP experiments is bound to the promoter of its target genes independently of their transcriptional activation through chemotherapeutic treatment. As Pediconi *et al.* (2003) showed, this could most probably be explained by posttranslational modifications of E2F1 that are necessary to promote active transcription. To further look into this matter, we would need to establish an antibody specific to acetylated E2F1 and thereby restrict the analysis to the active form of the transcription factor. Additionally, it could be tested whether RNA polymerase II in its active form

(Serine 2 phosphorylated) is more abundant around the transcriptional start site, in response to doxorubicin treatment, compared to untreated cells in a re-ChIP experiment, where the eluate of an E2F1 ChIP is used as starting material.

5.2. WRAP53 - A NATURAL ANTISENSE TRANSCRIPT OF P53 PREVENTS MRNA MATURATION

Natural antisense transcripts (NATs) are a class of non-coding RNAs that were shown to have an impact on the expression regulation of genes. It is estimated that in mammals about 70% of all transcripts have antisense partners that can alter the expression of the sense genes (Katayama *et al.*, 2005). For example, in about 70% of tumor samples, the antisense transcript of the tumor suppressor p15 (p15AS) is highly accumulated, whereas the tumor suppressor itself is silenced; in normal cells this is observed vice versa (Yu *et al.*, 2008). NATs have been proposed to regulate the expression of their target genes at several levels, including transcription, messenger RNA processing, splicing, stability, cellular transport, and translation (Lapidot and Pilpel, 2006). To understand the mechanisms of NAT regulation, Chen *et al.* (2005b) analyzed the expression profiles of sense and corresponding antisense transcripts on a genome-wide scale. This revealed that sense-antisense pairs tend to be co-expressed or inversely correlated more frequently, than would be statistically expected. Furthermore, they found that most of these pairs and their expression regulation is evolutionary conserved.

Within this study we observed that three topoisomerase II inhibitors, daunorubicin, doxorubicin, and epirubicin, induced the p53 mRNA expression in an E2F1 and TAp73 dependent manner. Two other topoisomerase II inhibitors, idarubicin and etoposide, were found to evoke a similar DNA damage response leading to the activation of the transcription factors E2F1 and TAp73, but, to our surprise, did not increase p53 mRNA expression levels (Figure 14, Figure 15). Over and above, we even found that etoposide treatment slightly decreased the

mRNA levels of p53 in U251 cells. Further analysis revealed that idarubicin and etoposide, but none of the other three analyzed topoisomerase II inhibitors, induced the expression of WRAP53-1α, a NAT encoded on chromosome 17 opposite of p53 (Figure 18). To elucidate the mechanism behind these controversial regulatory activities of highly related chemotherapeutic drugs, we on the one hand analyzed the pre-mRNA expression of p53 and on the other hand used siRNA mediated knock-down of WRAP53. Within the first experiment we found that the E2F1 and TAp73 dependent transcriptional activation of p53 occurred upon idarubicin treatment the same way, as upon treatment with any of the other anthracyclines (Figure 16, data not shown). This indicates that the maturation of p53 mRNA is affected upon idarubicin treatment, possibly mediated through the elevated expression of WRAP53-1α. The knock-down experiment of WRAP53 revealed that the used siRNAs targeted the mRNAs of most WRAP53 isoforms efficiently. Nevertheless, we also observed that transcripts, that either lack exon 2 and 8 or escape the knock-down by other means, are specifically induced in response to idarubicin treatment (Figure 19). Since siRNAs are believed to mainly act in the cytoplasm, absent nuclear export of the transcript should be considered as a possible way of inefficient knock-down of mRNAs, containing the siRNA target sequence. Additionally to the data we obtained in mutant p53 expressing cell lines, we observed that doxorubicin treatment strongly induces the expression of WRAP53-1α in the wt p53 expressing cell lines U2OS and HCT116 (Figure 20 C, data not shown), while the mRNA levels of p53 remained unaffected from the treatment (Figure 20 A).

When interpreting all these results from the tumor cells point of view: It appears advantageous for wt p53 expressing cells to circumvent the accumulation of p53 in response to DNA damage, in order to escape the induction of apoptosis. Elevated expression of WRAP53-1α could serve as one mechanism to achieve this. For mutant p53 expressing cells, on the contrary, this induction of

WRAP53-1α in response to doxorubicin is needless if not disadvantageous, since they induce a protein with oncogenic activities, leading to tumor progression and chemoresistance, rather than apoptosis. Nevertheless, this still owes a rational for the WRAP53-1α induction in response to idarubicin and etoposide in mutant p53 expressing cells.

The following NAT related mechanisms could serve to explain our data:

DNA METHYLATION AND HETEROCHROMATIN FORMATION:

Antisense-induced DNA methylation and silencing was described to play an important role for example in thalassaemia, where the haemoglobin 2 gene is efficiently silenced through the expression of an antisense transcript (Tufarelli *et al.*, 2003). Several studies have indicated that this is not mediated through the formation of RNA duplexes, but through the modification of chromatin structure or DNA methylation patterns (Lee and Lu, 1999; Wutz *et al.*, 1997). According to studies from Tufarelli *et al.* (2003) and Yu *et al.* (2008) antisense induced DNA methylation leading to efficient silencing of target genes should be considered a widespread mechanism of tumor suppressor silencing. Nevertheless, this concept stands in conflict with our observation that idarubicin treatment entails the same induction of p53 pre-mRNA synthesis, as it is observed in response to doxorubicin.

RNA MASKING:

Sense-antisense RNA duplex formation masks cis-regulatory elements within the p53 mRNA hindering proteins involved in polyadenylation, splicing, or nuclear export to bind to the pre-mRNA (Hastings *et al.*, 1997). This would in turn lead to less efficient maturation and thereby explain the observed phenotype of increased pre-mRNA levels that do not affect the overall mRNA levels of p53. Furthermore, this would explain the inefficient knock-down that was observed for WRAP53-1α, since the p53 mRNA is only exported into the cytoplasm after successful polyadenylation and splicing.

One central question that remains is how the inversely correlated transcription of p53 and WRAP53-1α is regulated. Collision of two RNA polymerase II enzymes is discussed as a possible mechanism of inverse regulation of antisense gene expression (Crampton *et al.*, 2006; Prescott and Proudfoot, 2002). Collision of RNA polymerase II with stalled topoisomerase II is not described so far. Nevertheless, we would like to suggest this to explain the generation of WRAP53-1α transcripts lacking exon 2. If, due to the collision, a shortened transcript of WRAP53 is released, it would resemble a natural antisense transcript to p53 and might then get stabilized in the nucleus through its interaction with the pre-mRNA or the DNA of the sense gene p53. This hypothesis is summarized in Figure 26.

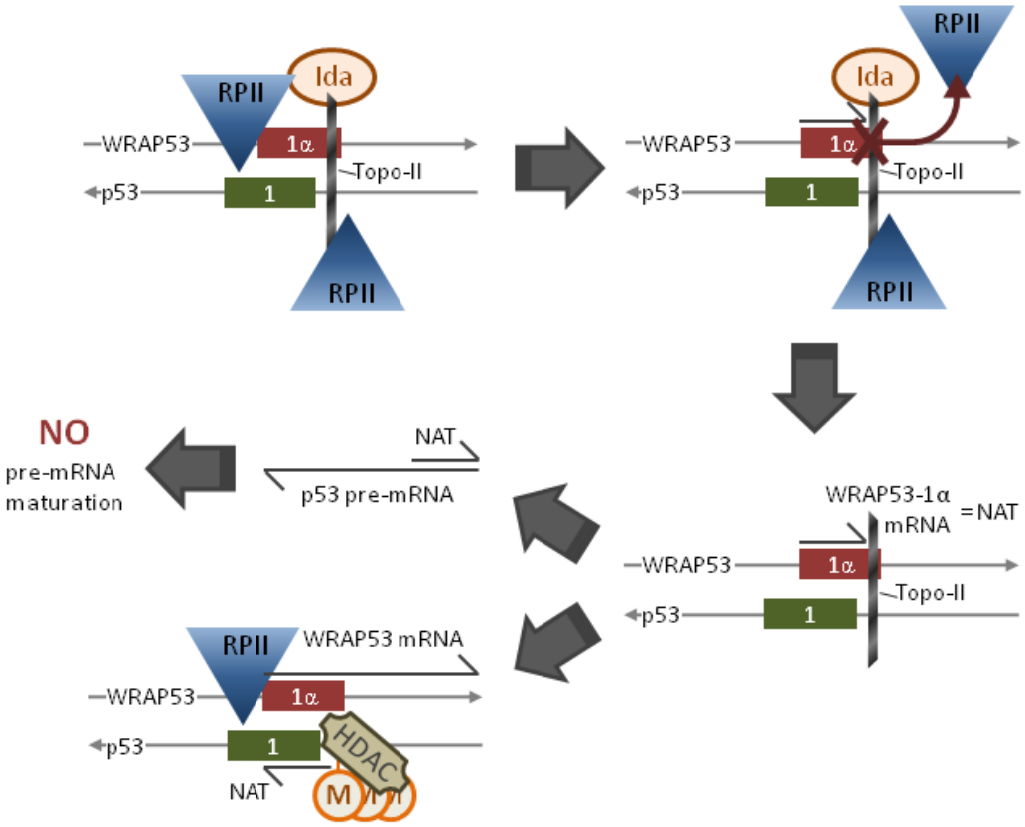


Figure 26: Model of the expression regulation within the *TP53* genomic locus.
Topoisomerase II (Topo-II) is stalled on the DNA through idarubicin (Ida). Thereby it prevents RNA polymerase II (RP-II) from binding to the p53 promoter, as well as elongation of WRAP53 transcription due to collision. The short WRAP53-1α mRNA acts as natural antisense transcript (NAT) on p53. This NAT could either bind to p53 pre-mRNA, preventing its further maturation, or NAT-DNA interactions could feature promoter methylation and heterochromatin formation, leading to the silencing of p53.

To experimentally elucidate the underlying mechanisms it would be important to first identify the WRAP53-1α transcript that is accumulating in response to treatment with these chemotherapeutic drugs and its cellular localization. Subcellular fractionation prior to RNA isolation would help to get insights into the localization of idrubicin induced WRAP53-1α transcripts. Additionally, RACE-PCR could be used to identify a potential truncated WRAP53-1α transcript. To investigate whether topoisomerases, inhibited through idarubicin or etoposide, get stalled within the *TP53* genomic locus at positions different from those where daunorubicin, doxorubicin, and epirubicin inhibited topoisomerases get stalled, ChIP technology could be used (Cashman and Kellogg, 2004). Furthermore, it could be tested whether HDAC inhibition would lead to similar impacts of all five topoisomerase II inhibitors on the p53 transcription and thereby rescue the defect in pre-mRNA maturation upon treatment with idarubicin or etoposide.

5.3. KINASES REGULATING MUTANT P53 EXPRESSION IN TUMOR CELLS

We found that the three kinases RPS6KL1, RPS6KB2, and RPSKB1 contribute to the expression regulation of mutant p53 in tumor cells (Figure 21, Figure 22, and Figure 23). We identified these kinases in a microscopy based siRNA screen and confirmed their roles in the performed follow-up experiments. The knock-down of the named kinases decreased the expression levels of mutant p53 in tumor cells that otherwise express the protein, without further stimuli, at high levels. Our data additionally suggest that the kinase activities of RPS6KB1 and -B2 are necessary for their impact on p53, rather than their simple abundance (Figure 25). For RPS6KL1 this can currently not be analyzed, since it is neither known, whether this protein exhibits intrinsic kinase activity, nor ways to alter it.

According to the data from Cho *et al.* (2005), some kinases of the RPS6K family directly phosphorylate p53 at Serine 15. Additionally, Melnikova *et al.* (2003) observed mutant p53 to be constitutively phosphorylated at Ser15 in

UV-induced skin tumor cells and correlated this with decreased susceptibility to Mdm2-mediated degradation. Taking these data together, it could be hypothesized that the knock-down or inactivation of the S6 kinases decreases the levels of mutant p53 phosphorylation, which in turn leads to the destabilization of the protein. Nevertheless, therefore the overall levels of posttranslationally modified mutant p53 would need to be high, even in the absence of DNA damage, a prerequisite that we did not observe in our studies (Figure 7A, B). Hence, we hypothesize that the mechanism, underlying this regulation of transformation induced accumulation of mutant p53, does not depend on posttranslational modifications and their impact on the susceptibility of p53 towards Mdm2, or other ubiquitin ligases. To further exclude this as a possible mechanism, we would like to investigate, whether ectopically over-expressed mutant p53 is regulated by the identified kinases. And, whether an additional mutation of known phosphorylation sites within p53 (Serine or Threonine to Alanine) would abolish this effect.

The presented results could also be explained with the help of reports published by Fu and Benchimol (1997) and Takagi *et al.* (2005), finding that p53 expression is regulated on the translational level in response to DNA damage. In the work of Fu and Benchimol (1997), the 3'UTR of p53 itself was identified to posses inhibitory activity on p53 translation. They further showed that γ-irradiation abolishes this translational inhibition. Whereas, Takagi *et al.* (2005) claims that irradiation leads to an increased binding of RPL26 to the 5'UTR of p53, which in turn promotes p53 mRNA association with heavy polysomes, augmenting the rate of its translation. Even though these data were obtained in wild type p53 expressing cells, in response to irradiation, we aimed to test whether RPL26 contributes to the regulation of mutant p53 expression in the absence of DNA damage. But, upon siRNA mediated knock-down of RPL26 in U251 cells neither the posttranslational modification of p53, nor its overall expression levels were affected (data not shown). This argues against

translational regulation of mutant p53 expression mediated by RPL26. Nevertheless, to determine the impact of translational regulation on mutant p53 accumulation mediated through the identified S6 kinases, it should be tested whether the amount of p53 mRNA associated with heavy polysomes changes upon the knock-down of the kinases.

The mTOR pathway was shown to contain sensors for nutrient and amino acid availability (Kim, 2009). Cells that lack essential amino acids often use autophagy to degrade cellular proteins, thereby increasing the pool of amino acids that can be used to translate new proteins of greater importance for their survival (Jung *et al.*, 2010). We would like to test, whether the highly accumulated mutant p53 protein might get degraded through autophagy upon RPS6K knock-down or starvation, since it was previously shown that Arginine deprivation, which was used in our experiments to inhibit mTOR signaling, induces autophagy (Savaraj *et al.*, 2010). Along that line, there are several ways to induce or block autophagy independently of mTOR that could be used to analyze the impact of this degradative pathway within the regulatory network of mutant p53 expression. According to Munafo and Colombo (2001), autophagy is efficiently blocked through treatment with 3-methyladenine or N-ethylmaleimide (NEM), while the incubation with the microtubule depolymerizing agent vinblastine could be used to accumulate autophagic vacuoles, by preventing their degradation. Furthermore, the knock-down of Belcin1, as a central regulator of autophagy could be used, to more specifically analyze the impact of this pathway on mutant p53 expression regulation (Liang *et al.*, 1999). Microscopic analysis of mutant p53 expression upon modulation of autophagy could be used to test, whether this pathway is involved in mutant p53 expression regulation in tumor cells. If this hypothesis turns out to be true, we will not have identified one of the tumor specific pathways that lead to the accumulation of mutant p53 at the first place. Nevertheless, this finding could help to decrease the expression levels of the oncogenic mutant p53 protein in

cancer cells and it should be tested, whether the induction of autophagy could be used to prevent tumor progression and metastasis formation.

5.3.1. METASTASIS FORMATION AS MUTANT P53 GAIN OF FUNCTION

It can be hypothesized that the activity of the mTOR pathway kinases is decreased in areas of the tumor that lack sufficient nutrient supply and elevated in the outer cells. According to the data we have obtained, this would result in high levels of accumulated mutant p53 in cells at the outer rim of the tumor cell mass and in close proximity to blood vessels. The fact that exactly these cells are the ones that detach from a primary tumor to form new metastases, pinpoints to the importance of finding ways to actively suppress the oncogenic gain of function of accumulated mutant p53. Rapamycin (Rapamune®), a small molecule that was originally isolated from *Streptomyces hygroscopicus* is FDA approved as immunosuppressant. This molecule, which can efficiently inhibit the mTOR pathway, is recently more and more described to have a tumor protective function (Guertin and Sabatini, 2005; Sudarsanam and Johnson, 2010). In many tumors the prognosis, especially in terms of tumor proliferation and metastasis formation, seems to be correlated with the activity of the mTOR pathway (Zhou and Huang, 2010). We believe that the expression of mutant p53 could be a mechanistic explanation for this observed correlation. To investigate the *in vivo* role of mutant p53 accumulation, sections of larger tumors, harboring a p53 point mutation, could be histochemically analyzed, in order to test whether the expression levels of mutant p53 are indeed higher at the outer rim of the tumor cell mass and whether they decrease in response to treatment with rapamycin.

5.3.2. CHEMORESISTANCE AS MUTANT P53 GAIN OF FUNCTION

One aspect that should be tested as a link between mutant p53 gain of function and the use of anthracyclines for chemotherapeutic treatment is the expression

of the multi-drug resistance gene 1 (MDR1). It was on the one hand shown by several groups that in malignancies, expressing high levels of mutant p53 protein, chemoresistance is often conferred through transcriptional activation of MDR1 (Blandino *et al.*, 1999; Brosh and Rotter, 2009; Bush and Li, 2002). It was on the other hand clinically observed that doxorubicin treatment often leads to multi-drug resistance, going along with increased MDR1 levels, as a side effect. Thereby it was further observed that idarubicin, despite its structural homology to the other anthracyclines is the only representative of its kind that lacks this side effect (Hargrave *et al.*, 1995; Lotfi *et al.*, 2002). These observations do not only reflect another example of different phenotypes conferred by the structurally almost identical anthracyclines doxorubicin and idarubicin. Rather, correlated with our data, it can be hypothesized that the clinically observed chemoresistance upon doxorubicin treatment is mediated by an accumulation of mutant p53 in the cells, conferred by increased expression of MDR1. Whereas idarubicin lacks the ability to induce this accumulation and thereby does not exhibit MDR1 over-expression and the observed side effect of chemoresistance.

5.4. WHICH CONSEQUENCES CAN BE DRAWN FROM THESE FINDINGS

It should be the aim of mutant p53 research to elucidate the mechanisms of its accumulation and to get more insights into the cellular concepts underlying the oncogenic gain of function. Within this study we obtained data indicating that the expression of mutant p53 is regulated on different levels depending on the stimuli that cause its accumulation. Bearing in mind the disadvantageous side effects of mutant p53 accumulation that were published by a number of groups (Barlev *et al.*, 2001; Di Agostino *et al.*, 2006; Lang *et al.*, 2004; Muller *et al.*, 2009; Strano *et al.*, 2007) it should be as well aimed to prevent this accumulation, or at least to decrease the expression levels.

We observed that the accumulation of mutant p53 is increased upon topoisomerase II inhibitor treatment. We further demonstrated that the

transcription of p53 is activated in response to this treatment, which is inversely regulated to the expression of the natural antisense transcript WRAP53. Therapeutically it should be considered to use idarubicin or etoposide more widely in the tumors expressing mutant p53. It could also be tested whether the transcription of WRAP53-1α can exogenously be stimulated to prevent the accumulation of mutant p53 in response to one of the other topoisomerase II inhibiting drugs, possibly through a combinational treatment with idarubicin or etoposide.

Regarding the accumulation of mutant p53 that occurs during cellular transformation, we found that inhibition of ribosomal S6 kinase activity decreases the expression of mutant p53 in the used cell lines. It was furthermore recently shown that mTOR plays a critical role in the regulation of tumor cell motility and cancer metastasis. It would now be important to analyze whether the mTOR activity in tumor cells promotes tumor progression through the oncogenic activities gained by p53 through its point mutation. Therapeutically, it should then be tested whether the metastatic gain of function of mutant p53 can be abolished by treatment with rapamycin.

SUMMARY

The tumor suppressor p53 in its mutant form was previously shown to massively accumulate in tumor cells. Furthermore, enhanced tumor progression, as well as chemoresistance were associated with its expression. Within this study, we observed that chemotherapeutic treatment with some, but not all topoisomerase II inhibitors, currently used in the clinics, leads to a further up-regulation of mutant p53 expression and thus might favor unwanted tumor progression of tumor cells that escape the apoptosis induction at the first place. The network to regulate the expression of mutant p53 includes different mechanisms in response to various stimuli. The mediators range from transcription factors, over non-coding RNAs, to kinases.

All topoisomerase II inhibitors that we tested within our study augmented mutant p53 transcription. We showed that this was mediated by several transcription factors, including E2F1 and its target gene TAp73, that itself is known to exhibit activities similar to wt p53. While it was previously shown that TAp73 binds to a responsive element with the p53 promoter we observed here for the first time that E2F1 also binds directly to the p53 promoter in close proximity to the transcriptional start site. This was first found using *in silico* methods and confirmed by chromatin immunoprecipitations.

Nowadays, non-coding RNAs are recognized as another level of gene expression regulation. Recently, it was identified that within the *TP53* genomic locus, a natural antisense transcript is encoded, partially overlapping with exon 1 of the p53 mRNA. We observed that idarubicin and etoposide, but none of the other topoisomerase II inhibitors, strongly induced the expression of this antisense transcript, WRAP53. Furthermore, it became evident that this

expression is inversely correlated with proper pre-mRNA maturation of p53. Therefore, we hypothesize that the expression of this natural antisense transcript efficiently inhibits p53 mRNA maturation, possibly through RNA masking. We further hypothesize that the inversely correlated expression of sense and antisense transcripts might be caused by the collision of RNA polymerase II with idarubicin- or etoposide-inhibited, stalled topoisomerase II.

The accumulation of mutant p53, as it is observed during tumor progression, seems to be mostly regulated on the protein level, where we identified the ribosomal S6 kinases to be involved in. We found that the kinase activity of RPS6KB2 is necessary, to regulate the amounts of mutant p53 protein, as it was determined by serum and amino acid starvation. The mechanistic details that form the basis of this regulation were not determined, but we would like to suggest several hypotheses to be investigated. While our data can be explained by translational defects that the knock-down or inhibition of RPS6KB2 might cause, we favor the model that the induction of autophagy in response to mTOR pathway deregulation causes an enhanced degradation of mutant p53. A role of direct phosphorylation of mutant p53 through RPS6KB2 can also not be excluded.

In conclusion, we found that tumor cells accumulate mutant p53 protein through the activity of kinases that transduce mTOR signaling. Surprisingly, some chemotherapeutics further enhance mutant p53 levels through an entirely different mechanism, i.e. the regulation of p53 sense and antisense transcription.

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CURRICULUM VITAE

PERSONAL INFORMATION

	Monika Bug
Adress	Am Hasengraben 5 37075 Göttingen Germany
Email	mbug@gwdg.de
Date of Birth	September, 12th 1982
Nationality	German

EDUCATION

	Georg August University Göttingen, Germany
2006 - 2010	PhD in the Department of Molecular Oncology Thesis: “The accumulation of mutant p53 in human cancer cells”
	Georg August University Göttingen, Germany
2005 - 2006	Molecular Biology (MSc/PhD program – Max-Planck International Research School)
	University of Lübeck, Germany
2002 - 2005	Molecular Life Science (BSc) Thesis in the Department of Biology: “Characterization of the cytosolic protein p97 binding to the membrane of rough endoplasmic reticulum during retrograde transport”

TEACHING

	Tutor of Lectures
2007 - 2010	“Apoptosis and Cancer” within the Max-Planck International Research School MSc/PhD Program “Molecular Biology”

Supervisor of Labrotations

2008 - 2009 Golnaz Aghazadeh: Master-student in “Molecular Medicine”, University of Göttingen
Anke Hellrung: Master-student in “Biomedizin”, University of Würzburg

Tutor of Advanced Methods Courses

2008 - 2009 “Assessing promoter activity by luciferase assays” within the Max-Planck International Research School MSc/PhD Program “Molecular Biology”

HONORS AND AWARDS

2005 - 2006 Stipend Max-Planck International Research School

2004 Student exchange scholarship at the University of New Mexico

2003 - 2006 Stiftung der deutschen Wirtschaft (Foundation of German Economy)

CONFERENCES

2009 First AACR International Conference on Frontiers in Basic Cancer Research, Boston, MA, USA (poster)

2007 PhD Symposium “Horizons in Molecular Biology” Göttingen, Germany (poster)

EXTRACURRICULAR ACTIVITIES

Student representative

2007 - 2009 Max-Planck International Research School MSc/PhD Program “Molecular Biology”, Georg August University Göttingen, Germany

PhD representative

2007 - 2009 Göttingen Center for Molecular Biosciences (GZMB), Georg August University Göttingen, Germany

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IN HUMAN CANCER CELLS

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Monika Bug

born in

Hanau

SEPTEMBER 2010

Affidavit

I hereby declare that this doctoral thesis has been written only by the undersigned and without any assistance from third parties.

Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Göttingen, 30.09.2010

Monika Bug

LIST OF PUBLICATIONS

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Bug M. and Dobbelstein M. **Anthracyclines induce the accumulation of mutant p53 through E2F1-dependent and -independent mechanisms.** (Submitted)

TABLE OF CONTENTS

ABSTRACT

The tumor suppressor p53 is mutated in more than 50% of all human solid tumors. This comprises mostly single residue missense point mutations that entail a loss of p53 tumor suppressor function. But at the same time, mutant p53 protein was shown to possess oncogenic activities, i.e. a gain of function, promoting invasion and chemoresistance. Mutant p53 specifically accumulates in advanced tumors, but not in normal tissues, engineered to contain a mutant p53 gene. This means that tumor specific changes evoke the accumulation of mutant p53 during tumor progression. Within this study we observed that mutant p53 accumulates even further, when tumor cells are exposed to some, but not all chemotherapeutic drugs. While the anthracyclines doxorubicin, daunorubicin and epirubicin led to the accumulation of mutant p53, the highly similar compound idarubicin did not. We found the expression of mutant p53 to be regulated at different levels: First, treatment with the topoisomerase II inhibitors daunorubicin, doxorubicin, epirubicin, idarubicin, and etoposide, evokes a DNA damage response that results in the activation of E2F1 and its target gene TAp73. Our data suggest that, upon these genotoxic treatments, E2F1 contributes to the transcriptional activation of mutant p53 pre-mRNA synthesis, both directly and through induction of TAp73. We further show for the first time that the transcription factor E2F1 associates with the promoter DNA of *TP53.* Second, among these chemotherapeutics that induce p53 transcription, we found two members to additionally induce a natural antisense transcript to p53, WRAP53. We further observed that the induction of WRAP53 coincides with impaired p53 mRNA maturation. We therefore hypothesize that the expressed antisense transcript interferes with p53 pre-mRNA stability or its nuclear export. Third, the accumulation that is inflicted on the cells during

carcinogenesis seems to be mostly regulated on the protein level. We performed a high-content siRNA screen, using single-cell based microscopy analysis, and thereby identified the ribosomal S6 kinases to be involved in mutant p53 expression regulation in advanced cancer cells. We believe that our findings should be considered for chemotherapy prescription, since we have shown that some topoisomerase II inhibitors augment mutant p53 expression and thus might favor unwanted tumor progression.

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ABBREVIATIONS

ABBREVIATION	FULL NAME
5'FU	5-fluorouracil
AA/ BAA	Acrylamide/ bisacrylamide
APS	Ammonium persulfate
AraC	Cyctosine arabinose
AraCTP	Cytidine triphosphate arabinose
ARF-BP1	ARF-binding protein 1
ATM	Ataxia-telengiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia-telengiectasia mutated and Rad3-related
Bax	Bcl2-associated X protein
BCLAF	B-cell lymphoma associated factor
BS	Binding site
BSA	Bovine serum albumin
°C	Degree Celsius
CBP	cAMP response element-binding protein binding protein
Cdc	Cell cycle division
Cdk	Cyclin dependent kinase
cDNA	complementary DNA
ChIP	Chromatin immunoprecipitation
Chk	Checkpoint kinase
CK1	Casein kinase 1
COP1	Constitutive photomorphogenesis protein 1
CPT	Camptothecin
Da	Dalton
Dauno	Daunorubicin
dCTP	Deoxycytidine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxynucleoside triphosphate
Dox	Doxorubicin
dTMP	Deoxythimidine monophosphate
dUMP	Deoxyuridine monophosphate
DTT	1,4-Dithiothreitol
DYRK	Dual specificity tyrosine-phosphorylation-regulated kinase
ECL	Enhanced chemoluminescence
Epi	Epirubicin
et al.	et altera
Etop	Etoposide
FCS	Fetal calf serum
g	Gram; Gravitational acceleration
GC rich	Guanine and Cytosine rich
h	Hour
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIPK2	Homeodomain-interacting protein kinase 2
Ida	Idarubicin
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
l	Liter
M	Molar
m	meter
Mdm	Mouse double-minute
min	Minute
MK2	Mitogen-activated protein kinase-activated protein kinase
M-MuLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
µ	Micro
NHEJ	Non-homologous end joining
NP-40	Nonidet P40
P-	Phospho-

PAGE	Polyacrylamide gel elektrophoresis
PBS	Phosphate buffered saline
PCAF	p300/ CBP associated factor
PCR	polymerase chain reaction
PDK	Phosphoinositide-dependent protein kinase
pH	Negative logarithm (base 10) of the molar concentration of dissolved protons
Pirh2	p53-induced protein, RING-H2 domain-containing
PUMA	p53-upregulated modulator of apoptosis
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNA Pol II	RNA polymerase II
RNAse	Ribonuclease
RPL27	Ribocomal protein L26
RPMI	Roswell Park Memorial Institute medium
RPS6K	Ribosomal protein S6 kinase
RREB	Ras-responsive element binding protein
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
sec, s	Second
SDS	Sodium dodecyl sulfate
siRNA	small interfering ribonucleic acid
Taq	*Thermus aquaticus*
TEMED	N,N,N´,N´- Tetramethylethylenediamine
TIP60	TAT-interacting protein, 60-kDa
Tris	tris(hydroxymethyl)aminomethane
TSS	Transcriptional start site
U	Unit of enzyme activity
UV	Ultra violet
V	Volt
w/v	weight per volume
WB	Westernblot = immunoblot
WRAP53	WD repeat-containing antisense to p53
wt	wild type

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Most cells of our body have the potential to divide, an essential process in the renewal of tissues and the scope of our immune system. Some cells proliferate rapidly, others rarely, but proliferation happens in a controlled fashion, with a large set of check points and back-up mechanisms (Pagano and Draetta, 1991). Due to exogenous stimuli like irradiation, exposure to toxins or other forms of stress, key players of this regulation cascade can be mutated and become dysfunctional. If the affected cell thereby gains a certain growth advantage, but is not recognized by the immune system and eliminated, it starts to proliferate in an uncontrolled fashion. Over time, more and more mutations accumulate in these rapidly dividing cells and, depending on the genes that were hit by mutations; this can lead to the development of malignancies.

When a tumor is diagnosed, it is often already in an advanced stage and needs to be treated by chemotherapy. Nowadays a number of chemotherapeutic agents are available; their mechanisms of action are diverse and often not completely understood yet. Depending on the cell type and the mutational spectrum, tumors are treated with different chemotherapeutic agents. Nevertheless, the mechanistic details and the cause of some side effects are widely unknown and a matter of current research.

2.1. CHEMOTHERAPEUTICS

The idea behind most chemotherapeutic drugs is that they trigger a DNA damage response in proliferating cells and subsequently lead to apoptosis (Johnstone *et al.*, 2002). Since tumor cells are normally proliferating faster than most other cells of our body, they are preferentially targeted. Nevertheless,

hematopoetic cells, gastrointestinal mucosal cells and hair are examples of rapidly dividing cells that often get affected by these drugs although their fast proliferation happens in a controlled fashion and is important for their proper function (Tannock, 1986).

Generally, chemotherapeutic drugs can be clustered in three groups according to their mechanism of action: nucleoside analogs, inhibitors of enzymes involved in replication and transcription, and drugs that directly damage the DNA (Pommier and Diasio, 2006).

2.1.1. NUCLEOSIDE ANALOGS

This group of drugs is also called 'antimetabolites' and either inhibits the formation of functional nucleotide triphosphates, or interferes with replication elongation (Daher *et al.*, 1990). All agents that belong to this class prevent efficient DNA synthesis and mostly affect the cells in S phase of the cell cycle. Examples are on the one hand 5-fluorouracil (5'FU), which inhibits the conversion of dUMP to dTMP and causes due to depletion of dTMP defects in DNA synthesis and cell division (Daher *et al.*, 1990). On the other hand, agents like Cytosine arabinose (AraC) affect replication elongation; AraC is recognized by DNA polymerase α as deoxycytosine, but the incorporation of AraCTP in the elongating DNA strand fails due to sterical hindrance resulting in the termination of DNA replication (Chrencik *et al.*, 2003).

2.1.2. ENZYME INHIBITORS

Enzymes with specific functions during replication are the polymerases, topoisomerases and helicases; these also reassemble the most common drug targets of this class. Polymerases are for example targeted by aphidicolin and foscarnet that block dCTP incorporation or pyrophosphate cleavage, respectively (Crumpacker, 1992; Sheaff *et al.*, 1991).

Topoisomerases are enzymes that relax supercoiled DNA by cleavage and religation (D'Arpa and Liu, 1989). The chemotherapeutic drugs camptothecin, etoposide and the anthracyclines daunorubicin, doxorubicin, epirubicin and idarubicin are well known representatives of this class. While camptothecin specifically acts on topoisomerase I, an enzyme that functions through single strand cleavage, and traps the cleavage intermediates (Pommier *et al.*, 2003), the other mentioned drugs are mainly known to act on topoisomerase II (Fortune and Osheroff, 2000). Even though all of these agents in the end lead to double strand breaks that trigger a DNA damage response and induce apoptosis. The topoisomerase II inhibitors additionally can interfere with other metabolic processes of the DNA, like transcription, DNA repair, and chromatin remodeling (Fortune and Osheroff, 2000). In contrast to camptothecin these drugs trap the cleavage intermediate, with the two enzyme subunits of 170 and 180 kDa covalently linked to the DNA. Thereby large protein-DNA adducts are produced that form steric blocks on the template DNA (Fortune and Osheroff, 2000).

The planar structure of anthracyclines additionally allows them to intercalate into DNA, preferentially in GC rich regions. This was shown to stabilize the duplex DNA and to prevent helicases from separating the strands (Bachur *et al.*, 1992).

Inhibitors that act independently of these enzymes, directly involved in replication, but still inhibit cell cycle progression interfere with cyclin-dependent kinases (Cdk) or the checkpoints. The Cdk inhibitors flavopiridol and roscovitine are competitive inhibitors of ATP binding and interfere at various steps in the cell cycle: G1/S transition (restriction point) through Cdk4/6, the activation of replication origins (S-phase) through Cdk2, and the inactivation of these replication origins by Cdk1-cyclin B complexes (De Falco and De Luca, 2010). Additionally, it was shown that these Cdk inhibitors inhibit RNA polymerase II and thereby transcription (Wesierska-Gadek and Krystof, 2009). Nevertheless,

currently most of these kinase inhibitors lack specificity. This also holds true for the checkpoint inhibitor 7-hydroxystaurosporine, a checkpoint kinase 1 (Chk1) inhibitor that was found to additionally inhibit Chk2 and phosphoinositide-dependent protein kinase 1 (PDK1) (Sato *et al.*, 2002; Yu *et al.*, 2002), as well as caffeine, the first drug identified to abrogate a cell cycle checkpoint by inhibiting Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and RAD3-related (ATR), but also a number of additional kinases (Sabisz and Skladanowski, 2008; Sarkaria *et al.*, 1999).

2.1.3. DNA DAMAGING DRUGS

Additionally to radiotherapy there are chemotherapeutic drugs that block the replication fork by inducing DNA template lesions, like DNA adducts, DNA strand breaks, and DNA protein crosslinks. The alkylating agents modify bases within the DNA, either through methylation of Guanine (methylmethanesulfonate), DNA-DNA crosslinks and DNA-protein crosslinks (cisplatin), or interstrand DNA crosslinks (cyclophosphamide) (DeNeve *et al.*, 1990; Hausheer *et al.*, 1989; Mirzayans *et al.*, 1988). In contrast to that, radiomimetic DNA cleaving agents like bleomycin and neocarcinostatin induce single- as well as double-strand breaks of the DNA (Goldberg, 1987; Huang *et al.*, 1981). As for most of the mentioned drugs, the induction of such DNA lesions triggers a DNA damage response, which signals from ATM or ATR down to the effectors p53, E2F1, cell division cycle 25 (CDC25) and others (Darzynkiewicz *et al.*, 2009).

2.2. THE DNA DAMAGE RESPONSE

Originally two different DNA damage pathways were identified. Their activation was observed depending on the kind of damage that was inflicted on the DNA. In response to double strand breaks ATM is recruited to the sites of DNA damage and gets activated, the signal is transduced by Chk2 which in turn

leads to the accumulation and activation of p53, E2F1 and other effectors (Lavin and Khanna, 1999). In contrast to that, ATR is activated by single strand breaks, the signal transduced by Chk1 and finally effectors like p53, Cdc25 and others get activated (Paulsen and Cimprich, 2007). Nowadays, there is a lot more crosstalk between the two pathways known and additional kinases at the levels of ATM, ATR as well as Chk1 and Chk2 were identified.

2.2.1. KINASES IN THE DNA DAMAGE RESPONSE

Not only the role of DNA-dependent protein kinase (DNA-PK), sensing DNA double strand breaks and lesions of non homologous end joining (NHEJ), amends the network of kinases activated in response to DNA damage (Danska and Guidos, 1997; Rathmell *et al.*, 1997), also p38 and its activation of mitogen-activated protein kinase-activated protein kinase 2 (MK2), as well as the recently identified cross talks between the pathways (Reinhardt *et al.*, 2007). The impact of phosphorylations involved in this network is immense and our knowledge about these is most probably far from being complete. The current view on central players within this network and their most prominent targets is summarized in Figure 1.

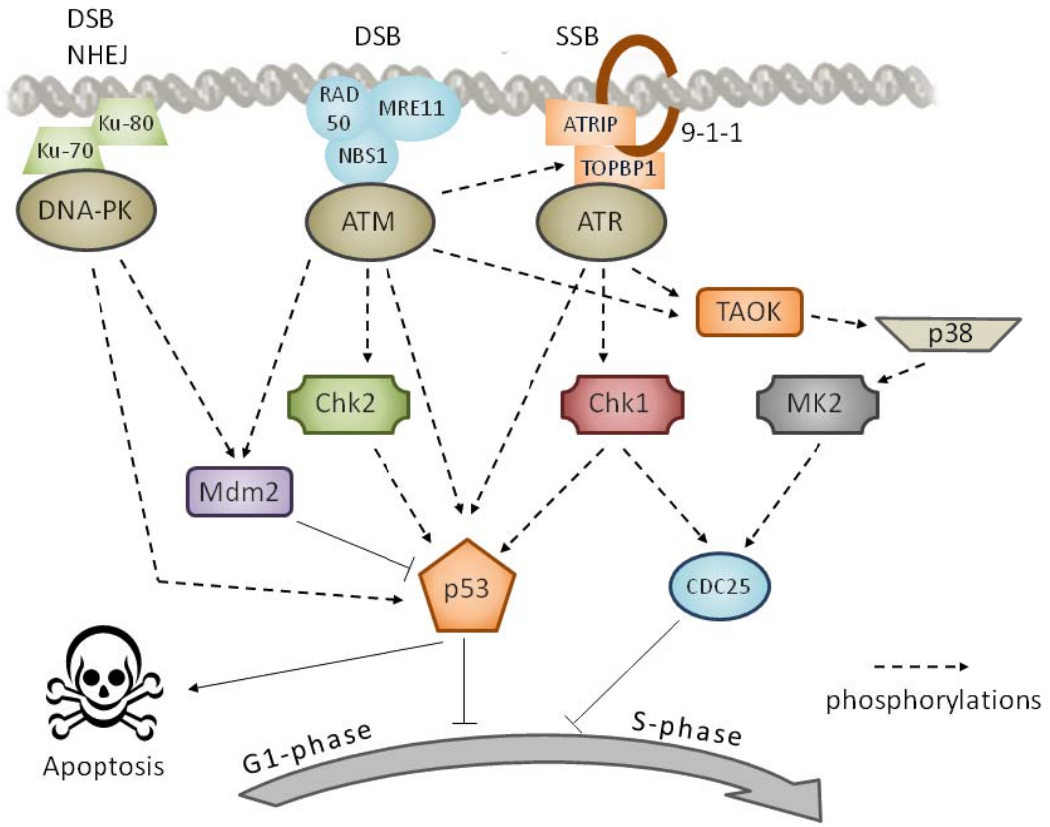


Figure 1: The role of kinases in the DNA damage response pathways.
Due to different stimuli sensor proteins get recruited to the site of DNA damage and trigger a DNA damage response. Double strand breaks (DSB) or lesions of non-homologous end joining (NHEJ) are recognized by the heterodimer Ku70/ 80 and lead to the recruitment and activation of DNA-dependent protein kinase (DNA-PK). This in turn activates p53, on the one hand through direct phosphorylation and on the other hand through phosphorylation of Mouse double-minute 2 (Mdm2). Additionally DSB can be sensed by the MRN complex (Meiotic recombination 11 (MRE11), Rad 50, and Nijmegen breakage syndrome 1 (NBS1)) that activates the transducer kinase Ataxia-telangiectasia mutated (ATM). ATM has a number of different phosphorylation targets that in turn all lead to the activation of p53: Mdm2, checkpoint kinase 2 (Chk2), p53 itself, TAO kinase (TAOK), and also DNA topoisomerase II-binding protein 1 (TOPBP1). The latter itself is part of the DNA single strand break (SSB) sensor complex and involved in the activation of Ataxia-telangiectasia and RAD3-related (ATR). Besides TOPBP1 also the Rad9-Hus1-Rad1 clamp complex (9-1-1) and ATR-interacting protein (ATRIP) contribute to the sensing of DNA single strand breaks and the activation of ATR. Similar to ATM, also ATR has a number of different phosphorylation targets that activate p53 and mark the cell cycle regulatory protein cell division cycle 25 (CDC25) for degradation: p53 itself, checkpoint kinase 1 (Chk1) and TAOK. The latter was found to phosphorylate and activate p38, which in turn phosphorylates and activates mitogen-activated protein kinase-activated protein kinase 2 (MK2), a kinases that was shown to directly phosphorylate CDC25A, leading to its destabilization.

2.2.2. E2F ACTIVITIES IN RESPONSE TO DNA DAMAGE

Since E2F1 was identified in 1987 by Kovesdi *et al.* the number of known E2F family members increased and currently comprises eight genes (E2F1 to 8), which give rise to nine distinct proteins (DeGregori and Johnson, 2006). The transcription factors can be categorized into three groups: E2F1 – 3A are mostly found as activating transcription factors that can get inactivated through their binding to the retinoblastoma protein (Rb). E2F4 and -5 are frequently detected in their inactive state, bound to one of the three pocket proteins (Rb, p107, or p130), but are generally categorized as weak activators. Finally, E2F6 – 8 are classified as transcriptional repressors, which do not interact with any of the pocket proteins (Trimarchi and Lees, 2002). The E2F proteins transactivate several Cdks, as well as cyclins and thereby contribute positively to cell cycle progression and cell proliferation.

Additionally to its cell cycle related functions, E2F1 was found to be an activator of the DNA damage response pathway. It was shown that over-expression of E2F1 leads to increased Chk2 mRNA, as well as protein levels (Rogoff *et al.*, 2004). Over and above, Stevens *et al.* (2003) reported that Chk2 phosphorylates E2F1 and thereby alters the DNA binding specificity of E2F1 from S-phase genes to the pro-apoptotic gene p73. These findings underscore the controversial activities that were implied to E2F1 in the literature. The transcription factor was originally identified as an oncogene, whose hyperactivation leads to uncontrolled cell proliferation, and was later on found to actively suppress tumorigenesis by inducing pro-apoptotic genes in response to DNA damage.

2.2.3. P53 IN THE DNA DAMAGE CASCADE

The tumor suppressor p53, as well as the two E3 ubiquitin ligases Mdm2 and Mdm4 are common phosphorylation targets of Chk1, Chk2, but also the

upstream components of the DNA damage signaling pathways ATM, ATR and DNA-PK (Figure 2) (Meek, 2009).

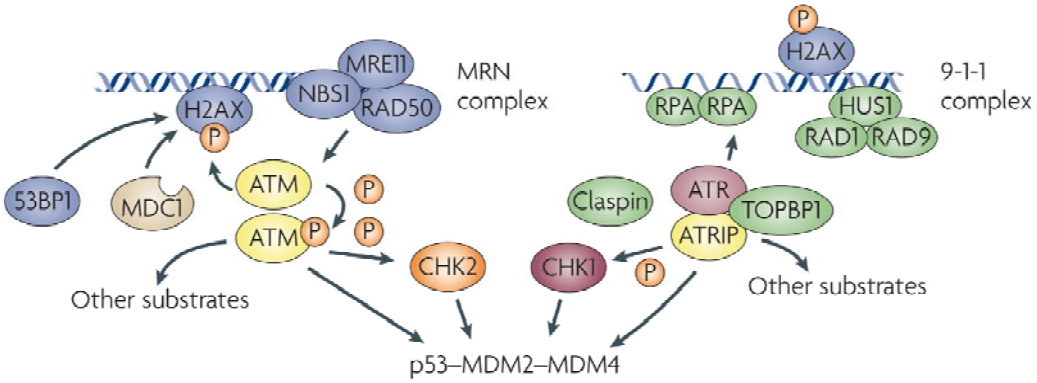


Figure 2: All DNA damage pathways converge at the point of p53 phosphorylation [taken from Meek (2009)].
The activation of Ataxia-telangiectasia mutated (ATM) and checkpoint kinase 2 (Chk2) in response to double strand breaks, sensed by proteins of the MRN complex (Meiotic recombination 11 (MRE11), Rad 50, and Nijmegen breakage syndrome 1 (NBS1)) results in the phosphorylation of p53 and its two antagonists Mouse double-minute 2 (Mdm2) and Mouse double-minute 4 (Mdm4). The same is achieved in response to single strand breaks, which are sensed by the 9-1-1 complex (RAD 9, RAD 1, and HUS 1) and transduced through the activation of the Ataxia-telangiectasia and RAD3-related (ATR) and checkpoint kinase 1 (Chk1) kinases.
H2AX: Histone variant; MDC1: Mediator of DNA damage checkpoint protein 1; 53BP1: p53-binding protein 1; RPA: Replication protein A; TOPBP1: DNA topoisomerase II-binding protein 1; ATRIP: ATR-interacting protein.

While p53 is activated and stabilized through these phosphorylations (Canman *et al.*, 1998), it was shown that its antagonists Mdm2 and Mdm4 get destructed (Maya *et al.*, 2001). Following from the above, p53 is stabilized in two ways in response to DNA damage, since Mdm2 in complex with Mdm4 is known to be the most prominent negative regulator of p53 (Meulmeester *et al.*, 2005; Montes de Oca Luna *et al.*, 1995). These ATM and ATR mediated phosphorylations trigger a cascade of additional posttranslational modifications of p53 that can tailor its response in an appropriate and proportionate manner according to the nature and intensity of the damage (Murray-Zmijewski *et al.*, 2008).

2.3. THE TUMOR SUPPRESSOR P53

Already in the early 90s the human p53 protein was identified to bind to the palindromic DNA sequence Pu-Pu-Pu-C-$^{A}/_{T}$-$^{T}/_{A}$-G-Py-Py-Py and its biological function as transcription factor was proposed (el-Deiry *et al.*, 1992; Kern *et al.*, 1991). The C-terminal oligomerization domain of the protein facilitates its tetramerization, which is essential for DNA binding as well as transcriptional activation of target genes (McLure and Lee, 1998). Nowadays, hundreds of genes regulated by p53 are known that can generally be classified upon their functions in cell cycle arrest, apoptosis, DNA repair, angiogenesis, and senescence (el-Deiry, 1998). The fine tuning of transcriptional activation through p53 mostly happens on the level of posttranslational modifications.

2.3.1. POSTTRANSLATIONAL MODIFICATIONS

The tumor suppressor p53 is known to be modified by all kinds of posttranslational modifications like phosphorylation, acetylation, methylation, neddylation, ubiquitination, and sumoylation. Most sites of modification as well as a number of modifying and demodifying enzymes are known so far (Olsson *et al.*, 2007). Nevertheless, the causes and consequences of the different modification patterns are not completely understood yet and a matter of current research.

PHOSPHORYLATION

Numerous Threonine and Serine residues, mainly within the transactivation domain of p53, have been identified as targets of phosphorylation by kinases like ATM, ATR, DNA-PK, Chk1, Chk2, CK1, JNK, HIPK2 and DYRK2 (Bode and Dong, 2004). These modifications often lead to the stabilization of the protein and hence to its activation in response to genotoxic and other forms of stress. Data from in vitro or over-expression studies indicate that phosphorylation at Serine 15 stimulates p53-dependent transactivation, growth arrest and apoptosis in response to DNA damage, whereas it is still under

debate whether phosphorylation of this site affects Mdm2 binding (Dumaz and Meek, 1999). Two groups established mouse models that express a mutant version of p53 where Serine 18 (corresponding to Serine 15 in humans) is replaced by Alanine and can therefore not be phosphorylated any longer. Thymocytes of these mice displayed a reduced induction of DNA damage mediated apoptosis, indicating that this phosphorylation in vivo contributes to the specific activation of target genes (Chao *et al.*, 2003; Sluss *et al.*, 2004).

ACETYLATION

The histone acetyltransferase (HAT) heterodimers CBP/p300 were found to acetylate p53 at Lysines 370, 372, 373, 381, and 382 (Gu and Roeder, 1997). In contrast, Lys320 and Lys305 in the nuclear localization domain of p53 are acetylated by PCAF and p300 respectively (Liu *et al.*, 1999). Some studies reported an enhancement of sequence-specific DNA-binding activity of acetylated p53, as well as more potent transcriptional activation of target genes (Barlev *et al.*, 2001; Sakaguchi *et al.*, 1998). Along that line, it was shown by two independent groups that acetylation of Lys120 of p53, by the MYST acetyltransferases MOF and TIP60, leads to the preferential induction of pro-apoptotic target genes such as PUMA and Bax, whereas the expression of other target genes like p21 and Mdm2 remains unaffected (Sykes *et al.*, 2006; Tang *et al.*, 2006). As the lysine residues within the C-terminal domain of p53 are also targets for ubiquitination, it was proposed that acetylation of these residues may promote the stabilization of p53 by interfering with proteasomal degradation (Brooks and Gu, 2003; Ito *et al.*, 2002).

To elucidate the impact of these acetylations in vivo, different mouse models were generated harboring up to 7 Lysine to Arginine mutations. Unfortunately these studies were not conclusive, since the phenotypes of these mice were very mild. The fact that various posttranslational modifications are conjugated to the same set of Lysines implies that the biological consequences, caused by

these, cancel each other out and therefore burrow the actual activities (Olsson *et al.*, 2007).

UBIQUITINATION

In contrast to the above it appeared to be very conclusive when mouse models were used to unravel the biological consequences of p53 ubiquitination. Montes de Oca Luna *et al.* (1995) generated a knock-out mouse line for Mdm2, the most prominent E3 ubiquitin ligase of p53. This resulted in embryonic lethality of the mice, a strong phenotype that was rescued by the additional knock-out of p53. These observations indicate that the lack of Mdm2-mediated p53 degradation leads to massive apoptosis and therefore to embryonic lethality of the mice. The E3 ubiquitin ligases COP-1, Pirh2, and ARF-BP1 were as well described to ubiquitinate p53 and to induce its proteasomal degradation (Chen *et al.*, 2005a; Dornan *et al.*, 2004; Leng *et al.*, 2003). Nevertheless, the above mentioned Mdm2 knock-out study suggests that in unstressed cells no additional E3 ubiquitin ligase is able to prevent the accumulation of p53 and its induction of apoptosis.

2.3.2. REGULATION OF P53 EXPRESSION

The expression levels of p53 are mainly regulated on the protein level. Mdm2, the above described essential p53 ubiquitin ligase, is itself one of the p53 target genes and thereby forms an autoregulatory feedback loop with the tumor suppressor (Freedman *et al.*, 1999). Mdm2 binds to the N-terminus of p53 and ubiquitinates it, either at C-terminal Lysines, or at Lysines within the DNA binding domain, this subsequently leads to the nuclear export or proteasomal degradation of the protein (Li *et al.*, 2003). Even though it seems to be an energetically unfavorable mechanism, the constant transcription, translation and proteasomal degradation of p53 allows the cell to rapidly react to various stress conditions, like DNA damage, oncogene activation, hypoxia and other inducers of the p53 network (Figure 3).

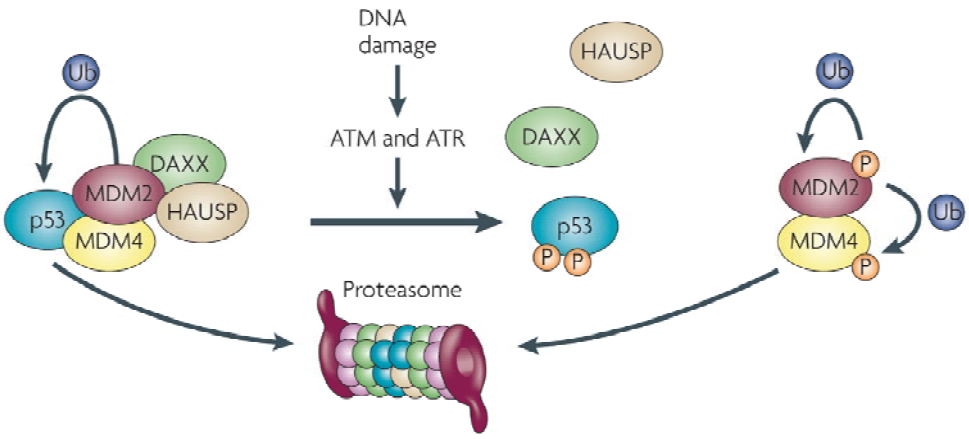


Figure 3: The fate of p53: between proteasomal degradation and DNA damage induced phosphorylation
[taken from Meek (2009)].
In unstressed cells the expression of p53 is kept at low levels. The E3 ubiquitin ligases Mouse double-minute 2 (Mdm2) and Mouse double-minute 4 (Mdm4), as well as the deubiquitinating enzyme Ubiquitin-specific protease (HAUSP) regulate its nuclear export and proteasomal degradation. In response to DNA damage, signaling cascades via Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and RAD3-related (ATR) lead to the phosphorylation of p53 and the E3 ligases Mdm2 and Mdm4. While p53 gets stabilized through these modifications, its antagonists get ubiquitinated (Ub) and subjected to proteasomal degradation.

Other kinds of p53 regulation received less attention. But, meanwhile it became evident that there are a few regulators that induce p53 expression through transcriptional activation. First, HOXA5 was found as a positive regulator of p53 transcription in response to DNA damage with the additional observation that its loss of mRNA expression in tumor samples is positively correlated with a loss of p53 mRNA expression (Raman *et al.*, 2000). Liu *et al.* (2007) reported that upon exposure to genotoxic stress, PKCdelta gets activated and interacts with the death-promoting transcription factor Btf (alias BCLAF) to co-occupy promoter elements within *TP53*. They furthermore reported that siRNA mediated knock-down of Btf suppresses p53-mediated apoptosis in response to DNA damage. Wang and el-Deiry (2006) found that p53 itself and, its structurally und functionally related family member, TAp73 are capable of regulating the expression of p53 on the mRNA level. They described three potential p53/ TAp73 responsive elements in the promoter region of p53, further identifying one of them to be essential using luciferase assays. Recently also

Ras-responsive element binding protein 1 (RREB1) was identified as potential transcriptional activator of p53 expression in response to DNA damage (Liu *et al.*, 2009).

In contrast to the forecited transcription factors, Mahmoudi *et al.* (2009) discovered an additional mechanism of p53 mRNA expression regulation. The natural antisense transcript to p53 (WRAP53) was found to mediate p53 mRNA stability in response to DNA damage. It was identified as a predicted gene within the *TP53* genomic locus on chromosome 17, encoded on the opposite strand of the tumor suppressor. The biological role of WRAP53 protein is completely unknown, whereas the specific over expression of certain transcripts was shown to increase p53 mRNA expression.

2.3.3. THE INTERPLAY OF P53/ P73 AND E2F1

In response to DNA damage p53 and E2F1 both get stabilized through phosphorylation by the same set of kinases: ATM, Chk1, and Chk2. The phosphorylation of E2F1 through Chk1 and Chk2 then leads to the induction of pro-apoptotic target genes like TAp73 (Stevens *et al.*, 2003). This is proposed to be a backup mechanism, when p53 is defective, since TAp73, a paralog of p53, is known to transactivate the same pro-apoptotic target genes as p53 (McKeon, 2004).

But, there is also direct cross-talk between the two transcription-factors p53 and E2F1 reported. On the one hand, deregulated E2F was found to directly transactivate the expression of $p14^{ARF}$, which inhibits Mdm2 and thereby leads to the stabilization and activation of p53 (Bates *et al.*, 1998). While in the absence of $p14^{ARF}$, E2F1 was found to stimulate p53 phosphorylation. Within the same study, it was claimed that this, most probably ATM or ATR dependent, posttranslational modification of p53 is crucial for E2F1-mediated apoptosis (Rogoff *et al.*, 2002).

2.3.4. MUTATIONS OF P53

In 1979 p53 was identified as a protein accumulated in the majority of the analyzed tumors and therefore characterized as tumor antigen (Crawford *et al.*, 1981; DeLeo *et al.*, 1979; Rotter *et al.*, 1980). Almost 10 years later Finlay *et al.* (1988) among others discovered that for all the work that was performed meanwhile a mutant p53 clone was used and that p53 in fact acts as a tumor suppressor. The mutations found in p53 do not reflect the classical spectrum known from other tumor suppressors, where frame shifts or large deletions mainly cause the loss of tumor suppressor activity. On the contrary, point mutations of single amino acids, as they are found in p53, are characteristic for oncogenes. Nevertheless, point mutations in oncogenes normally affect a small number of codons, encoding residues involved in their enzymatic activity, whereas the mutational spectrum of p53 ranges throughout the whole DNA binding domain of the protein, with a number of hotspot mutations that occur more frequently than others (Soussi and Lozano, 2005) (Figure 4).

A comprehensive list of published studies where p53 mutations have been analyzed by gene sequencing is provided on the IARC *TP53* database (http://www.iarc.fr/p53/). Evaluation of these data revealed that in about 70% of the reported studies the presence of a *TP53* mutation is significantly associated with bad prognosis, whereas only 5% of the studies reported a significantly good prognosis upon *TP53* mutation (Olivier *et al.*, 2005).

These observations indicate that cancer-associated mutant p53 isoforms are more than just relics of wt p53 inactivation and possess distinctive roles in tumor cells. Firstly, this can be achieved through dominant-negative effects over co-expressed wild type p53 proteins, forming mixed tetramers that are incapable of DNA binding and transactivation. Secondly, the generated mutant p53 protein might possess activities of its own, which could actively contribute to tumor progression.

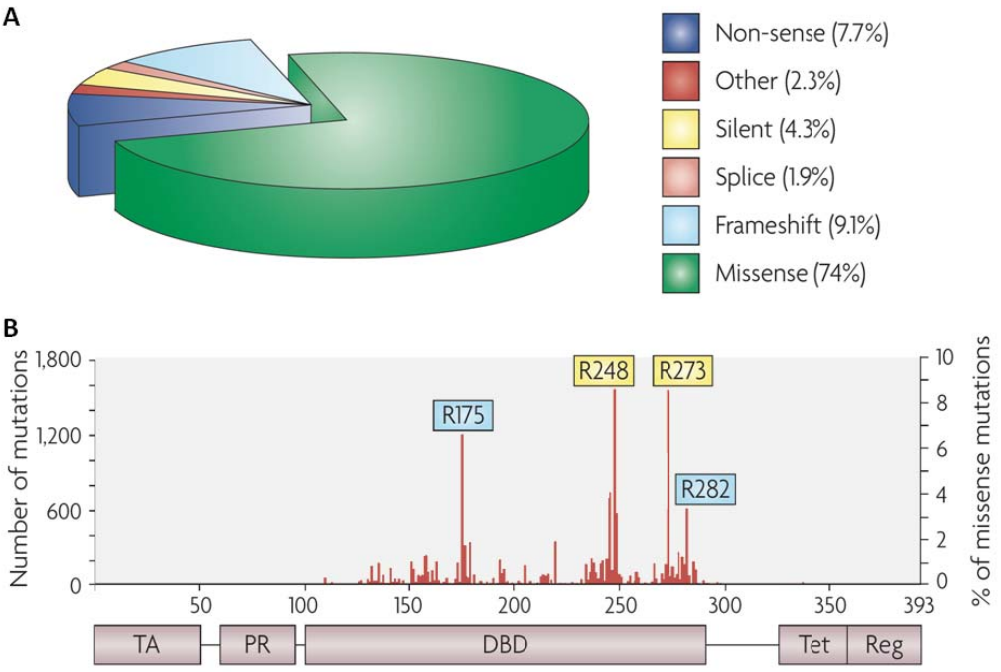


Figure 4: The tumor suppressor p53 is most frequently inactivated through a missense mutation within the DNA binding domain
[adopted from Brosh and Rotter (2009)].
A: Pie chart representing the different tumor-derived mutation types reported in the IARC *TP53* Mutation Database. **B:** Almost every amino acid of the central DNA binding domain of p53 is hit by a point mutation leading to p53 loss of function. Four amino acids at the positions 175, 248, 273, and 282 are more frequently hit and represent the so called hotspot mutations of p53.
TA: transactivation domain; PR: proline-rich domain; DBD: DNA binding domain; Tet: tetramerization domain; Reg: carboxy-terminal regulatory domain. Data derived from the IARC *TP53* Mutation Database version R13 (November 2008).

2.3.5. P53 GAIN OF FUNCTION

Mutant p53 gain of function (GOF) was described first by Dittmer *et al.* (1993). They showed that ectopic mutant p53 expression can transform p53-null cells, leading to increased colony formation in culture and raised numbers of tumors in mice. Meanwhile three hallmarks of transformation were described to promote mutant p53 gain of function:

GENOMIC INSTABILITY

Mutant p53 expression was shown to disrupt normal spindle checkpoint control leading to polyploid cells (Gualberto *et al.*, 1998). Additionally, mice with over-expressed mutant p53 were described to exhibit a high degree of genomic

instability with aberrant centrosome amplification, as well as chromosome translocations (Caulin *et al.*, 2007).

ANTIAPOPTOTIC SIGNALING

Mutant p53 can suppress c-myc induced apoptosis in leukemic cells and thereby allows the cell to benefit from the pro-proliferative effects of the oncogene, without inducing apoptosis at the same time (Lotem and Sachs, 1995). Additionally, mutant p53 expression decreases the induction of apoptosis in response to chemotherapeutics, as well as other kinds of DNA damage, thereby conferring chemoresistance on the tumor cells (Blandino *et al.*, 1999; Li *et al.*, 1998).

CELL MIGRATION AND INVASION

In vitro studies by Adorno *et al.* (2009) and Wang *et al.* (2009) indicated that mutant p53 can augment cell migration and invasion. Nevertheless, it is believed that this process is highly cell-context dependent and in many cases additional signals like oncogenic Ras or TGF-β are needed to unleash this gain of function activity. To estimate the biological relevance of these observations, data from different mouse models were used. Both, mutant p53 over-expressing cells intravenously inoculated into syngeneic mice and knock-in studies, where the endogenous wt p53 was replaced by its mutant variant, revealed that mutant p53 expression leads to the development of more aggressive, metastatic tumors. This supports the concept that mutant p53 gain of function actively contributes to tumor progression (Heinlein *et al.*, 2008; Pohl *et al.*, 1988).

The mechanistic understanding of the role of mutant p53 in tumor cells is still not complete, but the available reports offer some insights. Microarray analysis yielded a large list of genes regulated in their expression by mutant p53. Nevertheless, it was also shown that most mutant p53 variants cannot directly bind to the p53 responsive elements, since either the amino acids involved in direct DNA binding are mutated or the gained mutations lead to overall changes

in the conformation of the protein. Therefore, the effects of mutant p53 on the transcriptional regulation of other genes have to occur indirectly (Figure 5) (Oren and Rotter, 2010). First, mutant p53 was found in complex with its two family members p63 and p73, thereby inhibiting their transcription factor activities (Gaiddon *et al.*, 2001; Strano *et al.*, 2002). Second, mutant p53 was shown to bind to a number of other transcription factors, either leading to the repression of their activity, or recruiting transcriptional activators that facilitate the transcription of the downstream genes (Di Agostino *et al.*, 2006; Stambolsky *et al.*, 2010; Weisz *et al.*, 2007). Last but not least, it was shown that mutant p53 can bind specific DNA elements, such as matrix attachment regions, in a conformation dependent manner. This is proposed to block the binding of other transcription factors to adjacent binding sites, resulting in transcriptional inhibition (Gohler *et al.*, 2005).

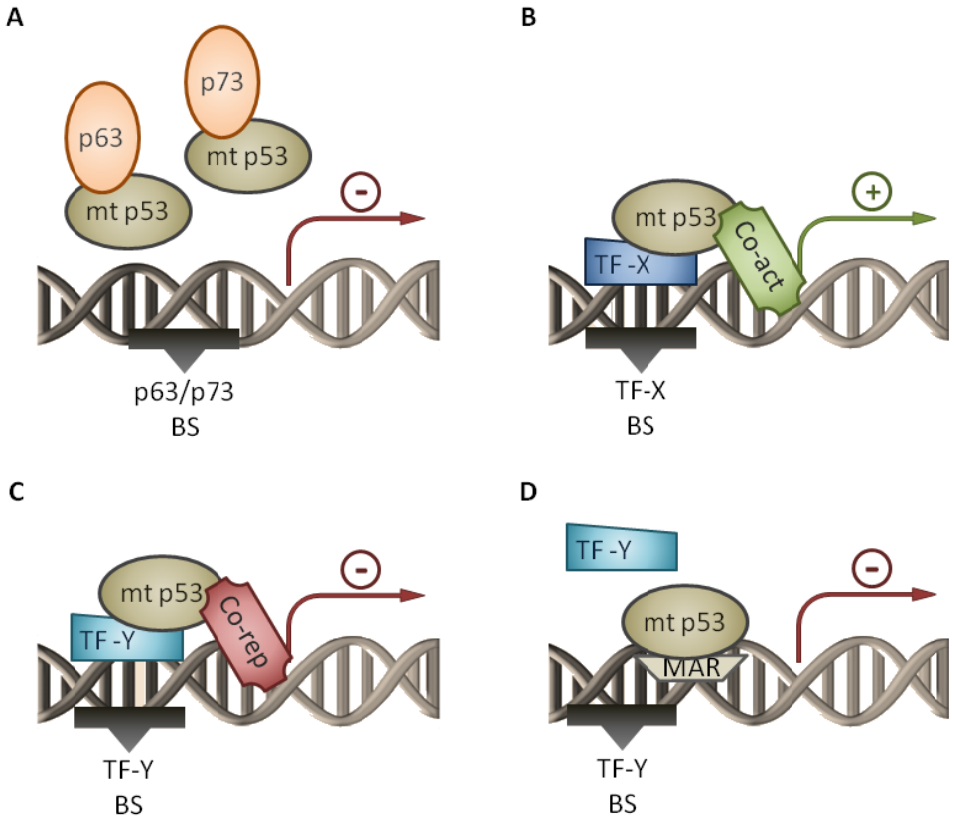


Figure 5: Transcriptional regulation by mutant p53 independently of its DNA binding activity.
A: Transcriptional inhibition through direct interaction and inactivation of its family members p63 and p73. **B:** Transcriptional activation of target genes through interaction with other sequence specific transcription factors (TF-X) and the recruitment of co-activators. **C:** Transcriptional repression of target genes through interaction with other sequence specific transcription factors (TF-Y) and the recruitment of co-repressors. **D:** Association with matrix-attachment regions (MAR) that partially overlap with other transcription factor binding sites and thereby prevent the binding and transactivation of the down-stream genes.
[Adopted from Oren and Rotter (2010)]

2.3.6. MUTANT P53 ACCUMULATION

Mutant p53 protein often accumulates in tumor cells and reaches steady-state levels that greatly exceed those of wt p53. Additionally, it was reported that mutant p53 is not intrinsically stable. This was on the one hand observed in primary cells derived from Li-Fraumeni syndrome patients, who carry germ line p53 mutations (Yin *et al.*, 1992), and on the other hand in the knock-in studies performed by Lang *et al.* (2004) and Olive *et al.* (2004), where mutant p53 protein was shown to only accumulate in tumors, but not in the surrounding tissue.

2.4. THE AIM OF THIS WORK:

THE MECHANISMS OF MUTANT P53 ACCUMULATION

During the last 30 years of p53 research, it was repeatedly shown that the tumor suppressor p53 strongly accumulates in response to chemotherapeutic treatment, going along with posttranslational modifications of the protein at various sites. A similar or even stronger accumulation of p53 is observed in tumor cells that express a mutant variant of the protein. Missense mutations of the protein were shown to not only abrogate its tumor suppressive activities, but also to actively promote oncogenic functions, ranging from genomic instability, over antiapoptotic signaling to increased metastasis and proliferation.

The question that arises from the above is whether the mechanisms leading to p53 accumulation in response to chemotherapeutic treatment, act synergistically with the generally observed augmentation of p53 expression in cancer cells harboring a p53 point mutation. Within this study we observed that such a synergism can lead to the further accumulation of mutant p53 in cancer cells upon treatment with some but not all chemotherapeutic drugs. Therefore we investigated the mechanistic details of mutant p53 accumulation on the one hand gained due to cellular transformation and on the other hand through chemotherapeutic treatment. We found that mutant p53 expression is regulated differently during these two processes causing its accumulation. To achieve the benefits of chemotherapeutic treatment and at the same time circumvent the undesired side effects of mutant p53 accumulation, it would be advantageous to use the obtained information for the development of new therapeutics that could be used in combination with classical chemotherapeutics.

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. TECHNICAL DEVICES

EQUIPMENT	COMPANY
Agitator, magnetic, heated (MR 3001)	Heidolph
Bioruptor (UCD-200TM-EX)	Diagenode
Blotting-chamber (EasyPhor Wet-Blotter)	BioZym
Centrifuge, mini (GMC-060 LMS)	Eppendorf
Centrifuge (5415R)	Eppendorf
Centrifuge (Megafuge 1.0 R)	Heraeus Instruments
ChemoCam Imager (ECL detection)	Intas
Countess	Invitrogen
Electrophoresis-System, for SDS-PAGE	Amersham Biosciences
Foil swelding machine	KRUPS
Freezer -20°C	Liebherr
Freezer -80°C	Heraeus Instruments
Heating block (HTB-1-131 HLC)	Haep Labor Consult
Thermomixer comfort	Eppendorf
Ice-machine (B100)	Ziegra
Incubator for cell cultures (Hera Cell 150)	Heraeus Instruments
Laminar flow cabinet (Hera Safe)	Heraeus Instruments
Light microscope (Axovert 40C)	Zeiss
Liquid Nitrogen Tank (LS 4800)	Taylor-Wharton
Microscope, fluorescent (AxioImager.Z1)	Zeiss
Microscope, automated (Pathway 855)	Becton, Dickinson and Company
PCR machine Thermocycler (T personal)	Biometra
pH-Meter (WTW-720)	WTW, Weilheim, DE
Pipet, electric (Portable-XP)	Drummond
Pipets 2.5, 20, 200, 1000 µl	Eppendorf
Power supply unit (Powerpack P25T)	Biometra

Power supply unit (Power-Pac Basic)	Biorad
Real-time PCR machine (Chromo4™)	Bio-Rad Laboratories
Real-time PCR machine (CFX96; C1000)	Bio-Rad Laboratories
Refrigerator 4°C	Liebherr
Rotator (PTR 300)	Grant Bio
Scales (Acculab ALC-6100.1, LE623S)	Sartorius
Shaker (DRS-12)	neo Lab
Shaker (Promax 2020)	Heidolph
Shaker (Rocky)	Schütt Labortechnik
Spectrophotometer NanoDrop (ND-1000)	PeqLab
UV-transilluminator (Intas UV system)	Intas
Vacuum pump	IBS Integra Biosciences
Vortex (Genie 2)	Scientific Industries
Water bath (TW 20)	Julabo Labortechnik

3.1.2. CONSUMABLE MATERIALS

CONSUMABLE	**COMPANY**
6- and 12- well cell culture plates (Cellstar)	Greiner-bio-one
96 well imaging plates (black)	BD-Falcon
96 well PCR plate (duo plate, skirted)	Sarstedt
Adhesive aluminum foil	Sarstedt
Cell scraper (16mm, 25mm)	Sarstedt
Centrifuge tubes (15ml, 50ml)	Sarstedt
Cryo Tube Vials (1.8ml)	Nunc
Gloves (Latex Safe Skin PFE)	Kimberly Clark
Micro tubes (0.5ml, 1.5ml, 2ml)	Sarstedt
Nitrocellulose, poresize: 0.2µM (Protran BA83)	Omnilab
Pasteur pipets, glass (230mm)	VWR international
Parafilm	Pechiney
Pipet tips with or without filter (20 µl, 200 µl, 1000 µl)	Sarstedt
Sealing tape (optically clear for 96 well PCR Duo plates)	Sarstedt
Syringe (1 ml)	BD Plastipak
Syringe needles (0,6 x 25mm)	BD Microlance
Tissue culture dish (CELLSTAR 60x15 mm)	Greiner-bio-one

Tissue culture dish (CELLSTAR 100x20 mm)	Greiner-bio-one
Tissue culture dish (CELLSTAR 145x20 mm)	Greiner-bio-one
Whatman paper (GB002)	Schleicher & Schuell

3.1.3. CHEMICALS

NAME	COMPANY
10 x Taq buffer with KCl (B38)	Fermentas
2-mercaptoethanol	Roth
2-propanol	Roth
25 mM $MgCl_2$ (R0971)	Fermentas
β-glycerol phosphate disodium salt pentahydrate (β-glycerophosphate)	Fluka
Ammonium persulfate (APS)	Roth
Bromphenol blue	Sigma-Aldrich
BSA	Roth
Calcium chloride ($CaCl_2$)	Roth
Camptothecin	Sigma-Aldrich
Chelex 100	Bio-Rad
Chloroform	Roth
Ciprobay 200	Bayer
Cycloheximide	Sigma-Aldrich
DAPI dilactate	Sigma-Aldrich
Di-sodiumhydrophosphate dihydrate (Na_2HPO_4 x H_2O)	Roth
Dithiothreitol (DTT)	Roth
dNTPs, 25 µM each (U1420)	Promega
Dulbecco's Modified Eagle's Medium (31600-091)	GIBCO/ Invitrogen
EDTA	Roth
Ethanol, >99.9%	Merck
Ethanol denatured, 99.8%	Roth
Ethidium bromide	Roth
Fetal Calf Serum (FCS)	GIBCO / Invitrogen
Formaldehyde, 37%	Roth
Glycerol	Roth
Glycine	Roth

GlycoBlue (AM9516)	Ambion
HEPES	Roth
Hydrochloric acid (HCl)	Roth
Immobilon western chemiluminescent HRP substrate	Millipore
Iodacetamide	AppliChem
L-glutamine	GIBCO / Invitrogen
Lipofectamine 2000	Invitrogen
Magnesium chloride ($MgCl_2$)	Roth
McCoy's Medium 5A	GIBCO / Invitrogen
Methanol	Roth
Milk powder, non fat	Roth
NEBuffer for M-MuLV reverse transcriptase (B0253)	NEB
N-ethylmaleimide	Sigma
N,N,N´,N´-Tetramethylendiamin (TEMED)	Roth
NP-40	USB
PBS tablets (18912-014)	GIBCO/ Invitrogen
Penicillin/Streptomycin	GIBCO / Invitrogen
pH-Solution 10,01	Roth
pH-Solution 4,01	Roth
pH-Solution 7,01	Roth
Ponceau S	Roth
Proteinase K (EO 0491)	Fermentas
Protein A sepharose CL-4B (17-0780-01)	GE Healthcare
Rotiphorese Gel 30 (30% acrylamide bisacrylamide solution; ratio 37.5:1)	Roth
Potassium chloride (KCl)	Roth
Potassium dihydrogen phosphate (KH_2PO_4)	Roth
RNase Inhibitor (M0307)	NEB
RPMI Medium 1640	GIBCO / Invitrogen
Sepharose CL-4B (17-0150-01)	Amersham
Sodium acetate	Roth
Sodium deoxycholate	AppliChem
Sodium dodecyl sulfate (SDS)	Roth
Sodium chloride (NaCl)	AppliChem
Sodium hydrogen carbonate ($NaHCO_3$)	Roth

Sodium hydroxide (NaOH)	Roth
Sonicated salmon sperm DNA (201190-81)	Stratagene
SuperSignal west femto maximum Sensitivity (34095)	Pierce
Tetracycline	Sigma
Trasylol (aprotinin 500.000 KIE)	Bayer
Trichostatin A	Sigma-Aldrich
Tris	Roth
Triton X-100	AppliChem
Trizol (15596-018)	Invitrogen
Trypanblue	Invitrogen
Trypsin-EDTA	GIBCO / Invitrogen

3.1.4. ENZYMES

NAME	COMPANY
M-MuLV Reverse Transcriptase (M0253)	NEB
Taq DNA polymerase	PrimeTec

3.1.5. CHEMOTHERAPEUTICS

NAME	COMPANY
5-Fluorouracil	SIGMA
Camptothecin	SIGMA
Daunorubicin	SIGMA
Doxorubicin	Santa Cruz
Epirubicin	SIGMA
Etoposide	SIGMA
Idarubicin	SIGMA

3.1.6. BUFFERS

BLOCKING SOLUTION	
PBS	
FCS	10%

CHIP BUFFER	
Tris-HCl pH 8.0	50 mM

NaCl	150 mM
EDTA pH 8.0	5 mM
NP40	0.5% (v/v)
Triton X-100	1% (v/v)

CHIP++ BUFFER

ChIP buffer	
Aprotenin/ Leupeptin	1 µg/ ml each
Pepstatin A	1 µg/ ml
Pefablock	1 mM

LAEMMLI BUFFER 6X

Tris-HCl pH 6.8	0.35 mM
Glycerol	30% (v/v)
SDS	10% (w/v)
DTT	9.3% (w/v)
Bromphenol blue	0.012% (w/v)

PBST

PBS	
Tween-20	0.1%(v/v)

RIPA-BUFFER

Tris-HCl pH 7.5	20 mM
NaCl	150 mM
Sodium deoxycholate	1% (w/v)
SDS	0.1% (w/v)
Triton-X 100	1% (v/v)
EDTA	10 mM
Trasylol	5 % (v/v) (equals 100,000 KIE)
pH was adjusted to 7.5 with 1M NaOH	

SDS RUNNING BUFFER (FOR SDS-PAGE)

Tris	25 mM
Glycine	192 mM
SDS	0.1% (w/v)

WESTERN SALTS

Tris	25 mM
Glycine	192 mM
SDS	0.02% (w/v)
Methanol	15%
pH was adjusted to 8.3 with HCl	

3.1.7. OLIGONUCLEOTIDES

siRNAs

NAME	ID	SEQUENCE (5' → 3')
N.C. 1		UNKNOWN
N.C. 2		UNKNOWN
BCLAF1	s18874	CAUUGAUCGCCGUAGAAAAtt UUUUCUACGGCGAUCAAUGtc
BCLAF1_2	s18875	CGCGAUUACAGAAAUAAUAtt UAUUAUUUCUGUAAUCGCGac
CBP	s3495	GAAUCUUUCCCAUAUCGAAtt UUCGAUAUGGGAAAGAUUCag
CBP_2	s3496	GGAUAUUGCUGUGGACGCAtt UGCGUCCACAGCAAUAUCCaa
HOXA5	s6765	GACUACCAGUUGCAUAAUUtt AAUUAUGCAACUGGUAGUCcg
HOXA5_2	s6766	CCAGUUGCAUAAUUAUGGAtt UCCAUAAUUAUGCAACUGGta
p300	s4696	CCACUACUGGAAUUCGGAAtt UUCCGAAUUCCAGUAGUGGat
p300_2	s4697	GCCUGGUUAUAUAACCGGAtt UCCGGUUAUAUAACCAGGCat
p53	s605	GUAAUCUACUGGGACGGAAtt UUCCGUCCCAGUAGAUUACca
PCAF	s16895	GGUACUACGUGUCUAAGAAtt UUCUUAGACACGUAGUACCta
PCAF_2	s16896	GGAGUUCGACAGAUUCCUAtt UAGGAAUCUGUCGAACUCCat
RPL26	s12203	GAAAUAUGUUAUCUACAUUtt AAUGUAGAUAACAUAUUUCtt
RPS6KA1	s12273	CACUGAUUCUGAAGGCGAAtt UUCGCCUUCAGAAUCAGUGtc
RPS6KA1_2	s12275	CCAUUGACUGGAAUAAGCUAtt UAGCUUAUUCCAGUCAAUGgt
RPS6KB1	s12283	GGUUUUUCAAGUACGAAAAtt UUUUCGUACUUGAAAAACCtt
RPS6KB1_2	s12284	GGACUAUGCAAAGAAUCUAtt UAGAUUCUUUGCAUAGUCCaa
RPS6KB2	s12286	CCCUUUUUCCGGCACAUGAtt UCAUGUGCCGGAAAAAGGGat

RPS6KB2_2	s12285	ACAUCAAACUGACCGACUUtt AAGUCGGUCAGUUUGAUGUgg
RPS6KL1	s38111	GGUACUUUGUGAGCGAGGAtt UCCUCGCUCACAAAGUACCtg
RPS6KL1_2	s38110	CGAUGUUAGUGAGGACUAUtt AUAGUCCUCACUAACAUCGcg
RREB1	s12354	CCAUCUCCUCUGAAACGUAtt UACGUUUCAGAGGAGAUGGag
RREB1_2	s12356	GGAGUUUGUUUGCAAGUAUtt AUACUUGCAAACAAACUCCtt
TIP60	s20630	GCAAGCUGCUGAUCGAGUUtt AACUCGAUCAGCAGCUUGCcg
TIP60_2	s20631	GGACGGAAGCGAAAAUCGAtt UCGAUUUUCGCUUCCGUCCtg
TP73	s14319	GCAAUAAUCUCUCGCAGUAtt UACUGCGAGAGAUUAUUGCct
TP73_2	s14320	CCACCAUCCUGUACAACUUtt AAGUUGUACAGGAUGGUGGtg
WRAP53_1	s30251	CCUCUGCUUUCAUCCCGAUtt AUCGGGAUGAAAGCAGAGGtg
WRAP53_2	s30252	GAAGCAAACGGGAGCCUUUtt AAAGGCUCCCGUUUGCUUCtt

PCR

NAME	**SEQUENCE (5' → 3')**
36B4_for	GATTGGCTACCCAACTGTTG
36B4_rev	CAGGGGCAGCAGCCACAAA
p53_for	ATGGAGAGAGCCGCAGTCAGATC
p53_rev	GGGAGCAGCCTCTGGCATTCTG
p53-Int1_for	GCCGAGACGGGCCATTCGTG
p53-Int1_rev	TCTCACCGCTCACCTGCCCA
WRAP53-Exon1a_for	CGGAGCCCAGCAGCTACC
WRAP53-Exon1a_rev	TTGTGCCAGGAGCCTCGCA
WRAP53-Exon2_rev	GTCCTGGTCTGAAGGACAGC
WRAP53-Exon7_for	GACTGCGAGGTCCGAGCCACATTTG
WRAP53-Exon8_rev	GAGCCATCATCCCAGGCATACAGAC
E2F1_for	CGGTGTCGTCGACCTGAACT
E2F1_rev	AGGACGTTGGTGATGTCATAGATG
TAp73_Exon1_for	GGGCTGCGACGGCTGCAG
TAp73_Exon3_rev	GATGTAGTCATGCCCTCCAGG

NOXA_for	GGACTGTTCGTGTTCAGCTCGC
NOXA_rev	GCCGGAAGTTCAGTTTGTCTCC

ChIP

NAME	SEQUENCE (5' → 3')
ChIP_p53-E2F1 BS-I_for	TGCACCCTCCTCCCCAACTCC
ChIP_ p53-E2F1 BS-I_rev	GCTCCCTGGACGGTGGCTCT
ChIP_p53-E2F1 BS-II_for	CCCGGGAGGAGAGGCGAACA
ChIP_ p53-E2F1 BS-II_rev	TGGGTCGCCCGCGAAATCTG
ChIP_p53+19.5kb_for	GCCACGGCTGGCACAAGGTT
ChIP_p53+19.5kb_rev	GCTGCCCCCACTTTCCTGGG
ChIP_p107-E2F1 BS_for	AGGCAGACGGTGGATGACAACAC
ChIP_p107-E2F1 BS_rev	TCAGCGTGGGGCTTGTCCTCGAA
ChIP_TP73-E2F1 BS_for	GAGCGCCGGGAGGAGACCTT
ChIP_TP73-E2F1 BS_rev	GCGGGCGTTAGCGCCTTTTT

3.1.8. ANTIBODIES

PRIMARY ANTIBODIES

NAME	DILUTION	APPLICATION	SOURCE	COMPANY
E2F1 KH95 and KH129	1:500 each	WB	mouse monoclonal	Santa Cruz Biotechnology
E2F1 KH20 and KH95	1µg	ChIP	mouse monoclonal	Upstate
p53 D0-1 HPR-conjugated	1:8000	WB	mouse monoclonal	Santa Cruz Biotechnology
p53 FL-393	1:500	IF	rabbit polyclonal	Santa Cruz Biotechnology
p53 (pSer15)	1:1000	WB	mouse monoclonal	Cell Signaling
p53 (acLys382)	1:1000	WB	rabbit polyclonal	Cell Signaling
RNA pol II	1µg	ChIP	rabbit polyclonal	Santa Cruz
β-actin	1:10000	WB	mouse monoclonal	Abcam

SECONDARY ANTIBODIES

NAME	DILUTION	APPLICATION	COMPANY
Donkey α-mouse IgG	1:10000	WB	Jackson ImmunoResearch

(H+L) HPR-conjugated			
Donkey α-rabbit IgG (H+L) HPR-conjugated	1:10000	WB	Jackson ImmunoResearch
Alexa Fluor 488 anti-rabbit	1:500	IF	Molecular Probes, Invitrogen
Alexa Fluor 594 anti-rabbit	1:500	IF	Molecular Probes, Invitrogen

3.1.9. EUKARYOTIC CELL LINES

NAME	SOURCE	P53 STATUS
5637	human bladder carcinoma	R280T
A431	human squamous cell carcinoma	R273H
HCT116	human colon carcinoma; p21 wt or $p21^{-/-}$	wt
U251	human glioma cells	R273H
U2OS	human osteosarcoma	wt

3.1.10. CELL CULTURE WORKING SOLUTIONS

DULBECCO'S MODIFIED EAGLE'S MEDIUM (DMEM -)

Dulbecco's Modified Eagle's Medium	10g
NaHCO3	3.7 g/L
HEPES	5.96 g/L
H_2O	ad 1L

The medium was filtered and stored at +4°C

DULBECCO'S MODIFIED EAGLE'S MEDIUM WITH SUPPLEMENTS (DMEM + FCS)

DMEM -	450 ml
FCS	10%
Penicillin/Streptomycin	50 U/mL
Tetracycline	2 µg/mL
L-glutamine	200 µM
Ciprobay 200	10 µg/mL

DMEM+FCS was and warmed up to +37°C directly before use

RPMI MEDIUM 1640 WITH SUPPLEMENTS (RPMI + FCS)

RPMI -	450 ml
FCS	10%
Penicillin/Streptomycin	50 U/mL
Tetracycline	2 µg/mL

L-glutamine	200 µM
Ciprobay 200	10 µg/mL

DMEM+FCS was and warmed up to +37°C directly before use

MCCOY'S MEDIUM 5A WITH SUPPLEMENTS (MCCOY'S + FCS)

McCoy's 5A -	450 ml
FCS	10%
Penicillin/Streptomycin	50 U/mL
Tetracycline	2 µg/mL
L-glutamine	200 µM

DMEM+FCS was and warmed up to +37°C directly before use

PBS BUFFER

PBS tablets	
H2O	500 ml

PBS for cell culture was autoclaved and stored at +4°C

CELL FREEZING SOLUTION

DMSO	10 %
FCS	90 %

3.2. METHODS

3.2.1. CELL BIOLOGY

MAINTENANCE OF CELL CULTURES

All cell lines were cultivated at 37°C in a humidified incubator with 5% CO_2 either in RPMI medium 1640 (A431, U251, 5637) or in Dulbecco's Modified Eagle's medium (DMEM) (U2OS), or McCoy's medium (HCT116 wt, HCT116 $p21^{-/-}$), all supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 µg/ml streptomycin, 200 µM Glutamine, 2 µg/ml tetracycline, and 10 µg/ml Ciprobay 200 (not for the HCT116 cells). Sub cultivation was performed every 3-4 days, as soon as the cells reached 70-80% of confluence. For passaging, the medium was removed; the cells were rinsed once with PBS and incubated at 37°C for a few minutes with 0.05% Trypsin-EDTA solution, to induce detachment from the culture dish. Upon neutralization of the Trypsin with fresh medium the cells were carefully resuspended and diluted 1:8 - 1:10 in fresh

medium. For experiments, the cell number was determined using trypanblue staining of living cells that was subsequently quantified using the Countess system. The required amount of cells was seeded into the corresponding culture dishes/ well plates. For long term storage, the cells were frozen in liquid nitrogen.

CELL FREEZING PROCEDURE

The cells from a 10 cm culture dish at 70-80% confluency were frozen in 1 cryovial. After trypsinization and dilution with fresh medium+FCS as described above, the cell suspension was centrifuged 10 min at 800 rpm. The supernatant was aspirated; the cell pellet was resuspended in 1ml cold cell freezing solution (10% DMSO in FCS) and transferred into pre-cooled cryovials. The vials were stored in -80°C for 2 days and then kept in a liquid nitrogen tank for long term storage.

To take frozen cells in culture, the vials were quickly thawed by hand and immediately transferred into a 15cm culture dish with prewarmed medium. After one day of incubation at 37°C, the medium was changed to remove the residual DMSO.

REVERSE SIRNA TRANSFECTION

Pre-designed or validated siRNAs from Applied Biosystems were used for all siRNA transfection experiments. Both the siRNAs and the transfection reagent Lipofectamine 2000 were diluted in cell culture medium without supplements and incubated for 5 minutes. The solutions were combined in an empty well-plate and incubated for additional 20 minutes to allow the siRNA-lipid-micelles to form. The cells were counted and the appropriate number of cells was added to the transfection mix and diluted with medium+FCS to the final volume of the corresponding well plate.

Different amounts of oligonucleotides, transfection reagent, cells and medium were used depending on the well sizes:

	siRNA + MEDIUM	LIPOF. 2000 + MEDIUM	CELL NUMBER	TOTAL VOLUME
6-well	30 pmol	2.7 µl	$2.5 - 3 \cdot 10^5$	2 ml
12-well	15 pmol	1.35 µl	$1.5 - 1.8 \cdot 10^5$	1 ml
96-well	1 pmol	0.25 µl	8000	100 µl

After 48 hours the cells were fixed for immunofluorescence staining or harvested for immunoblot analysis or RNA isolation.

LONG-TERM SIRNA TRANSFECTION:

The cells were reverse transfected as described above. 48 hours later the samples were trypsinized in the well plate and 25 - 33% of the cells were used for a second reverse transfection following the same protocol as above. After an additional incubation for 72 hours the cells were harvested for RNA isolation or immunoblot analysis.

CHEMOTHERAPEUTIC TREATMENT

Most treatments were performed for 24 hours; therefore the cells were either seeded about 12 hours before treatment or siRNA transfected 24 hours before treatment. The medium was removed from the cells and fresh medium containing the chemotherapeutic drug at the desired final concentration was added to the cells. The mock sample was treated with the same volume of dissolvent only.

	STOCK CONC.	FINAL CONC.	DISSOLVENT
5-Fluorouracil	0.3 M	500 µM	DMSO
Camptothecin	2.87 mM	2.87 µM	DMSO
Daunorubicin	4 mM	500 nM	H_2O
Doxorubicin	4 mM	500 nM	H_2O
Epirubicin	4 mM	500 nM	H_2O
Etoposide	20 mM	100 µM	DMSO
Idarubicin	4 mM	500 nM	H_2O

3.2.2. MOLECULAR BIOLOGY

TOTAL RNA ISOLATION

For the preparation of total RNA 0.5 - $1 \cdot 10^6$ cells per sample are needed (corresponds to one 6-well). The medium was aspirated and 800 µl Trizol (Invitrogen) was added to the cell layer for lysis. After 5 minutes at room temperature the lysates were transferred into microtubes and supplemented with 180 µl chloroform. The mixture was vigorously shaken and further incubated for 3 min at room temperature, followed by a centrifugation step (4°C, 16000 g, 20 min). The upper aqueous phase, containing RNA, was carefully transferred into a new microtube and supplemented with the same amount of isopropyl alcohol. The samples were mixed vigorously by hand and incubated for 3 min at room temperature and for 2 - 24 hours at -20°C. RNA was precipitated by centrifugation (4°C, 16000 g, 20 min), the pellet was washed with 70% ethanol, centrifuged (4°C, 9000 g, 10 min), air dried for 10 minutes at 37°C and resuspended in 30 µl nuclease free water.

QUANTIFICATION OF RNA

The RNA concentration was measured, using a NanoDrop spectrophotometer (PeqLab). The absorbance at 260 nm was used to determine the concentration, whereas the ratios 260:230 and 260:280 were used as indicators for the purity of the isolated RNA. The ratios 260:230 around 1.9-2.0 and 260:280 in the range of 2.0-2.1 were considered as ‘pure’ RNA. In case these values were appreciably lower, RNA was additionally purified, as described in the following section.

PURIFICATION OF RNA

The RNA sample (30µl) was mixed with 20µl H_2O, 1µl 125 mM EDTA, 1µl 3M sodium acetate and 70µl 100% ethanol. The samples were vortexed and incubated for 5 min at room temperature. After shock-freezing in liquid N2 the samples were centrifuged at 4°C for 20 min at 16000 g. The pellet was washed with 70 µl 70% ethanol and centrifuged (4°C, 10 min, 9000 g). The Supernatant

was aspirated and the pellet was air dried for 10 minutes at 37°C. The precipitated RNA was resuspended in 30µl nuclease free water and the concentration and purity of the RNA was determined, again using the NanoDrop spectrophotometer as above.

REVERSE TRANSCRIPTION

Reverse transcription was performed using moloney murine leukemia virus (M-MuLV)-derived reverse transcriptase (NEB). The following stock solutions were prepared and aliquots were kept at -20°C:

- Combined primer stock: 15µM random nonamers (N9) and 50µM oligo dT23VN
- Deoxyribonucleoside triphosphates (dNTPs): 2.5mM of each (dCTP, dATP, dTTP, dGTP) (Promega)

1µg of total RNA was used for the reverse transcription and diluted with nuclease free water to a final volume of 10µl. 2µl of the combined primer stock and 4µl dNTP mix were added to the diluted RNA. The samples were incubated for 5 minutes at 70°C. Meanwhile a master mix of 2µl NEBuffer for M-MuLV reverse transcriptase (NEB), 0.25µl RNase inhibitor (10U, NEB), 0.125µl M-MuLV reverse transcriptase (25U, NEB), and 1.625µl nuclease free water per sample was prepared. The transcriptase master mix was added to the RNA samples and incubated for 1 hour at 42°C. The enzyme was inactivated at 95°C for 5 min and the cDNA was diluted with 30µl nuclease free water. It was either directly used for real-time PCR or stored at -20°C. To control for genomic DNA contamination each reaction was also performed as noRT control, omitting the reverse transcriptase in the master mix.

REAL-TIME PCR

Real-time PCR (qPCR) was used to obtain semi-quantitative measurements of gene expression or to quantify the recovered sheared DNA from chromatin

immunoprecipitation experiments. The following master mix was prepared on ice and aliquoted in microtubes:

	FINAL CONC.	FILTERED
Tris-HCl pH 8.8	75 mM	✓
$(NH_4)_2SO_4$	20mM	✓
Tween-20	0.01 %	✓
$MgCl_2$	3 mM	✓
Triton X-100	0,25%	✓
Trehalose	300 mM	✓
Sybr Green	1:80000	
dNTPs	0.2 mM	
Taq-Polymerase	20U/ ml	

The tubes were shock-frozen in liquid N2 and kept at -20°C for up to 3 month.

For the PCR reaction 14µl master mix were mixed with 0.075µl of each primer (stock concentration: 100µM) and 9.85µl H_2O. This was prepared as a master mix and aliquoted into a 96-well PCR plate. 1µl cDNA/ sheared DNA was added into the corresponding wells. The plate was sealed, centrifuged (30sec, 600rpm) and the following PCR program was used for the specific gene amplifications:

STEP	TEMPERATURE	TIME	FLUORESCENT READ STEP	
1 - DNA melting	95°C	3 min		
2 - DNA melting	95°C	15 sec		39x
3 - primer annealing and elongation	60°C	1 min	X	
4 - melting curve	60°C - 95°C		every 0.5°C	

For the semi-quantitative analysis of target mRNA expression the C_t-values of the genes of interest as well as the reference gene 36B4 were obtained. The ΔΔCt-method was used to determine the relative expression of the analyzed target genes:

$$\Delta\Delta C_t = \frac{C_t \text{ (36B4, untreated)} - C_t \text{ (target gene, untreated)}}{C_t \text{ (36B4, treated)} - C_t \text{ (target gene, treated)}}$$

(Livak and Schmittgen, 2001)

For ChIP analysis a serial dilution of the input DNA was used to determine the relative amounts of target DNA in the input samples, as well as the IP samples. The recovered DNA is diagramed relative to the input DNA.

$$\% \text{ of input DNA} = \frac{\text{rel. amount of target DNA (IP sample)}}{\text{rel. amount of target DNA (input sample)}}$$

3.2.3. BIOCHEMISTRY AND IMMUNOLOGICAL METHODS

IMMUNOBLOT ANALYSIS

CELL HARVESTING AND LYSIS

Adherent cells were grown, treated, or transfected in a 12-well plate for immunoblot analysis. For harvesting they were scraped in the growth medium, transferred to a microtube and centrifuged (5 min, 1000 rpm). The cell pellet was resuspended and the cells were lysed in 60µl of RIPA/ 6x Laemmli buffer (1:1 mixture). The samples were incubated for 5 min at 95°C for protein denaturation. The samples were centrifuged (1 min, 13000 rpm) and stored at -20°C or directly used for SDS-PAGE.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was developed 1967 by (Shapiro *et al.*, 1967) to determine the molecular weight of proteins. The detergent SDS coats the denatured proteins and translates their molecular weight into a negative charge, therefore a sample buffer developed by (Laemmli, 1970) is widely used. An electric field is applied to the gel and the negatively charged proteins migrate towards the anode. Within the stacking gel the pores are large and therefore the proteins form a concentrated stack between the leading chloride ions and the trailing ion Glycine. As soon as the sample migrates into the resolving gel, which obtains a pH that is 2 units higher than that of the stacking gel and pores that are

restrictively small for the proteins, the sample starts to resolve according to the molecular weight of the proteins. Depending on the molecular weight of the protein of interest different percentages of acrylamide/ bisacrylamide (AA/ BAA) are used within the resolving gel. All SDS-PAGE experiments within this study were performed using 10% AA/ BAA.

CHEMICAL	STACKING GEL (5%)	RESOLVING GEL (10%)
Acrylamide/ bisacrylamide	850 µl	4.15 ml
Tris-HCl pH 6.8	625 µl	-
1.5 M Tris-HCL pH 8.8	-	3.15 ml
H_2O	3.4 ml	5 ml
10% SDS	50 µl	125 µl
10% APS	50 µl	75 µl
TEMED	10 µl	7.5 µl

The resolving gel was casted between two glass plates, separated by spacers (1mm thick) and covered by a layer of 2-propanol to prevent air contact for polymerization. The solidified gel was rinsed with water to remove any residual 2-propanol and the stacking gel was casted on top of the resolving gel. A comb, either with 10 or 15 teeth was inserted into the stacking gel before polymerization in order to form separated slots for sample loading.

After gel polymerization, 10 to 20 µl of cell lysate were loaded into the pockets of the stacking gel. Electrophoresis was performed at 15mA per gel until the samples migrated into the resolving gel, then it was increased to 20 mA per gel.

WESTERN BLOT

For immunodetection of the proteins they were transferred onto a nitrocellulose membrane (pore size: 0.2µM) after the separation through SDS-PAGE using the tankblot technique (Bittner *et al.*, 1980). A stack of sponges, filter papers, the gel, the membrane, filter papers, and sponges was prepared, all soaked in transfer buffer. This was then placed within the vertical blotting chamber, filled up with transfer buffer and again an electric field was applied. After blotting for 1 hour at 100V all proteins were bound to the nitrocellulose membrane. The

quality of the transfer was controlled through the reversible protein staining with Ponceau S solution.

IMMUNOSTAINING

For specific protein visualization after western blotting, membranes were subjected to immunostaining. First, membranes were blocked with a 5% non-fat milk solution in PBST (milk) for 1 hour followed by the incubation with primary antibody, diluted in milk for 2 hours at room temperature or overnight at 4°C. Subsequently, membranes were washed twice according to the following protocol: 3 times in PBST followed by 15 min in milk. To visualize the specifically bound primary antibodies the membranes were incubated with HPR-conjugated secondary antibodies for one hour. Washing was repeated the same way as described above. All washing and incubation steps were fulfilled with gentle shaking at room temperature, if not specified otherwise. For protein detection enhanced chemiluminescence solutions (ECL) were used and the signal was measured using the ChemoCam Imager (Intas). For quantification the LabImage 1D software (Intas) was used.

CHROMATIN IMMUNOPRECIPITATION

$1 \cdot 10^6$ U251 cells were seeded per 10 cm culture dish and treated 18 hours later with 500nM doxorubicin. 24 hours after treatment protein-DNA crosslinking was performed using 1.42% (v/v) formaldehyde in PBS for 15 min and stopped by the addition of Glycine to a final concentration of 138 mM for 5 min. After washing with PBS twice, the cells were scraped in 1ml ChIP++ buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 0,5% NP-40, 1% Triton X-100, Leupeptin (1µg/ml), Aprotenin (1µg/ml), Pepstatin A (1µg/ml), Pefabloc (1mM)), transferred into a microtube and centrifuged at 1000 rpm for 5 min. The Pellets were washed once with 1 ml ChIP++ buffer and resuspended in 300 µl of the same buffer. The lysates were sonicated in an icewater bath sonicator (Bioruptor) to shear the chromatin to a length of 500 – 1000 base pairs (3 times 10 minutes using 10 sec on/ off cycles at maximum power). The lysates were

diluted in ChIP++ buffer before pre-clearing for 1 hour at 4°C with 100µl sepharose, washed 3 times in ChIP buffer and finally resupended in ChIP++ buffer to achieve a 50% slurry. The samples were centrifuged at 12000 rpm, 10 min, at 4°C, and the supernatants were transferred to new microtubes. The pre-cleared chromatin was diluted with ChIP++ buffer according to the number of immunoprecipitations that were performed. 1 µg of antibody per 50 µl of lysate was used for the immunoprecipitation (IP), additionally 50µl of the precleared DNA were used as input control. The IP samples were further diluted with ChIP++ buffer up to 500 µl and incubated overnight at 4°C with rotation. The input samples (50 µl) were mixed with 1µl glycogen (Glycoblue) and 100µl 100% ethanol and placed at - 20°C overnight for DNA precipitation. Protein A sepharose (GE Healthcare) beads were incubated over night in a 15ml tube filled up with ChIP buffer to allow the beads to swell. At the same time 0.5g BSA and 100µl sheared salmon sperm DNA were added to block the beads and avoid unspecific precipitation. Blocked protein A sepharose was washed three times with ChIP buffer (centrifuged at 2000 rpm, 2 min, 4°C) and finally resuspended in ChIP++ buffer to get a 50% sepharose slurry. 30µl of this slurry were added to each immunoprecipitation reaction and samples were incubated for 2 hours at 4°C with rotation. Meanwhile the input samples were centrifuged (13000 rpm, 20 min, 4°C), and the DNA pellets were washed once with 500 µl of 70% ethanol before they were air dried for 10 min at 37°C. The immune-sepharose complexes were washed 8 times with 1 ml cold ChIP buffer, centrifuged for 2 min at 2000 rpm and 4°C. 100 µl 10% (w/v) Chelex 100 slurry was added to the washed beads and to the input DNA pellet. After brief vortexing the samples were heated to 95°C for 10 min. 30µg Proteinase K was added to each sample and incubated at 55°C for 30 min with shaking at 1000 rpm. For the inactivation of Proteinase K the samples were heated to 95°C for 10 min. All beads were precipitated by centrifugation (12000 rpm, 1min, 4°C) and the supernatants were carefully transferred into new tubes. For

quantification of the precipitated/ recovered DNA 1µl of the supernatant was used for qPCR analysis.

IMMUNOFLUORESCENCE

Cells were grown in 96-well imaging plates (BD Falcon). Prior to immunofluorescence staining the cells were fixed using 3.7% formaldehyde in PBS for 20 minutes. After fixation the cells were washed with PBS containing 50mM Glycine in order to inactivate residual free formaldehyde that could otherwise unspecifically cross-link the primary antibodies to proteins of the cells. Permeabilization was achieved through 10 minutes incubation with PBS containing 0.5% Triton X-100. All buffers that were used from this step on contained 0.2% Triton X-100 in order to keep the cells in a permeabilized state. Incubation for 10 minutes in blocking solution (10% FCS in PBS + 0.2% TX100) was performed to block all unspecific binding sites in the cells before they were incubated for 1 hour with the primary antibodies (for dilutions see 3.1.8). The remaining primary antibodies were washed away with blocking solution 3 times for 5 minutes. The secondary antibodies coupled to the fluorophores Alexa488 or Alexa546 were incubated in a 1:500 dilution in blocking solution for 45 minutes in the dark. A nuclear stain (Hoechst 33342 or Doxorubicin) was additionally used during this incubation. We observed that the previous treatment of the cells with red fluorescent chemotherapeutic drugs (Daunorubicin, Doxorubicin, Epirubicin, or Idarubicin) has an impact on the fluorescent signal of Hoechst 33342; therefore we used in these cases a high dose of doxorubicin (10µM) for nuclear stain. The free secondary antibodies were washed away with blocking solution for 5 minutes, PBS +0.2% TX100 for 5 minutes and with PBS for additional 5 minutes; all incubations were performed in the dark. Finally the cells were kept in 100µl PBS and the plate was sealed with aluminum foil.

The fluorescent pictures were taken, using the BD Pathways system. In each well at least 9 pictures were taken using a 10x or 20x magnification. On the

basis of the nuclear stain the single nuclei within each well were defined and the average fluorescent intensity of the immunodetected proteins in each of these nuclei was measured. The results are either presented as average intensity per well, or the single nuclei intensities in each well are plotted in histograms.

3.2.4. THE SCREEN

THE LIBRARY

For the kinase screen a siRNA library (Silencer Human Kinase siRNA Library V3) was obtained from Applied Biosystems containing 3 different siRNAs against each of the 719 kinases included in the library. The siRNAs were obtained lyophilized in 96 well plates, containing 8 empty wells that were used for internal controls. The 3 siRNAs targeting the same gene were always localized on different plates. Before transfection all siRNAs were dissolved in nuclease free water at a final concentration of 50µM and dilution plates containing 5µM of the siRNAs were prepared. All pipetting steps were performed by the Biomek 2000 (Beckmann Coulter).

TRANSFECTION

For the siRNA transfections in a 96 well format the Biomek 2000 was used. The robot was programmed according to the siRNA transfection protocol as it was described in section 3.2.1 (REVERSE SIRNA TRANSFECTION), but using 4.5 times more siRNA. The reason for this is that the library consists of Silencer siRNAs that are less efficient compared to the Silencer Select siRNAs that were used throughout the other experiments.

IMMUNOFLUORESCENCE STAINING AND ANALYSIS

48 hours after reverse siRNA transfection of the cells they were fixed and stained for immunofluorescence analysis as described in 3.2.3 (IMMUNOFLUORESCENCE). The polyclonal p53 antibody (FL-393) was used for the immunostaining at a dilution of 1:500 in combination with an anti-rabbit

secondary antibody coupled to the fluorophore Alexa488 at a dilution of 1:500. Hoechst 33342 was used as nuclear stain to identify the regions of interest during the analysis. In each well 12 pictures were taken at a 10x magnification covering different positions. For the analysis around 10000 nuclei per well were used.

DATA MINING

To identify the HITs statistical methods were applied to the average expression per well data. Therefore the fluorescent signals were normalized to the average signal of the plate, this compensates for differences within the staining procedure or the microscopy. To finally evaluate the impact of the single knock-downs on the expression of p53 Z-scores were determined:

$$z\text{-}score = \frac{x - \mu}{\sigma}$$

x = average intensity in the well; μ = average intensity of all wells;
σ = standard deviation of the intensities of all wells

The relative p53 expression intensities of the individual siRNAs as well as the sum of the three siRNAs targeting the same kinase were used for the z-score analysis. On the basis of these results the kinases that revealed the strongest down-regulation of mutant p53 expression were further analyzed on the basis of the following three criteria:

1) How many of the siRNAs revealed this down-regulation?
2) Do we see a peak-shift in the histograms of the p53 staining intensities, when comparing the three siRNAs to the negative control siRNAs?
3) Visual inspection of the microscopic raw data. Do we observe morphological changes or increased cytoplasmic staining upon knock-down?

RESULTS

Most chemotherapeutic agents induce a DNA damage response in the cells subsequently leading to apoptosis. This comprises the activation and stabilization of the tumor suppressor p53, mainly through posttranslational modifications (Murray-Zmijewski *et al.*, 2008). It is estimated that 50% of all human tumors carry a p53 mutation, accompanied by a strong accumulation of the mutant p53 protein. Since most of these mutations are substitutions of single amino acids, we expect that at least some of the enzymes that were found to be responsible for the modification of wild type p53 also affect the mutant variants of the protein in response to DNA damage. Therefore, we first investigated whether the evoked DNA damage response upon chemotherapeutic treatment influences the modification of mutant p53 and whether this goes along with a further stabilization of the protein.

4.1. THE ACCUMULATION OF MUTANT P53 UPON DOXORUBICIN TREATMENT

4.1.1. THE EXPRESSION LEVELS OF MUTANT P53 PROTEIN ARE ELEVATED IN RESPONSE TO DOXORUBICIN

U251 cells are derived from a glioma and harbor the hotspot p53 point mutation R273H. As most tumor cell lines, that express a mutated form of p53, these cells accumulate high levels of the protein. Nevertheless, we observed by immunoblotting that the expression levels of p53 in these cells get elevated even further, when treated with the chemotherapeutic drug doxorubicin (Figure 6A).

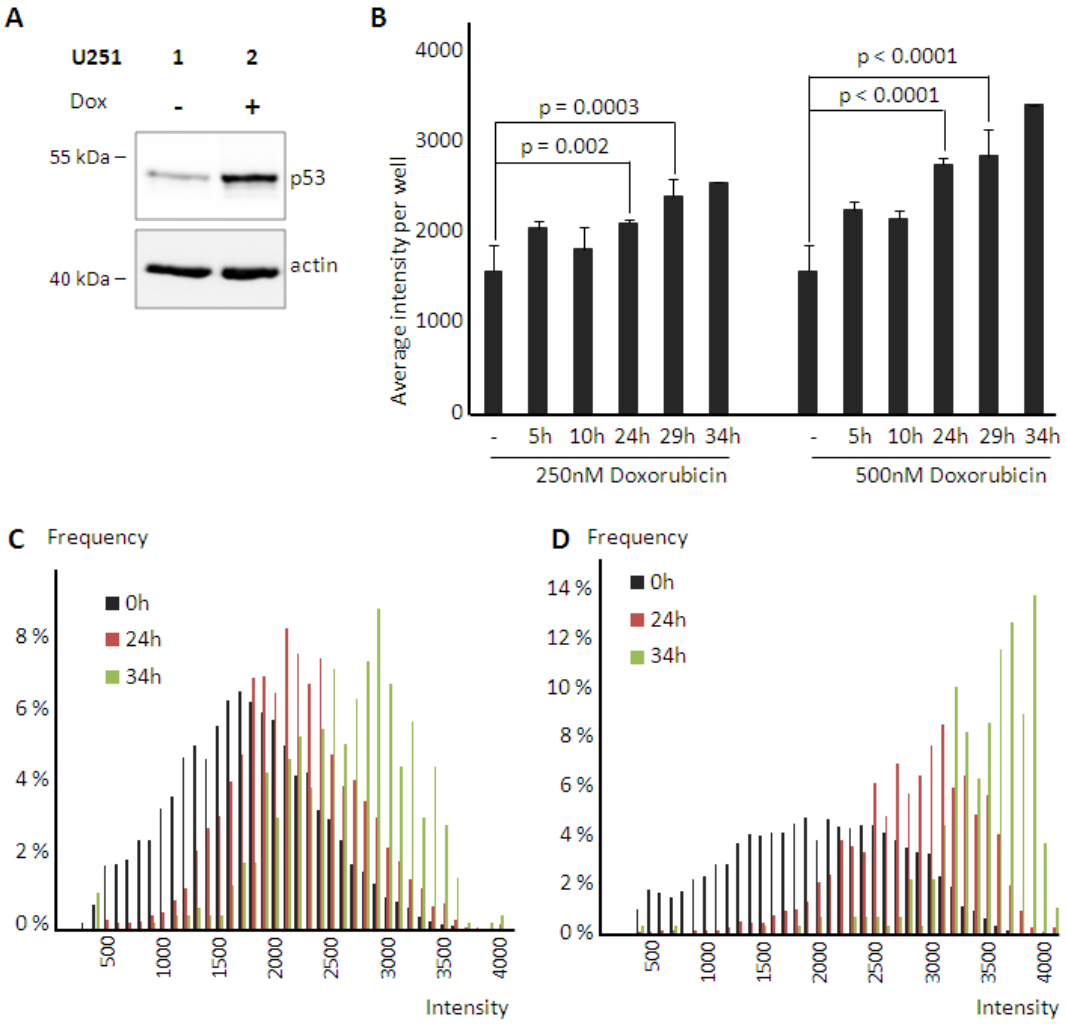


Figure 6: The accumulation of mutant p53 in response to doxorubicin treatment.
U251 cells (p53 R273H) were treated with 500nM doxorubicin for 24h. **A:** Total cell lysates were subjected to immunoblot analysis using antibodies against p53. Actin staining was used as loading control. **B-D:** Cells were fixed and stained for immunofluorescence analysis using antibodies against p53. Single nuclei were identified using Hoechst 33342 staining. The average p53-staining intensity was determined per nucleus. The experiment was performed in triplicate **B:** Means and standard deviations of the average p53-intensities per well are depicted. A Student's t-test (α=0.05) was performed for statistical analysis. **C, D:** Histograms of the p53-intensities per nucleus were generated for three different time-points at 250nM (**C**) and 500nM (**D**) final concentration of the drug.

This result was confirmed by quantitative immunofluorescence microscopy analyzing the p53 expression levels upon doxorubicin treatment in a time dependent manner at two different concentrations. The average expression of p53 was measured for each nucleus. In Figure 6B the mean intensities per well are diagramed, as they were determined in triplicate. The levels increased significantly after 29 hours of treatment using 250nM doxorubicin, as well as

after 24 hours of treatment with a final drug concentration of 500nM. The representation of the data in histograms (Figure 6C and D) shows that with both concentrations the majority of the cells accumulate their mutant p53 protein over time.

The mechanisms by which mutant p53 is generally stabilized in tumor cells are poorly understood. About the further accumulation of the protein upon chemotherapeutic treatment even less is known.

4.1.2. POSTTRANSLATIONAL MODIFICATIONS OF MUTANT P53 ARE INDUCED IN RESPONSE TO DOXORUBICIN TREATMENT, EVEN THOUGH THEY DO NOT DIRECTLY INFLUENCE ITS STABILITY

Kurz *et al.* (2004) showed that doxorubicin acts through the activation of the transducer kinase ATM. Additionally, it is known that ATM phosphorylates p53 at Serine 15, which in turn leads to the acetylation of Lysine 382 of p53 (Dumaz and Meek, 1999; Sakaguchi *et al.*, 1998). This suggests itself that also mutant p53 might get posttranslationally modified at these sites in response to doxorubicin treatment. We therefore analyzed the response of U251 cells to chemotherapeutic drug exposure by immunoblotting using antibodies against Serine 15 phosphorylated and Lysine 382 acetylated p53. We did not detect any modified p53 in untreated cells, but after incubation with doxorubicin for 24h the levels were dramatically increased (Figure 7A).

This result was confirmed using immunofluorescence analysis, quantifying the expression of Serine 15 phosphorylated p53 in individual cells treated with 500nM doxorubicin for 24 hours. The histogram of the obtained data clearly shows a peak shift towards higher intensities upon treatment (Figure 7B).

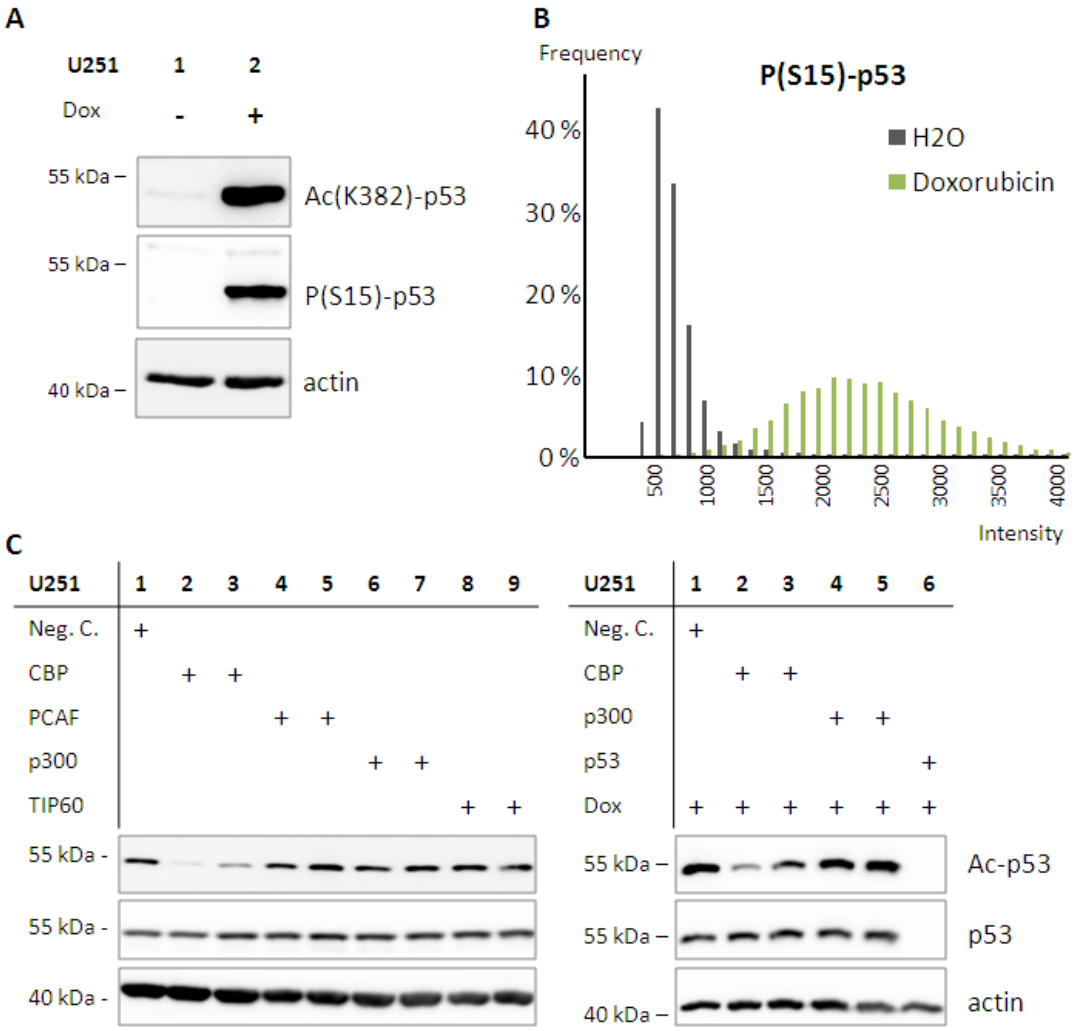


Figure 7: Mutant p53 gets posttranslationally modified upon doxorubicin treatment.
A: U251 cells were treated with 500nM doxorubicin for 24h. Total cell lysates were subjected to immunoblot analysis using antibodies against acetyl(Lys382)-p53 or phospho(Ser15)-p53. Actin staining was used as loading control. **B:** U251 cells were treated for 24 hours with 500nM doxorubicin. The cells were fixed and stained for immunofluorescence analysis using antibodies against phospho(Ser15)-p53. Single nuclei were identified using 10µM doxorubicin staining. The average p53-staining intensity per nucleus is diagramed in a histogram. **C:** U251 cells were reverse transfected with two different siRNAs per gene for 48 hours. As indicated, the samples in the right panel were additionally treated with 500nM doxorubicin for the last 24 hours. Whole cell lysates were subjected to immunoblot analysis using antibodies against acetyl(Lys382)-p53 and total p53. Actin staining served as loading control.

The impact of these modifications is unknown, but we cannot exclude that, as for wt p53, the protein gets stabilized through these modifications. To explore their functional significance with respect to the stabilization of the protein, we used siRNA mediated knock-down of known p53 acetyltransferases, as Ito *et al.* (2002) described their role in the regulation of wild-type p53 stability. 48

hours post transfection of U251 cells with siRNAs, targeting the 4 histone acetyl transferases CREB binding protein (CBP), p300/CBP-associated factor (PCAF), p300, and Lysine acetyl transferase 5 (KAT5 alias TIP60) total cell lysates were subjected to immunoblot analysis. Surprisingly, only the knock-down of CBP led to a clear reduction of Lys382-acetylated p53 in these cells. Nevertheless, this did not have any impact on the expression levels of total p53 protein (Figure 7C, left panel).

Since we have shown that the levels of Lys382-acetylated p53 are increased dramatically in response to doxorubicin (Figure 7A), we additionally investigated whether the knockdown of CBP and p300 impairs the further accumulation of mutant p53 protein levels upon doxorubicin treatment. But, we again did not detect any changes in the expression levels of total p53 protein (Figure 7, right panel). Therefore we conclude that the posttranslational modification of Lysine 382 of mutant p53 is not the primary regulator of its stability, neither in the default state of the cells, nor in response to chemotherapeutic treatment.

4.1.3. U251 CELLS DISPLAY AUGMENTED MRNA LEVELS OF P53 IN RESPONSE TO DOXORUBICIN TREATMENT

Apart from protein stability, many proteins are regulated in their expression on the transcriptional level. Even though there are only a few reports claiming that p53 gets differentially expressed due to transcriptional activation, it appears to be logic that if the general accumulation of mutant p53 is associated with increased half life of the protein the response to chemotherapeutic treatment happens to be regulated by other means. Therefore, we next aimed to test whether doxorubicin mediated accumulation of mutant p53 is caused by transcriptional activation of the gene. To investigate this, we isolated total RNA from U251 cells treated for 24 hours with 500nM doxorubicin. Strikingly,

quantitative real-time PCR analysis revealed that there was about 5 times more p53 mRNA upon doxorubicin treatment (Figure 8).

The activation of E2F1 through ATM, ATR and the checkpoint kinases Chk1 and -2 upon DNA damage leads to its stabilization and preferential transactivation of apoptotic target genes like TAp73 and NOXA (Hershko and Ginsberg, 2004). In our experiments the up-regulation of TAp73 mRNA is always used as a positive control for proper induction of the DNA damage response. It should be noted that the transcription of p53 mRNA in response to doxorubicin is surged as strong, as the well known E2F1 target gene TAp73 (Figure 8).

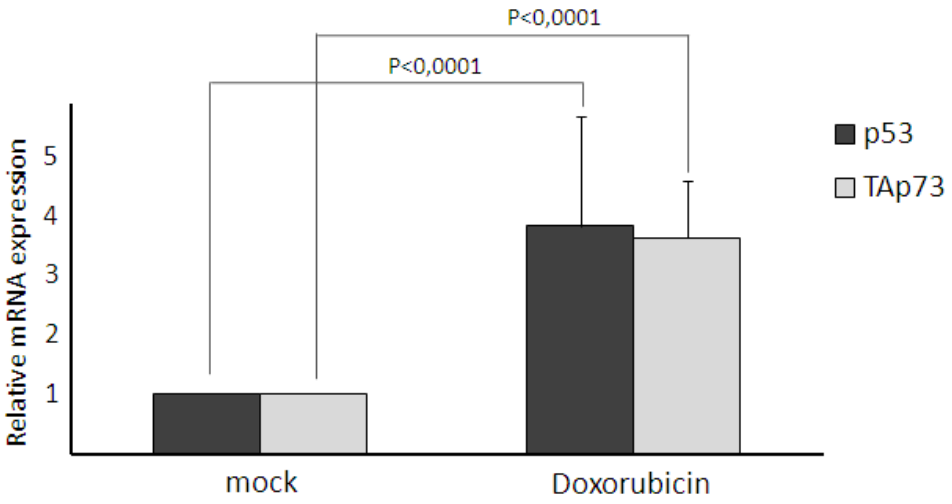


Figure 8: Doxorubicin induces the transcription of p53.
U251 cells were treated with 500nM doxorubicin for 24 hours. Total RNA was isolated and reverse transcribed into cDNA. Relative mRNA expression levels were determined using 36B4 as control gene. The mean and standard deviation of 8 independent replicates were plotted. For statistical analysis a Student's t-test was performed (α=0.05).

4.2. THE MECHANISMS OF P53 TRANSCRIPTIONAL REGULATION

It was previously reported that the two transcription factors HOXA5 and RREB1 contribute to the transcriptional regulation of p53. We confirmed that in our system HOXA5, as well as RREB1 contribute to the up-regulation of p53 mRNA in response to doxorubicin, as it was shown by Raman *et al.* (2000) and Liu *et al.* (2009) respectively (data not shown).

4.2.1. THE TRANSCRIPTION FACTORS E2F1 AND TAP73 ARE NECESSARY FOR THE INDUCTION OF P53 IN RESPONSE TO DOXORUBICIN

In addition to the above, Ren *et al.* (2002) published a ChIP-on-chip study where the promoter of p53 was found occupied by the transcription factor E2F4 four fold over average. Nevertheless, E2F4 is thought to be primarily involved in the repression of E2F-responsive genes (Dyson, 1998), whereas its homolog E2F1, which is known to be stabilized and activated in response to doxorubicin, is a potent transcriptional activator of its target genes (Dyson, 1998).

As a first step to determine whether E2F1 regulates the expression of p53, the messenger RNA levels of p53 were analyzed in response to doxorubicin treatment upon the previous knock-down of E2F1. The up-regulation of TAp73 transcription in this experiment occurred mainly through the activation of E2F1, as the increased transcription of TAp73 is abolished completely after knock-down of E2F1 with two different siRNAs (Figure 9, light grey bars). At the same time we observed that the knock-down of E2F1 diminishes the accumulation of p53 mRNA (Figure 9, dark grey bars) upon doxorubicin to a large extent even though the effect is not as strong, as it was observed for TAp73. The knock-down efficiencies for both siRNAs were very high as determined by qRT-PCR (Figure 9, upper chart) and immunoblot analysis (Figure 10C).

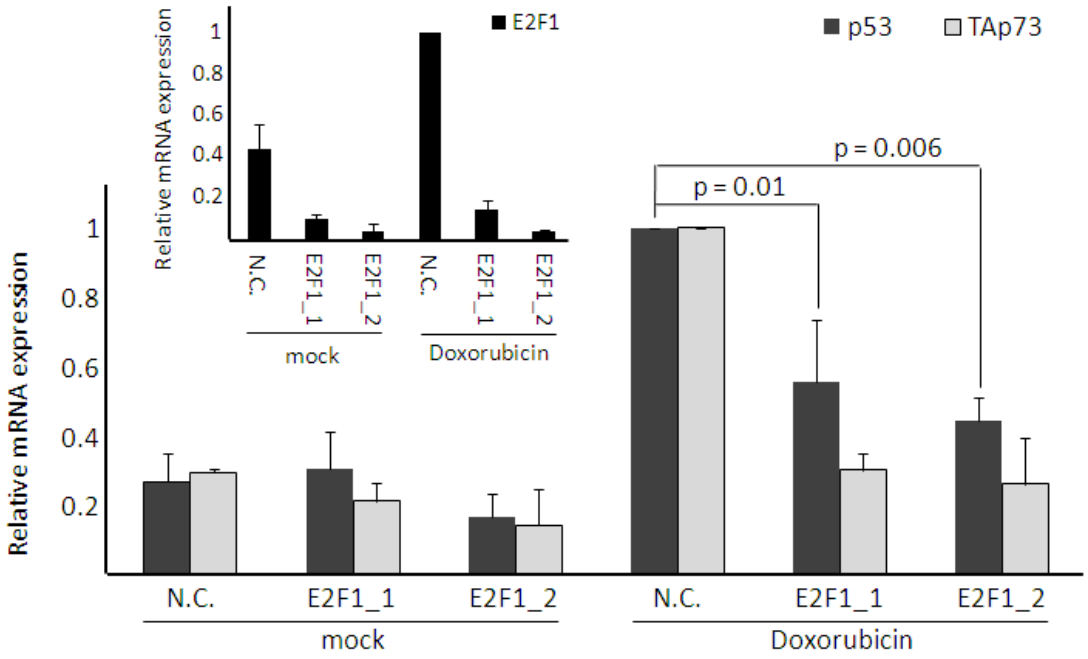


Figure 9: The knock-down of E2F1 alleviates the effects of doxorubicin on p53 transcription
U251 cells were transfected with siRNAs targeting E2F1 for 24 hours, before they were treated with 500nM doxorubicin for additional 24 hours. Total RNAs were reverse transcribed and quantified, relative to 36B4, by real-time PCR. The means and standard deviations of four biological replicates are depicted. For statistical analysis a Student's t-test was performed (α=0.05).

To further support the theory that the augmented transcription of p53 upon doxorubicin treatment is dependent on the transcription factor E2F1, we performed quantitative immunofluorescence microscopy, as well as immunoblotting. In both cases, we observed that also on the protein level the induction of p53 through doxorubicin treatment gets diminished by the knock-down of E2F1 (Figure 10).

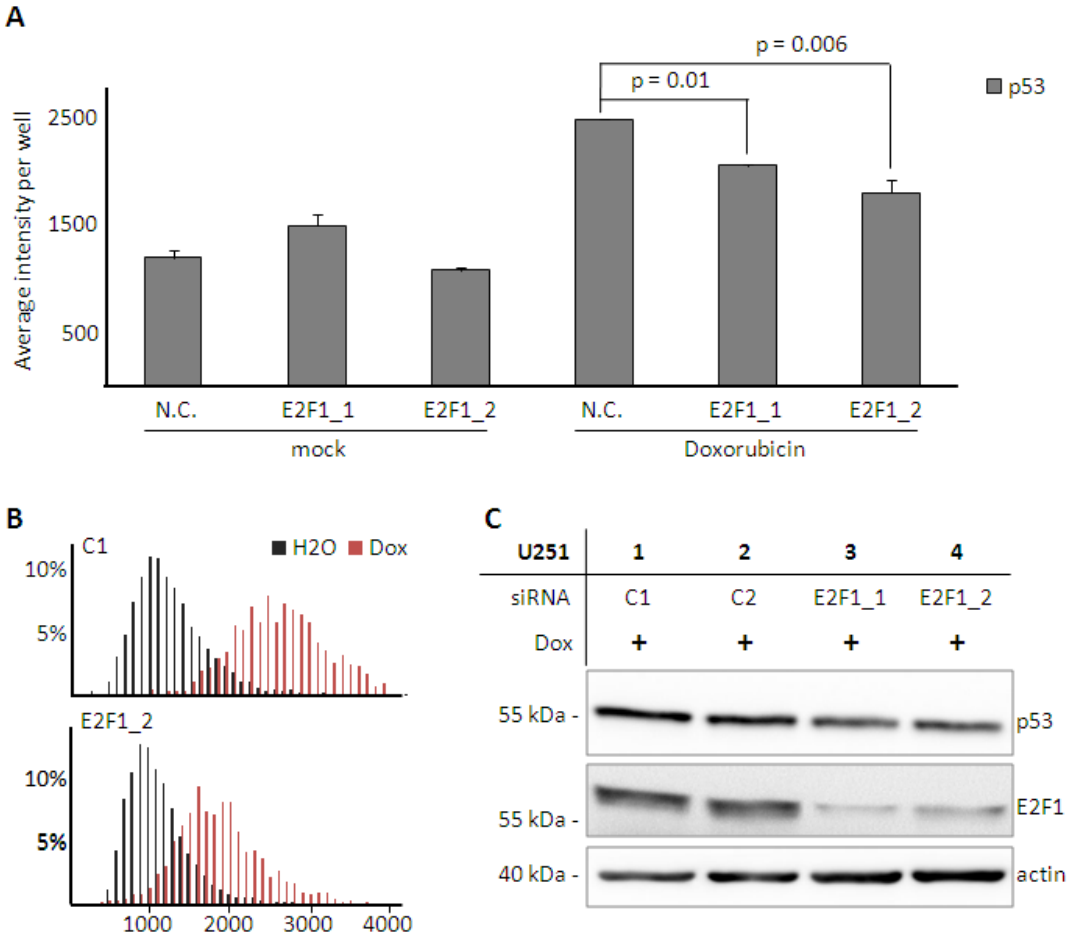


Figure 10: E2F1 contributes to the enhanced protein levels of mutant p53 upon doxorubicin.
U251 cells were transfected with two different siRNAs targeting E2F1 for 24 hours, and then they were treated for additional 24 hours with 500nM doxorubicin. **A, B:** The cells were fixed and stained for immunofluorescence microscopy. Single nuclei were identified using 10µM doxorubicin. The p53 staining was quantified per nucleus. **A:** The average intensity per well was determined in triplicate, means and standard deviations are depicted. For statistical analysis a Student's t-test was performed (α=0.05). **B:** Representation of the data in histograms. **C:** The cells were harvested for immunoblot analysis and stained for p53 as well as E2F1. Actin was used as a loading control.

Bearing in mind the observations of Wang and el-Deiry (2006) that p53 and TAp73 are capable of directly regulating the transcription of p53, the observed may in part be due to the E2F1 dependent induction of TAp73.

To investigate whether E2F1 is only indirectly acting on p53 through the co-regulation of its target gene TAp73 we used siRNA mediated knock-down of the p53 paralog. And indeed, TAp73 is as well necessary for the induction of p53 mRNA (Figure 11), even though it should be noted that the knock-down of

E2F1 had a stronger effect on the expression of p53 mRNA compared to the knock-down of p73, whereas the expression levels of TAp73 were unequivocally lower in the latter case. Therefore, we believe that E2F1 is acting on the transcriptional regulation of p53 not exclusively through TAp73.

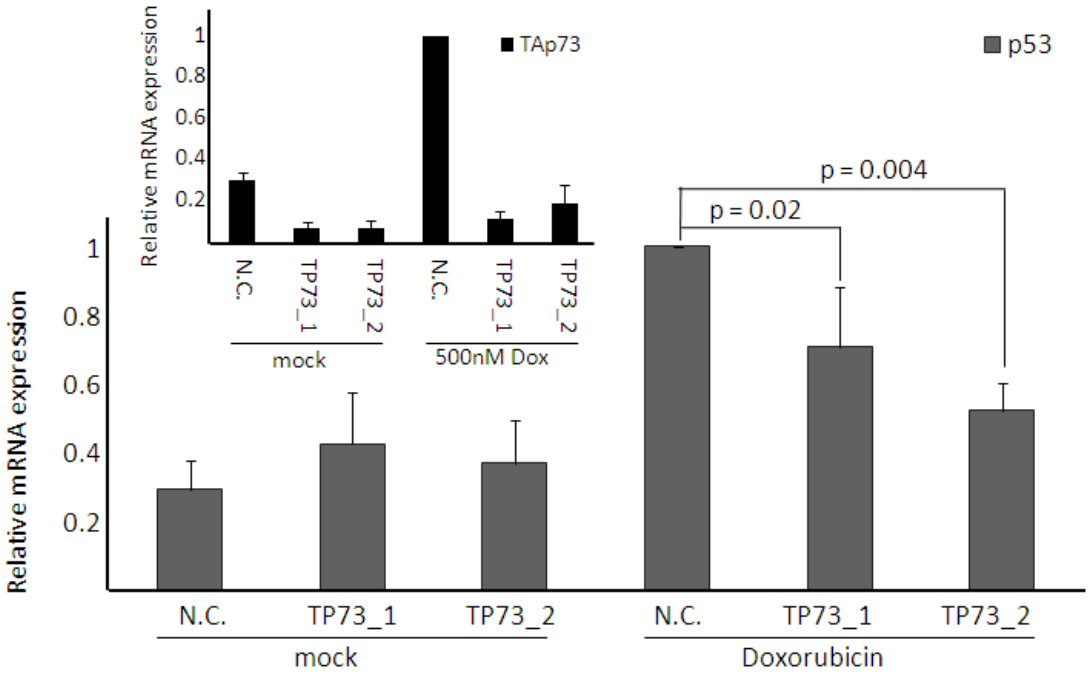


Figure 11: The induction of p53 transcription is partially dependent on p73.
U251 cells were treated with 500nM doxorubicin for 24 hours. The isolated RNA was reverse transcribed and the expression of p53 and TAp73 was quantified relative to 36B4 using real-time PCR. The experiment was performed in four biological replicates. The means and standard deviations were plotted. For statistical analysis a Student's t-test was performed (α=0.05).

4.3. E2F1 REGULATES P53 DIRECTLY AS A TRANSCRIPTION FACTOR

The finding that E2F1 knock-down has a stronger effect on the doxorubicin induced augmentation of p53 transcription, compared to p73, pinpoints to the additional involvement of E2F1 in the regulation of p53. To identify potential E2F1 binding sites and their biological relevance we first used bioinformatical tools to *in silico* predict potential binding sites that were then confirmed by chromatin immunoprecipitation analysis (ChIP).

4.3.1. *IN SILICO* IDENTIFICATION OF POTENTIAL E2F1 BINDING SITES WITHIN THE P53 PROMOTER REGION

E2F1 is an important transcription factor regulating the expression of various genes in response to its activation through DNA damage or other stimuli. The DNA sequence TTTSSCGC is described to be the canonical binding site motif for E2F1; nevertheless, some ChIP-on-chip studies revealed that a large proportion of E2F binding occurs at sites where this recognition sequence cannot be found (Bieda *et al.*, 2006). In collaboration with Martin Haubrock (Department of Bioinformatics, University of Göttingen) all these ChIP-on-chip data sets were used to generate a scoring matrix, helping to identify potential E2F1 binding sites (Table 1).

Table 1: Scoring matrix for the identification of potential E2F1 binding sites
Sequence information from known E2F1 binding site motifs were integrated to generate a scoring matrix for the identification of potential new E2F1 binding sites. The resulting consensus motif is displayed in the left column. N: any (A, C, G, or T); K: ketone (G or T); S: strong bonds (C or G)

Nucleotide / Consensus	A	C	G	T
N	1	4	3	5
K	0	1	5	7
T	2	0	0	11
S	0	7	6	0
S	0	5	8	0
C	0	10	3	0
G	0	3	10	0
C	0	8	4	1

This matrix was then applied to the genomic sequence around the transcriptional start site (TSS) of p53 in order to identify potential binding sites. In Figure 12A the determined scores are plotted against the genomic region. Two sites within the analyzed sequence revealed a score greater than 0.9 and

were therefore considered as potential E2F1 binding sites. First, E2F1 BS-I, at position 7590195-7592195 (hg19 chromosome 17) with the sequence ACTGGCGC revealed a score of 0.911, and second, E2F1 BS-II, at position 7590195-7592195 (hg19 chromosome 17) with the sequence TTTCGCGG resulted in a score of 0.954. Analysis of the evolutionary conservation of the predicted binding sites showed that E2F1 BS-I close to the TSS is highly conserved, whereas E2F1 BS-II even though displaying a higher score lacks this conservation (Figure 12B).

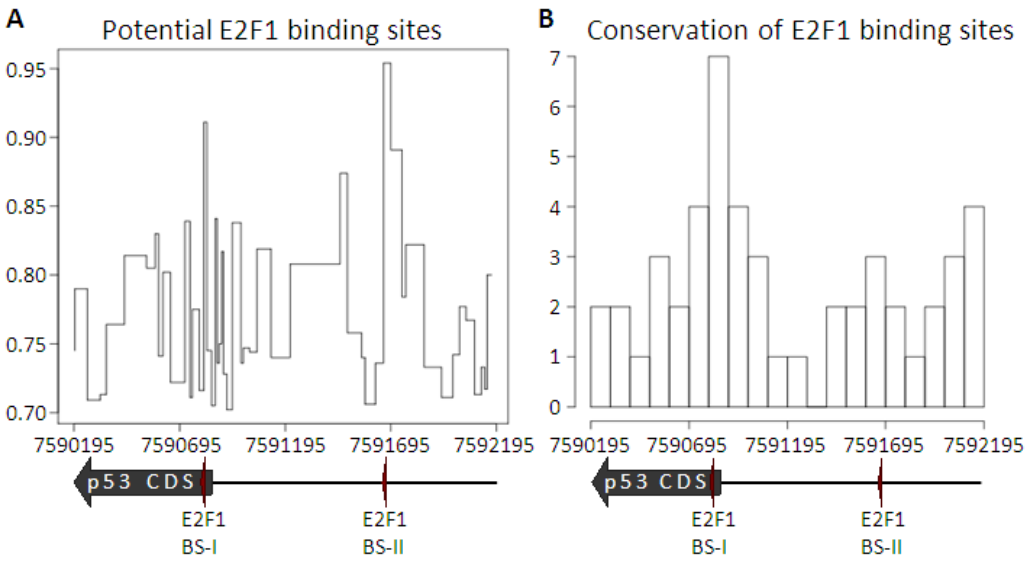


Figure 12: Bioinformatical analysis of the p53 promoter uncovers two E2F1 binding sites.
A scoring matrix (Table 1) to discover potential E2F1 binding sites was applied to the DNA sequence of the p53 promoter region (-1500 to +500 around the TSS). **A:** The calculated scores are plotted against the genomic region. **B:** The level of conservation within the analyzed genomic region is depicted. **A, B:** A schematic representation of the p53 gene locus, with the two binding motifs displaying the highest score and sequence conservation, is shown underneath both plots.

4.3.2. THE TRANSCRIPTION FACTOR E2F1 BINDS TO ONE OF THE POTENTIAL E2F1 BINDING SITES WITHIN THE P53 PROMOTER REGION

Within 2000 base pairs around the TSS of p53 two potential E2F1 binding sites were identified using bioinformatical tools. In order to confirm the biological relevance of these binding sites ChIP analysis was performed. E2F1 protein,

cross linked to its DNA binding elements, was precipitated using antibodies, generated against the transcription factor. ChIP-grade IgG antibodies were used for negative control precipitation. The recovered DNA was amplified by quantitative real-time PCR using primers spanning the two potential E2F1 binding sites on the p53 promoter. Additionally, primers spanning the well known E2F1 binding sites on the p107 and TAp73 gene loci were used as positive controls. Primers amplifying a region 19.5kb downstream of the p53 TSS, a region where no binding of E2F1 is expected, served as negative control. The data show that E2F1 is bound to the predicted E2F1 BS-I roughly 40bp downstream of the TSS, but not to the less conserved second potential binding site (Figure 13, upper panel).

As expected, E2F1 also associated with the promoters of its target genes p107 and TAp73. In contrast, the recovery of a distant fragment of the p53 genomic locus (p53 +19.5kb) was at the background level (similar to precipitation with non-specific IgG) (Figure 13, lower panel).

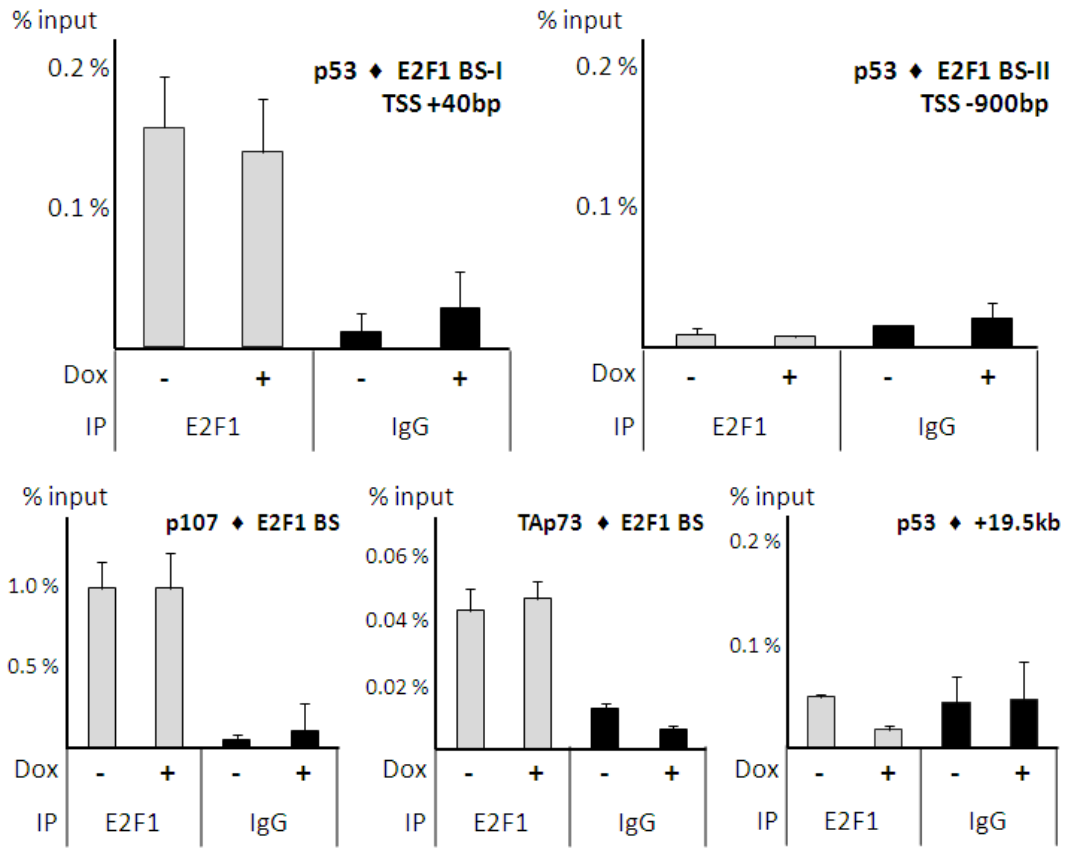


Figure 13: E2F1 is bound to one of the predicted binding sites under physiological conditions.
U251 cells were treated with 500nM doxorubicin for 24 hours. Cross linked DNA – protein complexes were precipitated using antibodies against E2F1 or IgG, as negative control. The recovered DNA was quantified by real-time PCR using primers flanking the two potential E2F1 binding sites, as well as two positive control sequences (p107 and TAp73) and a negative control region (p53 +19.5kb). The means and standard deviations of three replicates are depicted as percentile of the input DNA.

4.4. IS THE INCREASED TRANSCRIPTION OF P53 A GENERAL RESULT OF CHEMOTHERAPEUTIC TREATMENT?

4.4.1. THE INFLUENCE OF DIFFERENT CHEMOTHERAPEUTIC DRUGS ON THE TRANSCRIPTIONAL REGULATION OF P53 EXPRESSION

Treatment of U251 cells with the topoisomerase I inhibitor camptothecin (CPT), the topoisomerase II inhibitor etoposide, as well as the nucleoside analog 5-fluorouracil revealed that the induction of p53 transcription is specific to doxorubicin, rather than a general result of the evoked DNA damage response.

The treatment of the cells with CPT resulted in a very strong induction of NOXA, another target gene of E2F1, whereas the induction of TAp73 and p53 was not detected under these circumstances (Figure 14A). Also 5-fluorouracil (5'FU), as nucleoside analog, caused similar to CPT an induction of NOXA, but neither TAp73 nor p53 were elevated in their expression (Figure 14A).

Additionally, we tested etoposide, a chemotherapeutic drug more closely related to doxorubicin, since it also acts through the inhibition of topoisomerase II. In contrast to CPT and 5'FU this drug led to the induction of TAp73, as it was previously observed for doxorubicin. But, to our surprise, the levels of p53 messenger RNA remained unaffected (Figure 14A). Other than the mentioned induction of TAp73 transcription, we also observed that etoposide treatment mediates an increase in Serine 15 phosphorylation of p53 in U251 cells (Figure 14B). This could be explained by the evoked DNA damage response and the accompanied activation of ATM. Nevertheless, we observed in the same experiment that the total levels of p53 protein remain unchanged in response to etoposide treatment (Figure 14C).

In conclusion, these data show that the induction of TAp73 in the DNA damage response seems not to be sufficient to augment the transcription of p53, although we demonstrated before (4.2.1) that it is necessary.

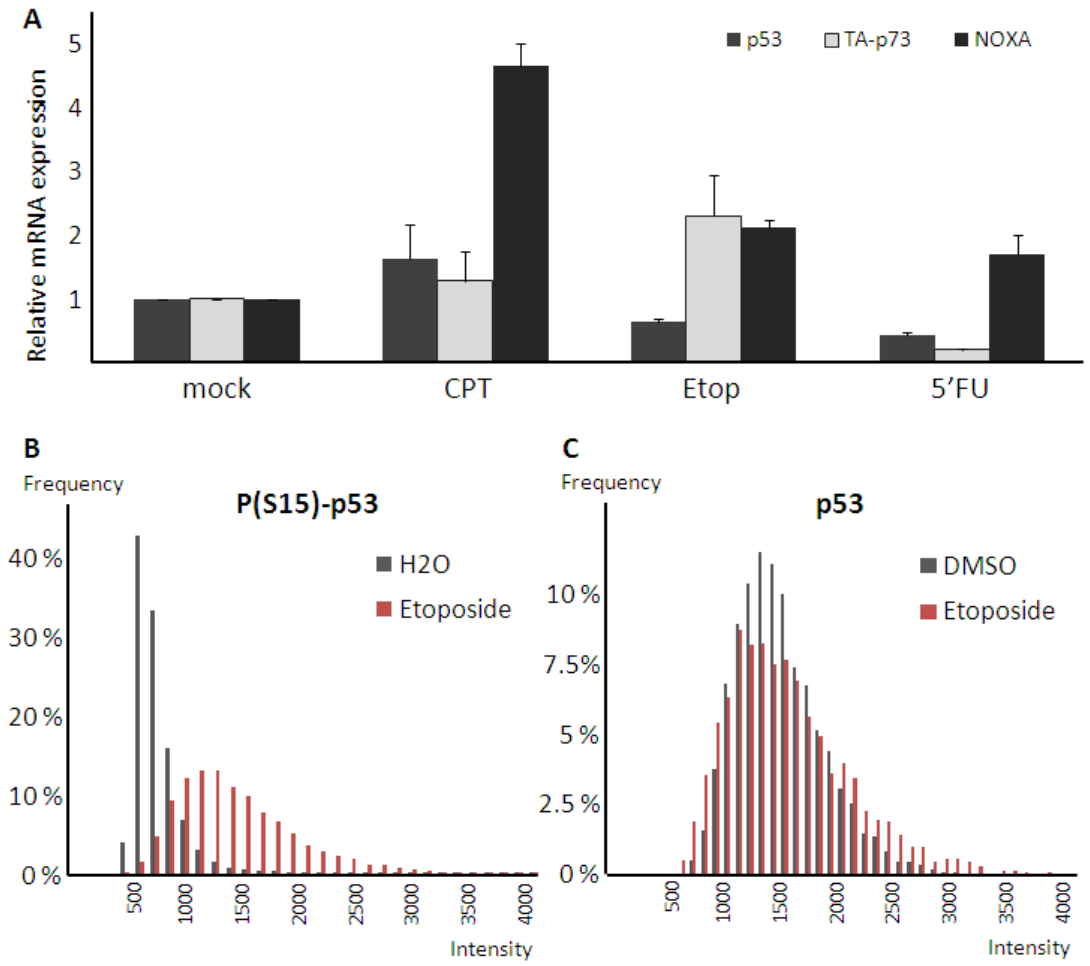


Figure 14: The induction of p53 is not simply caused by the inhibition of topoisomerase. **A:** U251 cells were treated with CPT (2,87µM), etoposide (100µM), or 5'FU (500µM) for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. The means and standard deviations of three biological replicates are depicted. **B, C:** U251 cells were treated with 100µM etoposide for 24 hours each. Upon fixation, the cells were stained for immunofluorescence analysis using antibodies against phospho(S15)-p53 (**B**) or total p53 (**C**). Single nuclei were identified using 10µM doxorubicin. The average fluorescence intensity per nucleus was determined. The results are represented in histograms.

4.4.2. THE IMPACT OF DIFFERENT ANTHRACYCLINES ON THE TRANSCRIPTIONAL REGULATION OF P53

The mechanism by which doxorubicin acts in the cell is controversially discussed in the literature, some reports claim that its ability to intercalate into DNA leads to decreased transcription through inhibition of helicase activity (Bachur *et al.*, 1992) or through DNA cross linking (Swift *et al.*, 2006), others claim that doxorubicin mainly functions by stalling of topoisomerase II on the

DNA and the stabilization of a reaction intermediate in which the DNA strands are cut and covalently linked to the enzyme (Tewey *et al.*, 1984a; Tewey *et al.*, 1984b). Additionally, there are mechanisms of free radical formation, DNA alkylation, direct membrane effects, and direct induction of apoptosis discussed (Gewirtz, 1999). The observation that etoposide, in contrast to doxorubicin, lacks the ability to induce p53 transcription, argues against the theory that the general inhibition of topoisomerase II activity is sufficient to induce the transcription of p53. In addition to doxorubicin, there are other anthracyclines currently used in the clinics, namely daunorubicin, epirubicin, and idarubicin. These drugs are described to have slightly different sequence specificities, but are all believed to interfere with DNA transcription through intercalation as well as topoisomerase II inhibition (Minotti *et al.*, 2004).

These 4 structurally related drugs share a common tetracyclic ring system containing an anthraquinone chromophore with a daunosamine moiety attached to the A-ring (C7), but they differ in their substitutions to this basic structure (Figure 15A, differences compared to doxorubicin are highlighted with red circles). Interestingly, analysis of their ability to induce the transcription of p53 revealed that dauno-, doxo-, and epirubicin induced p53 in a comparable manner. Whereas idarubicin, differing from daunorubicin only in a methoxy-group at C4 (D-ring), has no effect on p53 transcription, even though it induces TAp73 the same way as the other three compounds (Figure 15B).

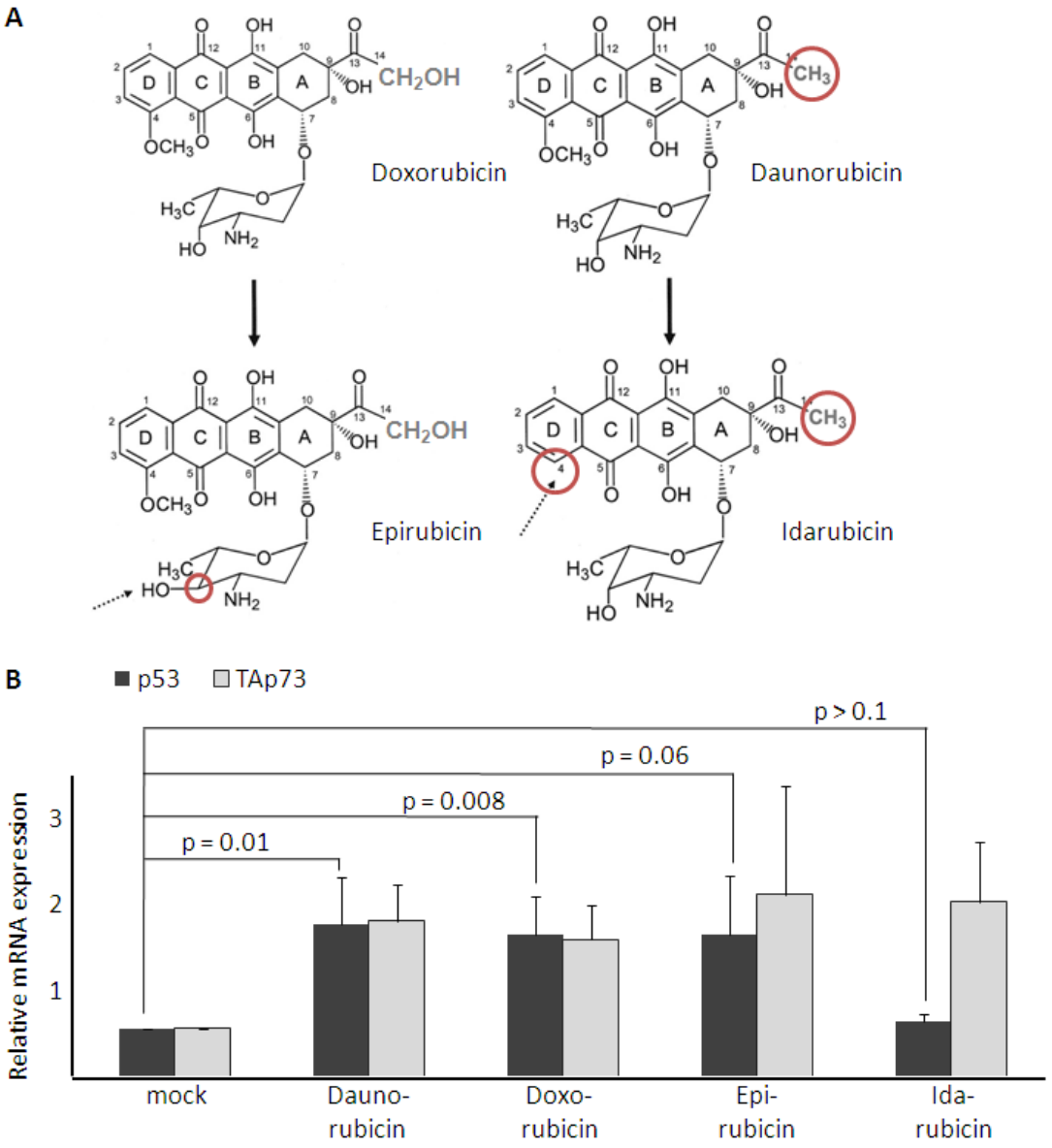


Figure 15: Three out of four anthracyclines activate the transcription of p53.
A: Chemical structure of the four anthracyclines doxorubicin, daunorubicin, epirubicin and idarubicin [Adopted from Minotti *et al.* (2004)]. Differences with respect to doxorubicin are highlighted with red circles. **B:** U251 cells were treated with 500nM dauno-, doxo-, epi-, or idarubicin for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. The experiment was performed in triplicate. The means and standard deviations of the three experiments are diagramed. For statistical analysis a Student's t-test was performed (α=0.05).

4.4.3. ANTHRACYCLINE MEDIATED ACTIVATION OF P53 TRANSCRIPTION

Quantification of the p53 pre-mRNA expression revealed that upon doxorubicin treatment not only the levels of mature p53 mRNA are elevated, which could as well be explained by increased mRNA stability, but also the direct product of

transcription, the pre-mRNA. This argues in favor of the hypothesis that the two transcription factors TAp73 and E2F1 facilitate the active transcription of the p53 gene (Figure 16).

Interestingly, we found that idarubicin, the anthracycline that is structurally related to doxorubicin, but not capable of augmenting the levels of p53 mRNA, induced the expression of p53 pre-mRNA the same way as the other anthracyclines (Figure 16; data not shown).

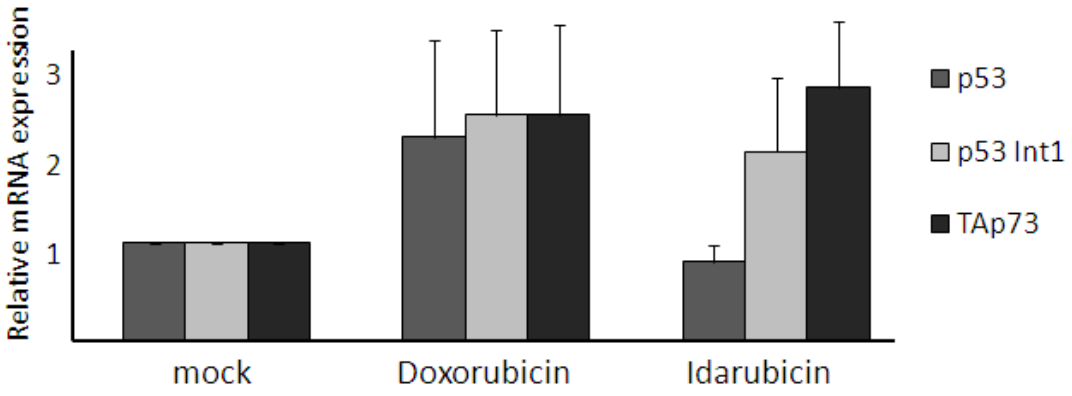


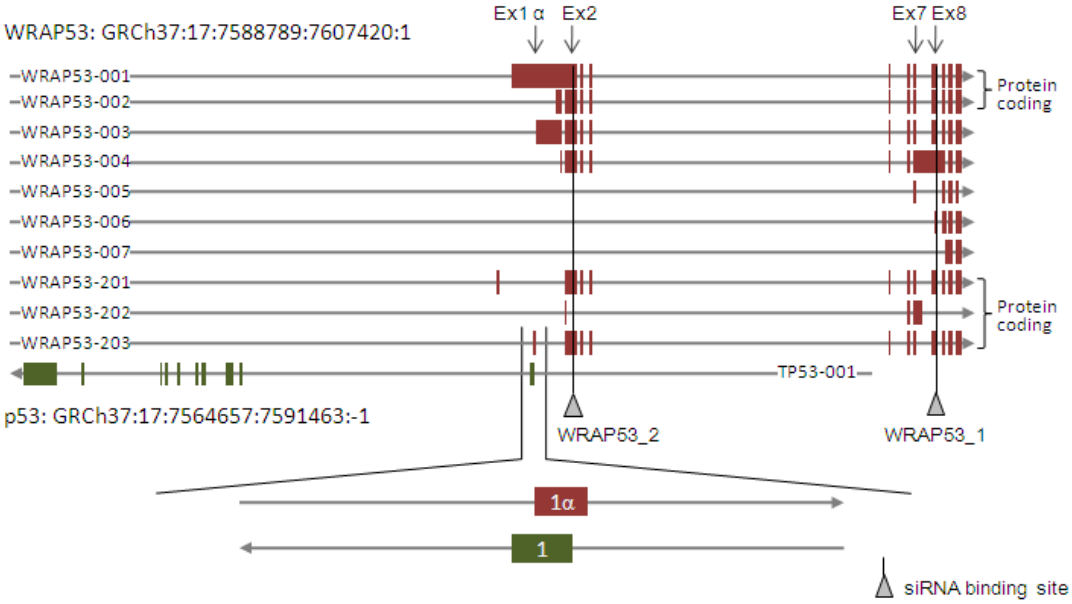
Figure 16: p53 pre-messenger RNA is elevated in response to all anthracyclines.
U251 cells were treated with 500nM doxorubicin or idarubicin for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. Means and standard deviations of three biological replicates are depicted.

4.5. WRAP53 AS REGULATOR OF P53 MRNA EXPRESSION

The recent finding of Mahmoudi *et al.* (2009) that the pre-mRNA stability of p53 is regulated through a natural antisense transcript prompted us to investigate whether the expression of the described WRAP53 (WD repeat containing, antisense to p53) gene contributes to the regulation of p53 in response to anthracyclines.

WRAP53 was identified as a gene located immediately upstream of *TP53* on the opposite strand. Mahmoudi *et al.* (2009) found at least 17 variants of this gene, generated through alternative splicing, as well as three different TSS. In Figure 17 the 10 transcripts listed in the Ensembl database were aligned with the p53 gene. Two of the transcripts (WRAP53-001 and WRAP53-203) contain

exon 1α, which is overlapping with a large portion of exon 1 of *TP53*. So far the function of WRAP53 protein is unknown; whereas it is claimed by Mahmoudi *et al.* (2009) that exon 1α of the WRAP53 mRNA contributes to the induction of p53 in response to DNA damage.



Transcript \ Primer	WRAP53 1α	WRAP53 1α-2	WRAP53 7-8
WRAP53-001	X	—	X
WRAP53-002	—	—	X
WRAP53-003	—	—	X
WRAP53-201	—	—	X
WRAP53-203	X	X	X

Figure 17: Schematic representation of the *TP53* genomic locus on chromosome 17
The 10 transcripts of WRAP53 listed in the Ensembl database are aligned with *TP53*. Both genes are encoded within the same genomic locus on opposite strands of the DNA. Exon 1α of WRAP53 and exon 1 of p53 overlap with the majority of their sequence, as depicted in the zoom-in. A table of the transcripts amplified using three different primer pairs is depicted in the lower part.

To explore the functional significance of WRAP53 in the context of anthracycline induced transcription of p53, we analyzed its mRNA expression using three different primer pairs: WRAP53 7-8 is used to amplify the majority of the transcripts independent of the TSS; WRAP53 1α is used to amplify both transcripts containing exon 1α; and WRAP53 1α-2 is used to specifically amplify WRAP53-203 (an overview of the transcripts targeted by the primer pairs is listed in Figure 17, lower part).

In response to doxorubicin we observed a slight up-regulation of WRAP53 1α. In contrast to that, our analysis yielded a massive induction of the WRAP53 transcripts containing exon 1α in response to idarubicin (Figure 18A), the anthracycline that was shown before to induce the pre-mRNA levels of p53 (4.4.3), but keeps the levels of mature mRNA low (4.4.2). An even stronger induction of WRAP53 1α was observed in response to etoposide treatment (Figure 18B), the topoisomerase II inhibitor that as well induced the DNA damage response through TAp73, but failed to augment the expression levels of p53 (4.4.1).

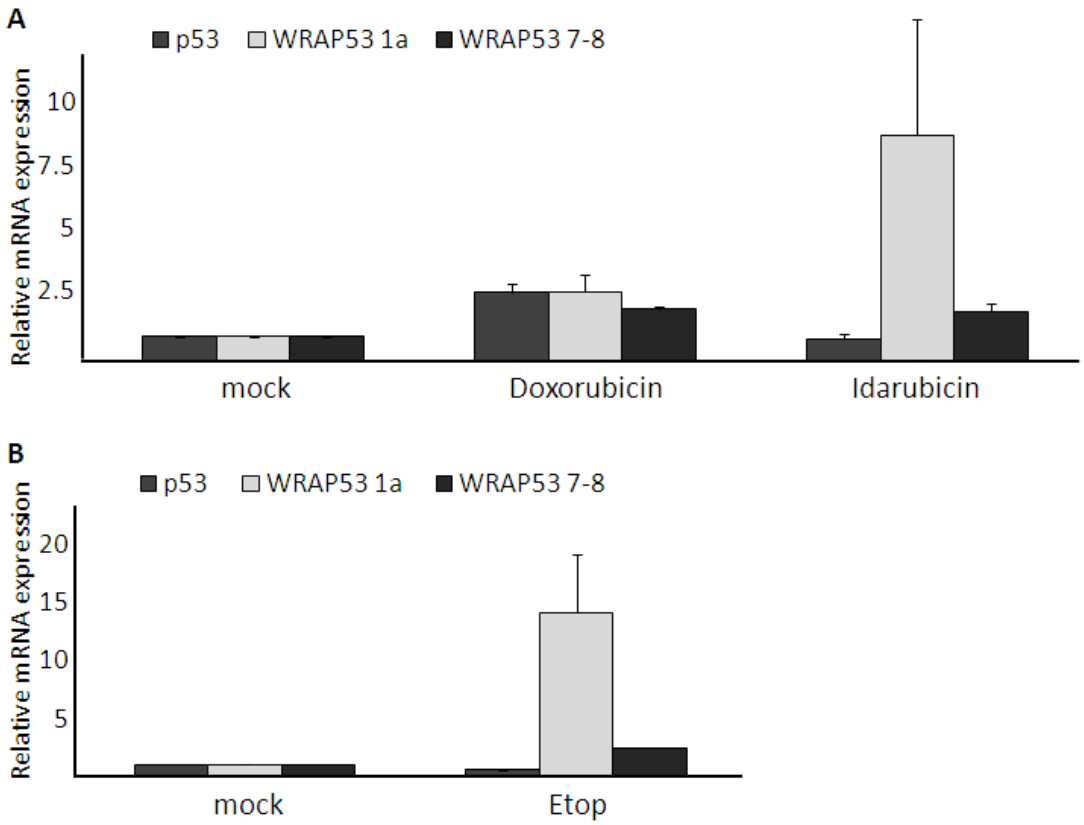


Figure 18: The natural antisense transcript of p53 is expressed antagonistic to p53 itself. U251 cells were treated with 500nM doxorubicin or idarubicin (**A**) or 100µM etoposide (**B**) for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. The depicted diagram represents the means and standard deviations of four biological replicates (**A**), experiment **B** was performed in triplicate.

The total levels of WRAP53 are slightly induced in response to any kind of DNA damage that was subjected to the cells, but remained the same for idarubicin

and etoposide, when compared to doxorubicin treatment (Figure 18A, B; black bars). These data show a clear correlation between the induction of WRAP53 transcripts containing exon 1α and the diminished response of p53 mRNA expression to E2F1 activation. Nevertheless, it should be mentioned that these data stand in contrast to the study published by Mahmoudi *et al.* (2009), where WRAP53 was shown to stabilize p53 mRNA in response to DNA damage.

Quantification of the p53 mRNA levels, upon siRNA mediated knock-down of WRAP53 (the siRNA target sites are depicted in Figure 17) and subsequent treatment with idarubicin was used to elicit the role of WRAP53 in the regulation of p53. To our surprise, the levels of p53 mRNA remained low upon knock-down of the antisense transcript WRAP53 and subsequent idarubicin treatment (Figure 19, white bars). Nevertheless, when we checked for the knock-down efficiency of the used siRNAs, we observed a discrepancy depending on the primer pair that was used for the analysis. Quantification of WRAP53 mRNA using primers to amplify either all transcripts, or specifically WRAP53-203 led to a reduction upon siRNA transfection of about 90%. Whereas, the usage of primers amplifying all transcripts containing exon 1α revealed that almost 60% of mRNA escaped the knock-down (Figure 19). A possible explanation for this discrepancy is that a WRAP53 transcript exists that contains exon 1α, but lacks exon 2 and 8, where the used siRNAs bind. Alternatively, it could be suggested that due to RNA masking or inhibited nuclear export, this WRAP53 transcript escapes the siRNA mediated knock-down.

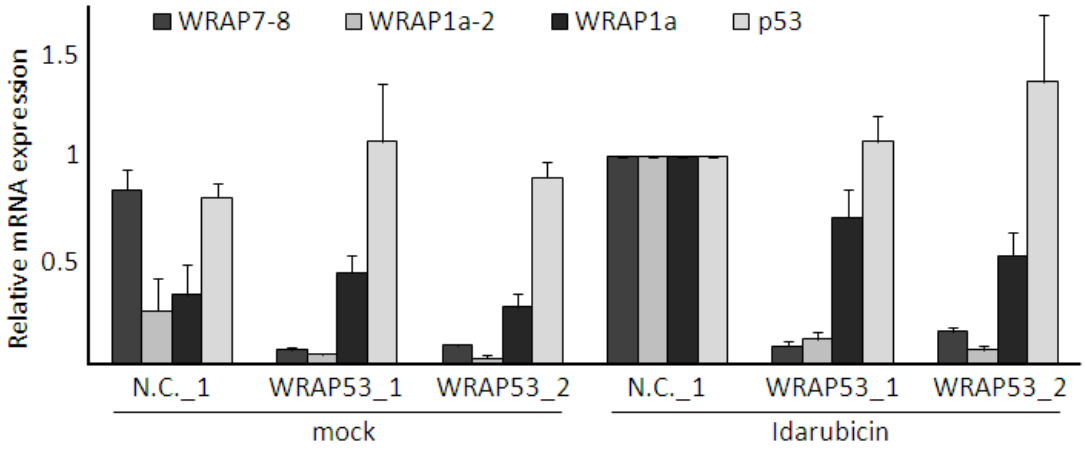


Figure 19: Not all transcripts of WRAP53 are targeted by the used siRNAs.
U251 cells were long-term transfected with two different siRNAs targeting WRAP53. Total RNA was isolated and reverse transcribed. Quantitative real-time PCR was used to determine the expression of the different WRAP transcripts as well as p53 mRNA. 36B4 was used as reference gene. Means standard deviations of three biological replicates are diagramed.

4.6. IS THE OBSERVED TRANSCRIPTIONAL REGULATION OF P53 THROUGH TOPOISOMERASE II INHIBITORS LIMITED TO U251 CELLS?

An important feature of tumor derived cell lines is their individual collection of mutations, rendering their physiological balance between different pathways. Thus, the behavior of cell lines might be different when they get exposed to certain stimuli. To substantiate that the presented mechanisms of p53 transcriptional regulation have general validity, we analyzed the effect of doxorubicin treatment using additional cell lines, also harboring different p53 mutations, as well as wild type p53.

A431 cells, derived from an epidermoid carcinoma (p53 R273H) and the bladder carcinoma cell line 5637 (p53 R280T) displayed an up-regulation of p53 mRNA levels in response to doxorubicin. In both cases the transcription of p53 is induced even stronger than that of TAp73, the gene that served throughout the study as a positive control for the triggered DNA damage response (Figure 20A). In contrast to these results we observed that the osteosarcoma cell line U2OS, that was used as a representative of wt p53

expressing cells, responded to doxorubicin treatment with an invariant expression of p53 mRNA (Figure 20A). Similar data were obtained for the colon carcinoma derived cell line HCT116 that as well expresses wt p53 (data not shown).

According to the general knowledge of the pathways within the p53 network, we hypothesized that a negative feedback loop from p53, via the cyclin dependent kinase inhibitor p21 and the Retinoblastoma protein, to E2F1 explains the decreased response of p53 mRNA expression to doxorubicin treatment. Of note, due to p53 loss of function, this feedback loop is permanently silenced in cells expressing mutant p53. To investigate the impact of the mentioned feedback loop in U2OS cells, we used siRNA mediated knock-down of p21, to intercept the pathway. The treatment of U2OS cells with doxorubicin after silencing of p21 resulted in the same slight up-regulation of p53 mRNA levels, as it was observed upon control siRNA transfection (Figure 20B). This indicates that the mentioned negative feedback loop, from transcriptionally active p53 via p21 to E2F1, is not causing the diminished response of p53 mRNA expression to doxorubicin treatment.

The result, we obtained in U2OS cells upon doxorubicin treatment, reminded us of, what we have seen in U251 cells with idarubicin before, the levels of TAp73 increase, but there is almost no change in the p53 expression. This prompted us to investigate, whether the expression of the p53 natural antisense transcript WRAP53 might again be involved in the regulation of p53. Strikingly, this theory was approved, treatment of U2OS cells with doxorubicin leads as well to the up-regulation of WRAP53 1α (Figure 20C) and thereby resembles another example of inverse correlation between the expression of opposing transcripts from the *TP53* locus.

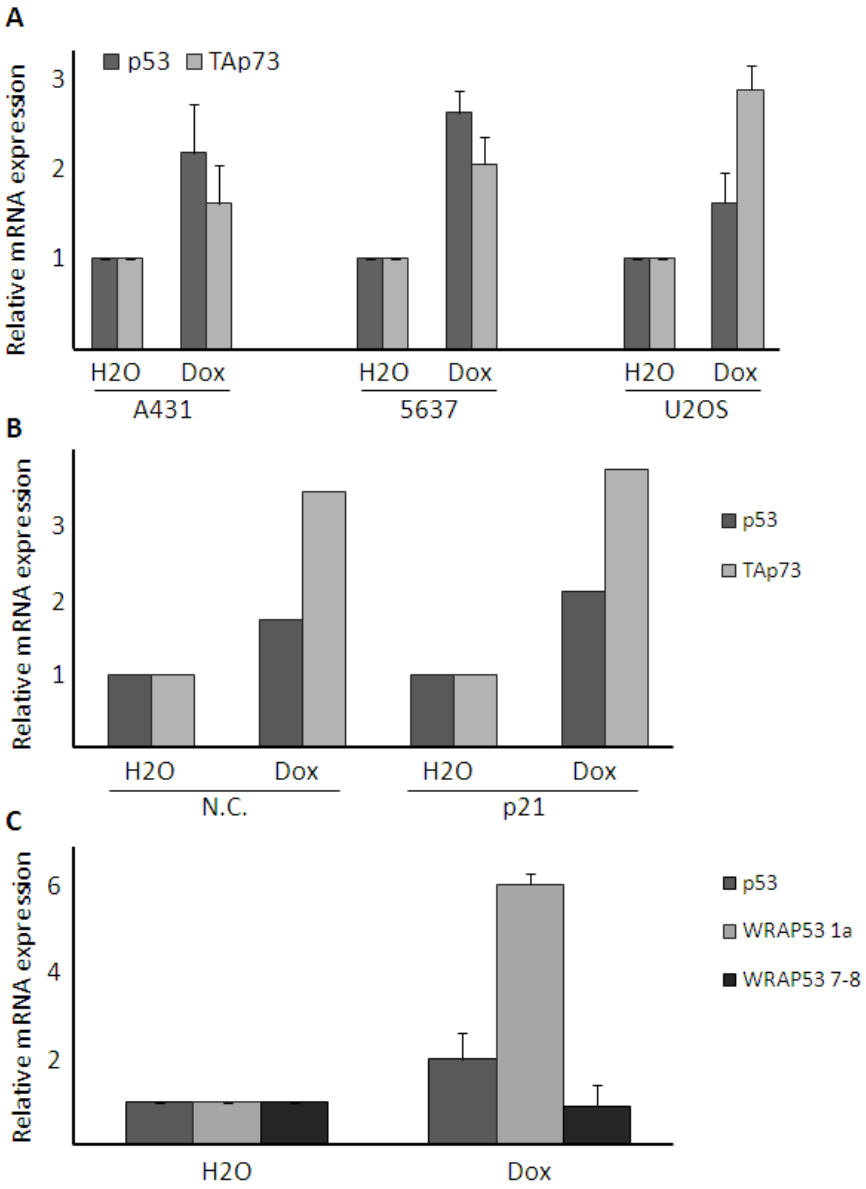


Figure 20: The discovered mechanisms of p53 regulation also apply to other cell lines
A: A431, 5637, and U2OS cells were treated with 500nM doxorubicin for 24 hours. **B:** U2OS cells were transfected with siRNAs as indicated for 48 hours. The last 24 hours the samples were additionally treated with 500nM doxorubicin. **C:** U2OS cells were treated with 500nM doxorubicin for 24 hours. **A-C:** Total RNA was isolated and subjected to reverse transcription. The levels of p53, TAp73, and WRAP53 were quantified relative to 36B4 using real-time PCR. Means and standard deviations of the experiments are diagramed.

Having elucidated important parts of mutant p53 expression regulation in response to different chemotherapeutics, the question remains whether these pathways also contribute to the general regulation of mutant p53 accumulation, as it is observed during tumor progression. The finding that E2F1, as well as TAp73 knock-down keeps the expression of p53 mRNA and protein levels more

or less constant in the absence of doxorubicin (4.2.1) pinpoints to the requirement of additional mechanisms regulating the expression of mutant p53.

4.7. A KINASE SCREEN DISCLOSES FURTHER CANDIDATES INVOLVED IN THE EXPRESSION REGULATION OF MUTANT P53 IN TUMOR DERIVED CELLS

Already 30 years ago, p53 was identified as a protein frequently accumulated in tumor cells and served as a diagnostic marker (Crawford *et al.*, 1981; DeLeo *et al.*, 1979; Rotter *et al.*, 1980). Until now, it is not clear why mutant p53 accumulates so strongly, whenever it is mutated. For many years, it was believed that the loss of p53 function goes along with low levels of Mdm2, the E3 ubiquitin ligase that is on the one hand a direct target gene of p53, but on the other hand its most important inducer of proteasomal degradation. This was a perfect explanation for the increased half-life of mutant p53 until Lang *et al.* (2004) and Olive *et al.* (2004) generated transgenic mouse models harboring various p53 hotspot mutations. Using these mouse models, they demonstrated that mutant p53 is specifically accumulated in the cells of advanced tumors, but remained at low levels in the surrounding tissue. These observations prompted us to investigate which pathways, imbalanced through tumor specific mutations, contribute to the expression regulation of p53.

The mutational spectrum in cancer cells mostly affects the pathways regulating cell cycle progression as well as DNA damage response, in order to keep a cell proliferating and alive. These pathways involve a series of constitutive phosphorylation events as to multiply the signal. Kinases, the enzymes that perform all these phosphorylations, are therefore central players and common targets of deregulation in the progression of tumor formation.

Investigating, whether these imbalanced pathways entail the accumulation of mutant p53, we performed a high content siRNA screen in 5637 cells. The so far known mutational spectrum of this bladder carcinoma derived cell line is

limited to the p53 mutation R280T and a nonsense mutation within the Rb gene. In addition to that, we found the cells to be siRNA transfected with a very high efficiency and perfectly shaped for single-cell based immunofluorescence analysis. The *Silencer* Human Kinase siRNA Library V3 targets 719 human kinases and kinase subunits with three individual siRNAs per gene, including validated siRNAs for more than half of the targets.

After 48 hours of siRNA transfection the cells were fixed and stained for single cell based immunofluorescence analysis. Hoechst 33342, as a nuclear stain, was used to define the individual nuclei of the cells. The average p53 staining in each of these nuclei was measured and used to calculate the mean expression of p53 upon each of the individual siRNA transfections. For the final hit determination Z-scores were calculated (Figure 21).

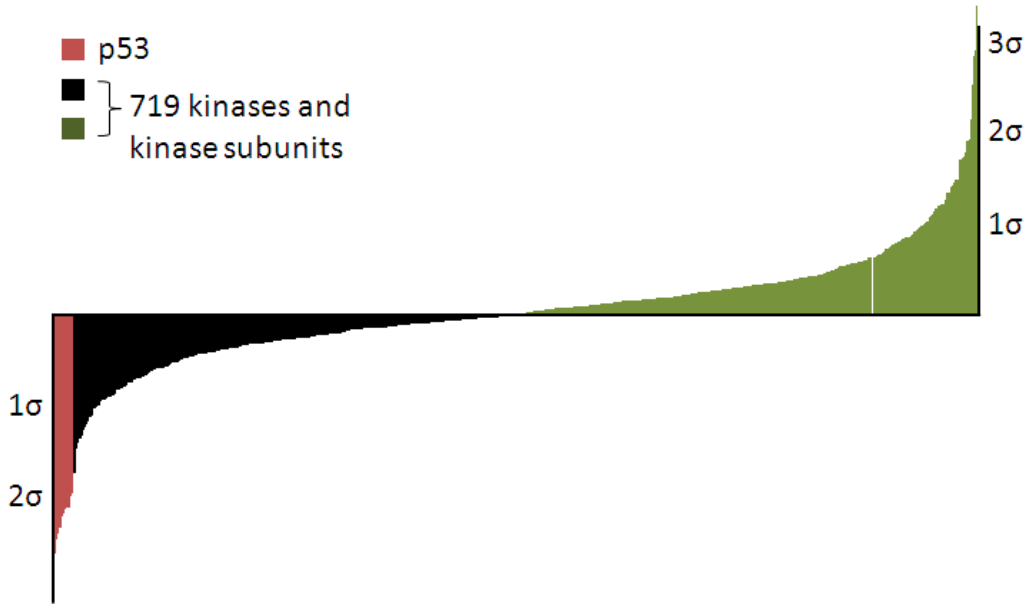


Figure 21: The influence of 719 human kinases on the expression of mutant p53 protein. 5637 cells were transfected with siRNAs targeting 719 human kinases and kinase subunits. Each target was silenced by three different siRNAs in individual wells. 48 hours after transfection the cells were fixed and stained using antibodies against p53. Hoechst 33342 was used to identify the individual nuclei within which the average expression levels of p53 were determined. Z-scores were assigned to each of the targeted kinases as a measure of p53 induction/ repression. For detailed description of the analysis please see 3.2.4. On each plate two wells were transfected with siRNAs targeting p53 itself as positive control for the down-regulation of mutant p53; the results of these controls are depicted in red.

The knock-down of 18 kinases revealed a Z-score lower than -1.1. These targets were considered as potential hits and analyzed in more detail. For 4 of these kinases it turned out that only one of the siRNAs had a striking effect, whereas the other two did not influence the expression of p53 at all. The chances that the effects of these kinases are caused by an off-target effect are very high and the targets were excluded from further analysis.

We assigned the remaining 14 hits to the pathways or cellular processes, where they were previously found to be involved in (Table 2).

Table 2: Hits from the screen.
The knock-down of the listed genes displayed a down-regulation of p53 in 5637 cells. Three siRNAs per gene were used, in column 3-5 the individual scores are listed and in column 6 the score of the average signal determined for the three siRNAs. The last column assigns the identified kinase to the pathway in which it is known to act.
PI5K: Phosphatidylinositol 5-kinase; PI3K: Phosphatidylinositol 3-kinase; mTOR: mammalian target of Rapamycin; PKC: Protein kinase C

	Kinase	Score A	Score B	Score C	Ø Score	Pathway
1	PIP5K1A	-1,32	-1,65	-1,75	-1,58	PI5K
2	PIK3C2G	-1,52	-1,58	-1,56	-1,55	PI3K → mTOR
3	RPS6KL1	-1,48	-1,56	-1,53	-1,52	PI3K → mTOR
4	PIM2	-1,28	-1,55	-1,64	-1,49	Cell proliferation, Meiosis
5	PIM3	-1,28	-1,08	-1,91	-1,43	Cell proliferation, Meiosis
6	PIP5K1C	-1,08	-1,49	-1,56	-1,38	PI5K
7	PGK2	-1,65	-0,65	-1,78	-1,36	Glycolysis, testis-specific
8	PINK1	-1,84	-1,76	-0,47	-1,35	Mitochondrial stress response
9	PIK3R3	-1,40	-1,14	-1,39	-1,31	PI3K → mTOR
10	PIK3CB	-1,20	-1,34	-1,25	-1,26	PI3K → mTOR
11	PIP5K2B	-0,73	-1,04	-2,02	-1,26	PI5K
12	PIP5K1B	-1,28	-0,99	-1,46	-1,24	PI5K
13	PKN2	-0,75	-1,34	-1,47	-1,19	PKC related, function unknown
14	RPS6KB2	-0,84	-0,84	-1,81	-1,16	PI3K → mTOR

Besides the two kinases PGK2 (Phosphoglycerate kinase 2) and PKN2 (Protein kinase N2) that are either very tissue specific or functionally unknown, three groups of kinases remained, as potential targets to be followed up on. First, the

PIM kinases, they are as well largely unknown in their function, but it became evident that two out of three paralogs appeared within the hit list. Second, the PI5K, they are represented by four members in the hit list, nevertheless, so far they are rather known to regulate cellular polarity and membrane trafficking, processes, in which the regulation of p53 expression would not be expected to happen. Third, the PI3K → mTOR pathway, it is represented by three members of the PI3K family and two members downstream of mTOR. Additionally, it should be mentioned that PINK1 (PTEN induced kinase 1) gets activated by members of the PI3K → mTOR pathway, even though its described function is so far restricted to the mitochondrial stress response in Parkinsons disease. Only very recently, it was observed by Morimoto *et al.* (2010) that the up-regulation of PINK1 expression in amyotrophic lateral sclerosis patients is positively correlated with the phosphorylation and stabilization of wt p53, suggesting that there is a link between the two pathways.

Taking all these information together, we decided to follow up on RPS6KL1 and –B2. These are two kinases that act quite far downstream in a pathway that is represented by 5 kinases within the hit list. In addition to this, there is one report claiming that some members of the large protein family of RPS6 kinases directly phosphorylate wild type p53, which was shown using an *in vitro* kinase assay (Cho *et al.*, 2005).

The S6 kinases were named according to their primary function of phosphorylating the ribosomal protein S6. The protein family consists of 10 members that can be grouped in three functionally more related subfamilies. First the RSKs (Ribosomal S6 kinases) consisting of RPS6KA1, RPS6KA2, RPS6KA3, and RPS6KA6, second the MSKs (Mitogen- and stress-activated kinases) with RPS6KA4 and -5, and third the S6Ks (S6 kinases) namely RPS6KB1 and RPS6KB2. Additionally, RPS6KC1 and RPS6KL1, as structurally related, but functionally mostly unknown kinases, belong to this family. Similar as described earlier for the E2Fs, also the RPS6 kinases fulfill

partially opposing activities and it can therefore not be expected that all 10 members of the protein family have an impact on the mutant p53 expression regulation.

4.7.1. VALIDATION OF THE IDENTIFIED HITS: RPS6KB2 AND RPS6KL1

RPS6KL1 and RPS6KB2 were identified in the performed kinase screen as potential regulators of mutant p53 expression in the tumor cell line 5637 by quantitative immunofluorescence microscopy. This is a very sensitive method that was on the one hand enabling us to detect kinases that have slight effects on the expression of mutant p53, but on the other hand also requires optimization until the effects can get validated by less sensitive methods like immunoblotting.

First, we tried to find another cell line, still harboring a p53 mutation, but expressing higher levels of the identified kinases, in order to observe whether S6 kinases have an even stronger impact on p53 levels in such a system. Nakamura *et al.* (2008) published the two glioma cell lines U251 and U373 to express detectable levels of the kinases RPS6KB1 and -2. Since RPS6KL1 is largely unknown and the available antibodies fail to specifically detect the protein, we could not optimize the cell line with respect to the expression level and activity of this kinase, which originally showed a stronger effect on the regulation of p53. In order to confirm that the knock-down of RPS6KB2, as well as RPS6KL1, contributes to the expression regulation of p53 in U251 cells we performed immunofluorescence analysis. The experiment was carried out the same way, as in the screen, but using U251 cells. This revealed that U251 cells are a good model system to analyze the impact of S6 kinases on the expression regulation of mutant p53. The impact of RPS6KL1 on mutant p53 levels was as well confirmed under these circumstances (Figure 22).

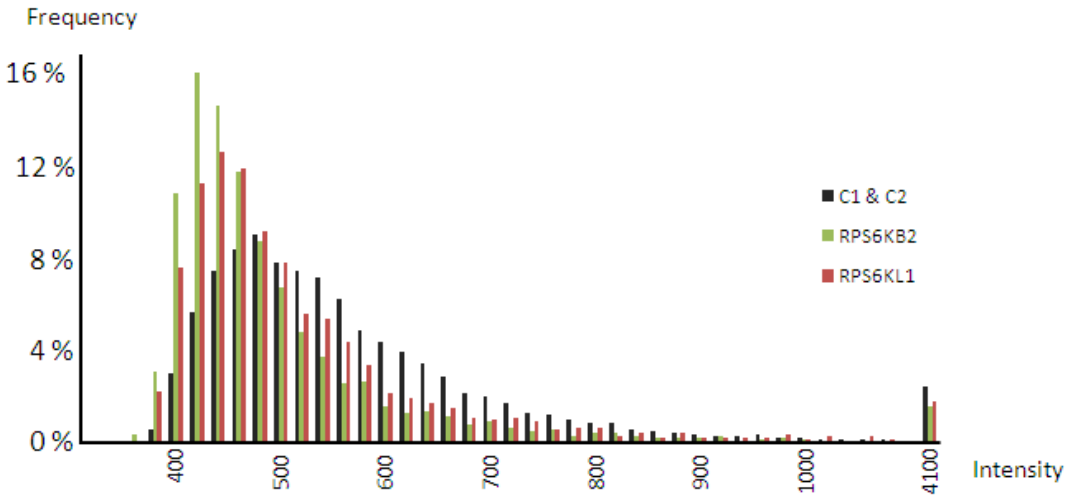


Figure 22: The knock-down of RPS6KB2 and RPS6KL1 decreases the expression levels of mutant p53 protein in U251 cells.
U251 cells were transfected with siRNAs against RPS6KB2 and RPS6KL1 for 48 hours. The fixed cells were stained for immunofluorescence analysis using antibodies recognizing total p53. Hoechst 33342 was used to identify the individual nuclei. The average expression of p53 per nucleus was determined; the data are diagramed in a histogram.

Taking into account that mutant p53 proteins in tumor cells have a much longer half-life than wild type p53, we further optimized the assay with respect to the duration of the knock-down. Using a double siRNA transfection protocol (3.2.1 LONG-TERM SIRNA TRANSFECTION), we confirmed the role of RPS6KB2 and RPS6KL1, as well as RPS6KB1, in the regulation of mutant p53 expression (Figure 23). The knock-down was performed for 5 days before the cells were harvested for immunoblot analysis. The structurally related kinase RPS6KA1 was used as an additional negative control, since it was found in the screen to keep the expression levels of p53 constant compared to non-targeting scrambled siRNAs.

The knockdown efficiency was monitored indirectly, by staining for the expression of Serine 235/236 phosphorylated S6, a well known target of the analyzed kinases RPS6KB1 and -2, due to the lack of specific antibodies recognizing the S6 kinases. This way, we observed that for RPS6KB1, as well as RPS6KB2 the first siRNAs mediated a stronger knock-down, compared to the second. This reflects the same pattern, as it is observed for the expression

of mutant p53. The knock-down of RPS6KL1 as well diminished the expression of mutant p53 to a large extent. Unfortunately, we could not monitor the knock-down efficiency of the protein, since there are neither specific antibodies available, nor a well characterized substrate that could be used for this analysis (Figure 23).

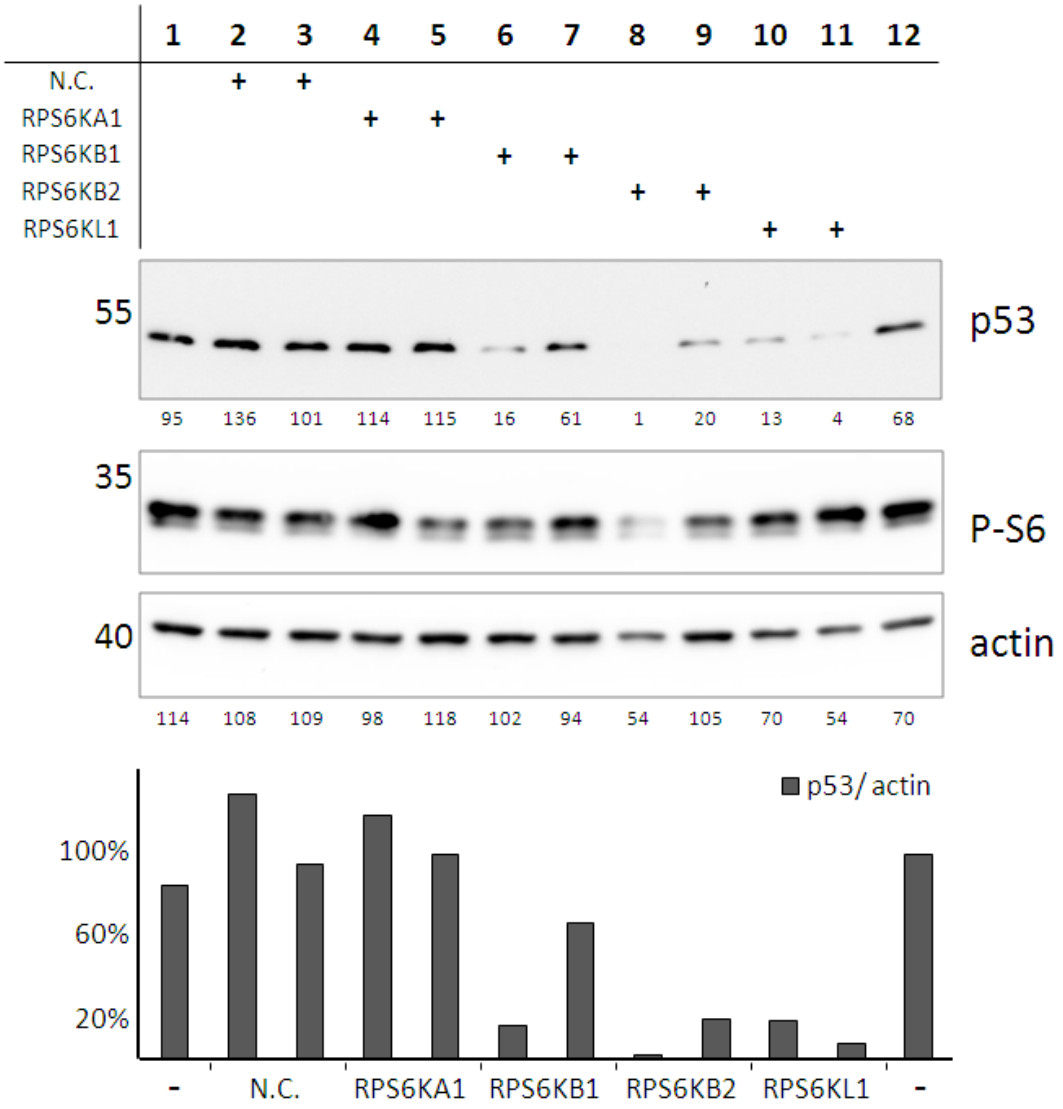


Figure 23: The S6 kinases RPS6KB2 and -B1, as well as RPS6KL1 contribute to the regulation of mutant p53 expression in tumor cells
U251 cells were double-transfected with siRNAs targeting four members of the RPS6 kinase family. Five days after the first transfection the cells were harvested and whole cell lysates were subjected to immunoblot analysis. The expression of p53, P(Ser 235/236)-S6, and actin was detected. The blots were quantified using LabImage 1D (lower part).

To test whether the knockdown of the identified kinases affects mutant p53 expression on the protein level, and not, as previously observed, on the transcriptional level, we isolated total RNA from cells upon siRNA mediated knockdown of the S6 kinases. This experiment clearly revealed that all three RPS6 kinases, that were shown to have an impact on mutant p53 expression, do not change its mRNA levels (Figure 24). This strongly argues, in line with previous observations, that the accumulation of mutant p53 during tumor progression happens on the protein level.

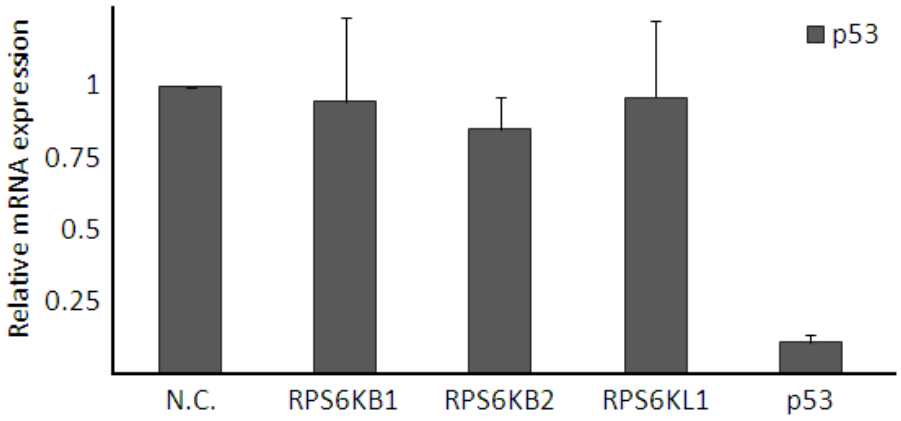


Figure 24: S6 kinases do not regulate the expression of p53 on the transcriptional level. U251 cells were siRNA transfected for 48 hours. Total RNA was isolated and quantified relative to 36B4 using real-time PCR. Means and standard deviations of four biological replicates were diagrammed.

Other than the depletion of S6 kinases, we also investigated, whether modulation of their activity would result in decreased expression of mutant p53. As described by Nobukuni *et al.* (2005) and Hidayat *et al.* (2003), inactivation of the kinases can be achieved by the withdrawal of FCS and the two amino acids Arginine and Lysine and can be reverted by 30min incubation with fresh medium containing FCS (Figure 25; P-S6 staining). The levels of mutant p53 decreased as well upon starvation and recovered after 30min of incubation in full medium supplemented with 10% FCS (Figure 25).

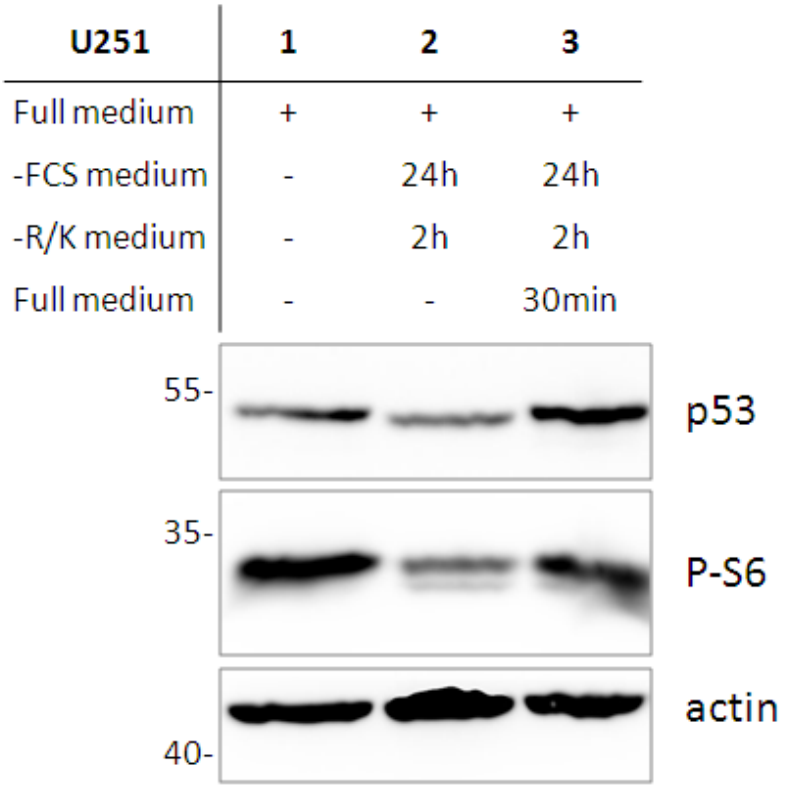


Figure 25: Serum starvation abolishes S6 kinase activity and leads to the down-regulation of mutant p53 expression.
U251 cells were seeded in 12 wells. After the cells attached the medium was changed, and the cells were kept in serum deprived medium. 24 hours later the medium was again changed to Arg/ Lys free medium without FCS, to further starve the cells. After 2 hours the cells were supplemented with fresh medium containing 10% FCS for 30 minutes. The cells were harvested and whole cell lysates were subjected to immunoblot analysis, using antibodies against p53 and P(Ser235/ 236)-S6. Actin staining was used as loading control.

This experiment argues that the protein level of p53 is regulated through the activity of S6 kinases and not simply through their abundance. Nevertheless, we don't know yet, whether this is a direct activity of the S6 kinases, as it was claimed by Cho *et al.* (2005) or, whether it still might be indirectly affected by other intermediates.

DISCUSSION

It is characteristic for most tumor cells that they proliferate rapidly in an uncontrolled fashion. The mechanistic principle behind most chemotherapeutic agents takes advantage of this feature, by preferentially inducing apoptosis in rapidly dividing cells. In most cases this is achieved through the induction of a DNA damage response, going along with the stabilization and activation of the tumor suppressor p53. This, depending on the severity and the nature of the damage, leads to cell cycle arrest or apoptosis. Sequence analysis of thousands of tumor samples revealed that p53 is mutated in more than 50% of all human solid tumors. In contrast to other tumor suppressors, p53 is mainly inactivated through single point mutations within the central DNA binding region of the protein. This mutation leads not only to the loss of tumor suppressor activity, but at the time confers oncogenic properties to the expressed gene product. According to different studies, this comprises increased chemoresistance, as well as a higher frequency of metastasis formation. It has previously been shown that knock-in mice, harboring one of the p53 hotspot mutations, differ from p53 null mice with respect to the frequency of metastasis formation (Lang *et al.*, 2004; Olive *et al.*, 2004). Histochemical analysis of the tumors revealed that mutant p53 was specifically over-expressed in high grade tumors, whereas its expression was at the background level in the surrounding tissue (Terzian *et al.*, 2008). The molecular signals that cause this accumulation are not known so far, nevertheless, studies of various groups indicate that Mdm2, as well as other E3 ubiquitin ligases like Cop1, ARF-BP1, and CHIP (C terminus of Hsc70-interacting protein) become inactivated due to the deregulation of tumor specific pathways (Lukashchuk and Vousden, 2007).

Within this study we identified three different levels of mutant p53 expression regulation. Due to different stimuli, either DNA damage responsive transcription factors were identified as central players of mutant p53 expression regulation, or the natural antisense transcript of p53, recently identified by Mahmoudi *et al.* (2009), or kinases involved in the PI3 kinase → mTOR pathway.

5.1. TRANSCRIPTIONAL REGULATION OF MUTANT P53 EXPRESSION IN RESPONSE TO CHEMOTHERAPEUTIC TREATMENT

In response to genotoxic stress, conferred by chemotherapeutic agents, like the three anthracyclines daunorubicin, doxorubicin, and epirubicin, the expression of mutant p53 in tumor cells is augmented due to increased transcription (Figure 15). Within the performed experiments, we have shown that the transcription factors HOXA5, RREB1, TP73, and E2F1 are all necessary for the observed up-regulation in response to treatment with the named anthracyclines, while they have no impact on the basal transcriptional expression regulation of mutant p53 in the absence of DNA damage (Figure 9, Figure 11, data not shown). The transcription factor E2F1 was further shown, to directly bind to a, so far not described binding site within the p53 promoter (Figure 13). Interestingly, ChIP analysis revealed no differences in E2F1 binding to the promoter of p53 upon doxorubicin treatment. Nevertheless, for the well known DNA damage responsive E2F1 target gene TAp73, we as well observed invariant E2F1 binding regardless of doxorubicin treatment. These data indicate that the E2F1 that we recover in our ChIP experiments is bound to the promoter of its target genes independently of their transcriptional activation through chemotherapeutic treatment. As Pediconi *et al.* (2003) showed, this could most probably be explained by posttranslational modifications of E2F1 that are necessary to promote active transcription. To further look into this matter, we would need to establish an antibody specific to acetylated E2F1 and thereby restrict the analysis to the active form of the transcription factor. Additionally, it could be tested whether RNA polymerase II in its active form

(Serine 2 phosphorylated) is more abundant around the transcriptional start site, in response to doxorubicin treatment, compared to untreated cells in a re-ChIP experiment, where the eluate of an E2F1 ChIP is used as starting material.

5.2. WRAP53 - A NATURAL ANTISENSE TRANSCRIPT OF P53 PREVENTS MRNA MATURATION

Natural antisense transcripts (NATs) are a class of non-coding RNAs that were shown to have an impact on the expression regulation of genes. It is estimated that in mammals about 70% of all transcripts have antisense partners that can alter the expression of the sense genes (Katayama *et al.*, 2005). For example, in about 70% of tumor samples, the antisense transcript of the tumor suppressor p15 (p15AS) is highly accumulated, whereas the tumor suppressor itself is silenced; in normal cells this is observed vice versa (Yu *et al.*, 2008). NATs have been proposed to regulate the expression of their target genes at several levels, including transcription, messenger RNA processing, splicing, stability, cellular transport, and translation (Lapidot and Pilpel, 2006). To understand the mechanisms of NAT regulation, Chen *et al.* (2005b) analyzed the expression profiles of sense and corresponding antisense transcripts on a genome-wide scale. This revealed that sense-antisense pairs tend to be co-expressed or inversely correlated more frequently, than would be statistically expected. Furthermore, they found that most of these pairs and their expression regulation is evolutionary conserved.

Within this study we observed that three topoisomerase II inhibitors, daunorubicin, doxorubicin, and epirubicin, induced the p53 mRNA expression in an E2F1 and TAp73 dependent manner. Two other topoisomerase II inhibitors, idarubicin and etoposide, were found to evoke a similar DNA damage response leading to the activation of the transcription factors E2F1 and TAp73, but, to our surprise, did not increase p53 mRNA expression levels (Figure 14, Figure 15). Over and above, we even found that etoposide treatment slightly decreased the

mRNA levels of p53 in U251 cells. Further analysis revealed that idarubicin and etoposide, but none of the other three analyzed topoisomerase II inhibitors, induced the expression of WRAP53-1α, a NAT encoded on chromosome 17 opposite of p53 (Figure 18). To elucidate the mechanism behind these controversial regulatory activities of highly related chemotherapeutic drugs, we on the one hand analyzed the pre-mRNA expression of p53 and on the other hand used siRNA mediated knock-down of WRAP53. Within the first experiment we found that the E2F1 and TAp73 dependent transcriptional activation of p53 occurred upon idarubicin treatment the same way, as upon treatment with any of the other anthracyclines (Figure 16, data not shown). This indicates that the maturation of p53 mRNA is affected upon idarubicin treatment, possibly mediated through the elevated expression of WRAP53-1α. The knock-down experiment of WRAP53 revealed that the used siRNAs targeted the mRNAs of most WRAP53 isoforms efficiently. Nevertheless, we also observed that transcripts, that either lack exon 2 and 8 or escape the knock-down by other means, are specifically induced in response to idarubicin treatment (Figure 19). Since siRNAs are believed to mainly act in the cytoplasm, absent nuclear export of the transcript should be considered as a possible way of inefficient knock-down of mRNAs, containing the siRNA target sequence. Additionally to the data we obtained in mutant p53 expressing cell lines, we observed that doxorubicin treatment strongly induces the expression of WRAP53-1α in the wt p53 expressing cell lines U2OS and HCT116 (Figure 20 C, data not shown), while the mRNA levels of p53 remained unaffected from the treatment (Figure 20 A).

When interpreting all these results from the tumor cells point of view: It appears advantageous for wt p53 expressing cells to circumvent the accumulation of p53 in response to DNA damage, in order to escape the induction of apoptosis. Elevated expression of WRAP53-1α could serve as one mechanism to achieve this. For mutant p53 expressing cells, on the contrary, this induction of

WRAP53-1α in response to doxorubicin is needless if not disadvantageous, since they induce a protein with oncogenic activities, leading to tumor progression and chemoresistance, rather than apoptosis. Nevertheless, this still owes a rational for the WRAP53-1α induction in response to idarubicin and etoposide in mutant p53 expressing cells.

The following NAT related mechanisms could serve to explain our data:

DNA METHYLATION AND HETEROCHROMATIN FORMATION:

Antisense-induced DNA methylation and silencing was described to play an important role for example in thalassaemia, where the haemoglobin 2 gene is efficiently silenced through the expression of an antisense transcript (Tufarelli *et al.*, 2003). Several studies have indicated that this is not mediated through the formation of RNA duplexes, but through the modification of chromatin structure or DNA methylation patterns (Lee and Lu, 1999; Wutz *et al.*, 1997). According to studies from Tufarelli *et al.* (2003) and Yu *et al.* (2008) antisense induced DNA methylation leading to efficient silencing of target genes should be considered a widespread mechanism of tumor suppressor silencing. Nevertheless, this concept stands in conflict with our observation that idarubicin treatment entails the same induction of p53 pre-mRNA synthesis, as it is observed in response to doxorubicin.

RNA MASKING:

Sense-antisense RNA duplex formation masks cis-regulatory elements within the p53 mRNA hindering proteins involved in polyadenylation, splicing, or nuclear export to bind to the pre-mRNA (Hastings *et al.*, 1997). This would in turn lead to less efficient maturation and thereby explain the observed phenotype of increased pre-mRNA levels that do not affect the overall mRNA levels of p53. Furthermore, this would explain the inefficient knock-down that was observed for WRAP53-1α, since the p53 mRNA is only exported into the cytoplasm after successful polyadenylation and splicing.

One central question that remains is how the inversely correlated transcription of p53 and WRAP53-1α is regulated. Collision of two RNA polymerase II enzymes is discussed as a possible mechanism of inverse regulation of antisense gene expression (Crampton *et al.*, 2006; Prescott and Proudfoot, 2002). Collision of RNA polymerase II with stalled topoisomerase II is not described so far. Nevertheless, we would like to suggest this to explain the generation of WRAP53-1α transcripts lacking exon 2. If, due to the collision, a shortened transcript of WRAP53 is released, it would resemble a natural antisense transcript to p53 and might then get stabilized in the nucleus through its interaction with the pre-mRNA or the DNA of the sense gene p53. This hypothesis is summarized in Figure 26.

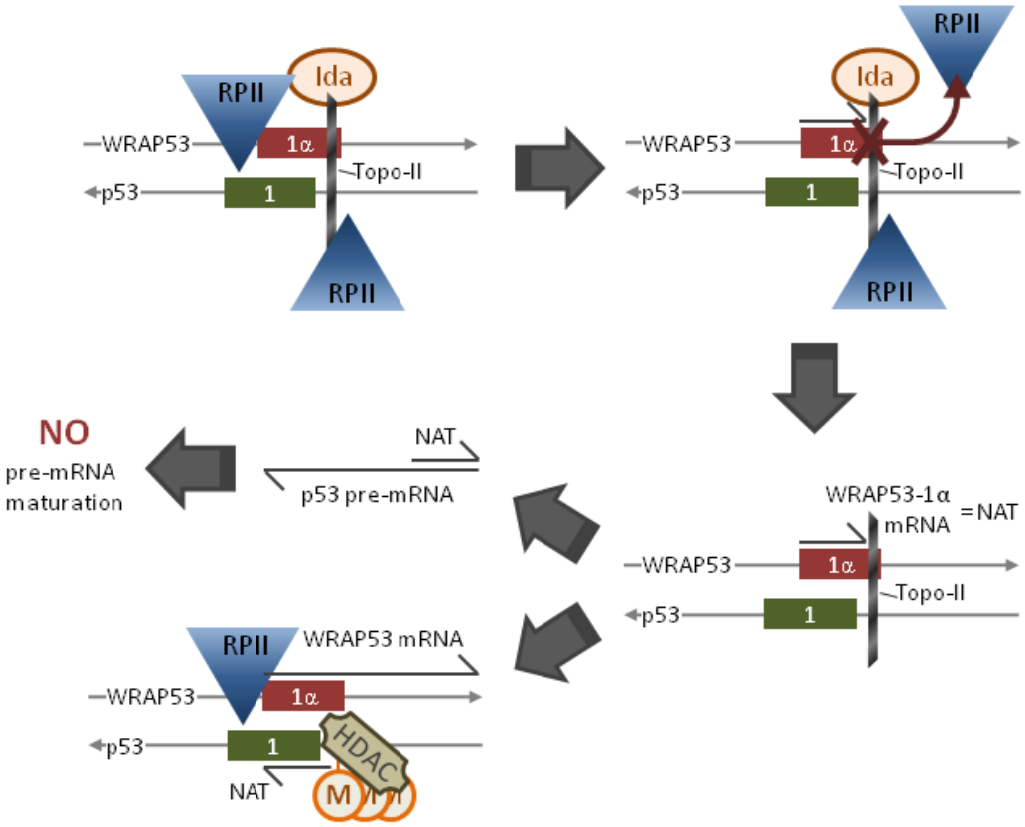


Figure 26: Model of the expression regulation within the *TP53* genomic locus.
Topoisomerase II (Topo-II) is stalled on the DNA through idarubicin (Ida). Thereby it prevents RNA polymerase II (RP-II) from binding to the p53 promoter, as well as elongation of WRAP53 transcription due to collision. The short WRAP53-1α mRNA acts as natural antisense transcript (NAT) on p53. This NAT could either bind to p53 pre-mRNA, preventing its further maturation, or NAT-DNA interactions could feature promoter methylation and heterochromatin formation, leading to the silencing of p53.

To experimentally elucidate the underlying mechanisms it would be important to first identify the WRAP53-1α transcript that is accumulating in response to treatment with these chemotherapeutic drugs and its cellular localization. Subcellular fractionation prior to RNA isolation would help to get insights into the localization of idrubicin induced WRAP53-1α transcripts. Additionally, RACE-PCR could be used to identify a potential truncated WRAP53-1α transcript. To investigate whether topoisomerases, inhibited through idarubicin or etoposide, get stalled within the *TP53* genomic locus at positions different from those where daunorubicin, doxorubicin, and epirubicin inhibited topoisomerases get stalled, ChIP technology could be used (Cashman and Kellogg, 2004). Furthermore, it could be tested whether HDAC inhibition would lead to similar impacts of all five topoisomerase II inhibitors on the p53 transcription and thereby rescue the defect in pre-mRNA maturation upon treatment with idarubicin or etoposide.

5.3. KINASES REGULATING MUTANT P53 EXPRESSION IN TUMOR CELLS

We found that the three kinases RPS6KL1, RPS6KB2, and RPSKB1 contribute to the expression regulation of mutant p53 in tumor cells (Figure 21, Figure 22, and Figure 23). We identified these kinases in a microscopy based siRNA screen and confirmed their roles in the performed follow-up experiments. The knock-down of the named kinases decreased the expression levels of mutant p53 in tumor cells that otherwise express the protein, without further stimuli, at high levels. Our data additionally suggest that the kinase activities of RPS6KB1 and -B2 are necessary for their impact on p53, rather than their simple abundance (Figure 25). For RPS6KL1 this can currently not be analyzed, since it is neither known, whether this protein exhibits intrinsic kinase activity, nor ways to alter it.

According to the data from Cho *et al.* (2005), some kinases of the RPS6K family directly phosphorylate p53 at Serine 15. Additionally, Melnikova *et al.* (2003) observed mutant p53 to be constitutively phosphorylated at Ser15 in

UV-induced skin tumor cells and correlated this with decreased susceptibility to Mdm2-mediated degradation. Taking these data together, it could be hypothesized that the knock-down or inactivation of the S6 kinases decreases the levels of mutant p53 phosphorylation, which in turn leads to the destabilization of the protein. Nevertheless, therefore the overall levels of posttranslationally modified mutant p53 would need to be high, even in the absence of DNA damage, a prerequisite that we did not observe in our studies (Figure 7A, B). Hence, we hypothesize that the mechanism, underlying this regulation of transformation induced accumulation of mutant p53, does not depend on posttranslational modifications and their impact on the susceptibility of p53 towards Mdm2, or other ubiquitin ligases. To further exclude this as a possible mechanism, we would like to investigate, whether ectopically over-expressed mutant p53 is regulated by the identified kinases. And, whether an additional mutation of known phosphorylation sites within p53 (Serine or Threonine to Alanine) would abolish this effect.

The presented results could also be explained with the help of reports published by Fu and Benchimol (1997) and Takagi *et al.* (2005), finding that p53 expression is regulated on the translational level in response to DNA damage. In the work of Fu and Benchimol (1997), the 3'UTR of p53 itself was identified to posses inhibitory activity on p53 translation. They further showed that γ-irradiation abolishes this translational inhibition. Whereas, Takagi *et al.* (2005) claims that irradiation leads to an increased binding of RPL26 to the 5'UTR of p53, which in turn promotes p53 mRNA association with heavy polysomes, augmenting the rate of its translation. Even though these data were obtained in wild type p53 expressing cells, in response to irradiation, we aimed to test whether RPL26 contributes to the regulation of mutant p53 expression in the absence of DNA damage. But, upon siRNA mediated knock-down of RPL26 in U251 cells neither the posttranslational modification of p53, nor its overall expression levels were affected (data not shown). This argues against

translational regulation of mutant p53 expression mediated by RPL26. Nevertheless, to determine the impact of translational regulation on mutant p53 accumulation mediated through the identified S6 kinases, it should be tested whether the amount of p53 mRNA associated with heavy polysomes changes upon the knock-down of the kinases.

The mTOR pathway was shown to contain sensors for nutrient and amino acid availability (Kim, 2009). Cells that lack essential amino acids often use autophagy to degrade cellular proteins, thereby increasing the pool of amino acids that can be used to translate new proteins of greater importance for their survival (Jung *et al.*, 2010). We would like to test, whether the highly accumulated mutant p53 protein might get degraded through autophagy upon RPS6K knock-down or starvation, since it was previously shown that Arginine deprivation, which was used in our experiments to inhibit mTOR signaling, induces autophagy (Savaraj *et al.*, 2010). Along that line, there are several ways to induce or block autophagy independently of mTOR that could be used to analyze the impact of this degradative pathway within the regulatory network of mutant p53 expression. According to Munafo and Colombo (2001), autophagy is efficiently blocked through treatment with 3-methyladenine or N-ethylmaleimide (NEM), while the incubation with the microtubule depolymerizing agent vinblastine could be used to accumulate autophagic vacuoles, by preventing their degradation. Furthermore, the knock-down of Belcin1, as a central regulator of autophagy could be used, to more specifically analyze the impact of this pathway on mutant p53 expression regulation (Liang *et al.*, 1999). Microscopic analysis of mutant p53 expression upon modulation of autophagy could be used to test, whether this pathway is involved in mutant p53 expression regulation in tumor cells. If this hypothesis turns out to be true, we will not have identified one of the tumor specific pathways that lead to the accumulation of mutant p53 at the first place. Nevertheless, this finding could help to decrease the expression levels of the oncogenic mutant p53 protein in

cancer cells and it should be tested, whether the induction of autophagy could be used to prevent tumor progression and metastasis formation.

5.3.1. METASTASIS FORMATION AS MUTANT P53 GAIN OF FUNCTION

It can be hypothesized that the activity of the mTOR pathway kinases is decreased in areas of the tumor that lack sufficient nutrient supply and elevated in the outer cells. According to the data we have obtained, this would result in high levels of accumulated mutant p53 in cells at the outer rim of the tumor cell mass and in close proximity to blood vessels. The fact that exactly these cells are the ones that detach from a primary tumor to form new metastases, pinpoints to the importance of finding ways to actively suppress the oncogenic gain of function of accumulated mutant p53. Rapamycin (Rapamune®), a small molecule that was originally isolated from *Streptomyces hygroscopicus* is FDA approved as immunosuppressant. This molecule, which can efficiently inhibit the mTOR pathway, is recently more and more described to have a tumor protective function (Guertin and Sabatini, 2005; Sudarsanam and Johnson, 2010). In many tumors the prognosis, especially in terms of tumor proliferation and metastasis formation, seems to be correlated with the activity of the mTOR pathway (Zhou and Huang, 2010). We believe that the expression of mutant p53 could be a mechanistic explanation for this observed correlation. To investigate the *in vivo* role of mutant p53 accumulation, sections of larger tumors, harboring a p53 point mutation, could be histochemically analyzed, in order to test whether the expression levels of mutant p53 are indeed higher at the outer rim of the tumor cell mass and whether they decrease in response to treatment with rapamycin.

5.3.2. CHEMORESISTANCE AS MUTANT P53 GAIN OF FUNCTION

One aspect that should be tested as a link between mutant p53 gain of function and the use of anthracyclines for chemotherapeutic treatment is the expression

of the multi-drug resistance gene 1 (MDR1). It was on the one hand shown by several groups that in malignancies, expressing high levels of mutant p53 protein, chemoresistance is often conferred through transcriptional activation of MDR1 (Blandino *et al.*, 1999; Brosh and Rotter, 2009; Bush and Li, 2002). It was on the other hand clinically observed that doxorubicin treatment often leads to multi-drug resistance, going along with increased MDR1 levels, as a side effect. Thereby it was further observed that idarubicin, despite its structural homology to the other anthracyclines is the only representative of its kind that lacks this side effect (Hargrave *et al.*, 1995; Lotfi *et al.*, 2002). These observations do not only reflect another example of different phenotypes conferred by the structurally almost identical anthracyclines doxorubicin and idarubicin. Rather, correlated with our data, it can be hypothesized that the clinically observed chemoresistance upon doxorubicin treatment is mediated by an accumulation of mutant p53 in the cells, conferred by increased expression of MDR1. Whereas idarubicin lacks the ability to induce this accumulation and thereby does not exhibit MDR1 over-expression and the observed side effect of chemoresistance.

5.4. WHICH CONSEQUENCES CAN BE DRAWN FROM THESE FINDINGS

It should be the aim of mutant p53 research to elucidate the mechanisms of its accumulation and to get more insights into the cellular concepts underlying the oncogenic gain of function. Within this study we obtained data indicating that the expression of mutant p53 is regulated on different levels depending on the stimuli that cause its accumulation. Bearing in mind the disadvantageous side effects of mutant p53 accumulation that were published by a number of groups (Barlev *et al.*, 2001; Di Agostino *et al.*, 2006; Lang *et al.*, 2004; Muller *et al.*, 2009; Strano *et al.*, 2007) it should be as well aimed to prevent this accumulation, or at least to decrease the expression levels.

We observed that the accumulation of mutant p53 is increased upon topoisomerase II inhibitor treatment. We further demonstrated that the

transcription of p53 is activated in response to this treatment, which is inversely regulated to the expression of the natural antisense transcript WRAP53. Therapeutically it should be considered to use idarubicin or etoposide more widely in the tumors expressing mutant p53. It could also be tested whether the transcription of WRAP53-1α can exogenously be stimulated to prevent the accumulation of mutant p53 in response to one of the other topoisomerase II inhibiting drugs, possibly through a combinational treatment with idarubicin or etoposide.

Regarding the accumulation of mutant p53 that occurs during cellular transformation, we found that inhibition of ribosomal S6 kinase activity decreases the expression of mutant p53 in the used cell lines. It was furthermore recently shown that mTOR plays a critical role in the regulation of tumor cell motility and cancer metastasis. It would now be important to analyze whether the mTOR activity in tumor cells promotes tumor progression through the oncogenic activities gained by p53 through its point mutation. Therapeutically, it should then be tested whether the metastatic gain of function of mutant p53 can be abolished by treatment with rapamycin.

SUMMARY

The tumor suppressor p53 in its mutant form was previously shown to massively accumulate in tumor cells. Furthermore, enhanced tumor progression, as well as chemoresistance were associated with its expression. Within this study, we observed that chemotherapeutic treatment with some, but not all topoisomerase II inhibitors, currently used in the clinics, leads to a further up-regulation of mutant p53 expression and thus might favor unwanted tumor progression of tumor cells that escape the apoptosis induction at the first place. The network to regulate the expression of mutant p53 includes different mechanisms in response to various stimuli. The mediators range from transcription factors, over non-coding RNAs, to kinases.

All topoisomerase II inhibitors that we tested within our study augmented mutant p53 transcription. We showed that this was mediated by several transcription factors, including E2F1 and its target gene TAp73, that itself is known to exhibit activities similar to wt p53. While it was previously shown that TAp73 binds to a responsive element with the p53 promoter we observed here for the first time that E2F1 also binds directly to the p53 promoter in close proximity to the transcriptional start site. This was first found using *in silico* methods and confirmed by chromatin immunoprecipitations.

Nowadays, non-coding RNAs are recognized as another level of gene expression regulation. Recently, it was identified that within the *TP53* genomic locus, a natural antisense transcript is encoded, partially overlapping with exon 1 of the p53 mRNA. We observed that idarubicin and etoposide, but none of the other topoisomerase II inhibitors, strongly induced the expression of this antisense transcript, WRAP53. Furthermore, it became evident that this

expression is inversely correlated with proper pre-mRNA maturation of p53. Therefore, we hypothesize that the expression of this natural antisense transcript efficiently inhibits p53 mRNA maturation, possibly through RNA masking. We further hypothesize that the inversely correlated expression of sense and antisense transcripts might be caused by the collision of RNA polymerase II with idarubicin- or etoposide-inhibited, stalled topoisomerase II.

The accumulation of mutant p53, as it is observed during tumor progression, seems to be mostly regulated on the protein level, where we identified the ribosomal S6 kinases to be involved in. We found that the kinase activity of RPS6KB2 is necessary, to regulate the amounts of mutant p53 protein, as it was determined by serum and amino acid starvation. The mechanistic details that form the basis of this regulation were not determined, but we would like to suggest several hypotheses to be investigated. While our data can be explained by translational defects that the knock-down or inhibition of RPS6KB2 might cause, we favor the model that the induction of autophagy in response to mTOR pathway deregulation causes an enhanced degradation of mutant p53. A role of direct phosphorylation of mutant p53 through RPS6KB2 can also not be excluded.

In conclusion, we found that tumor cells accumulate mutant p53 protein through the activity of kinases that transduce mTOR signaling. Surprisingly, some chemotherapeutics further enhance mutant p53 levels through an entirely different mechanism, i.e. the regulation of p53 sense and antisense transcription.

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CURRICULUM VITAE

PERSONAL INFORMATION

	Monika Bug
Adress	Am Hasengraben 5 37075 Göttingen Germany
Email	mbug@gwdg.de
Date of Birth	September, 12th 1982
Nationality	German

EDUCATION

	Georg August University Göttingen, Germany
2006 - 2010	PhD in the Department of Molecular Oncology Thesis: “The accumulation of mutant p53 in human cancer cells”
	Georg August University Göttingen, Germany
2005 - 2006	Molecular Biology (MSc/PhD program – Max-Planck International Research School)
	University of Lübeck, Germany
2002 - 2005	Molecular Life Science (BSc) Thesis in the Department of Biology: “Characterization of the cytosolic protein p97 binding to the membrane of rough endoplasmic reticulum during retrograde transport”

TEACHING

	Tutor of Lectures
2007 - 2010	“Apoptosis and Cancer” within the Max-Planck International Research School MSc/PhD Program “Molecular Biology”

Supervisor of Labrotations

2008 - 2009 Golnaz Aghazadeh: Master-student in “Molecular Medicine”, University of Göttingen
Anke Hellrung: Master-student in “Biomedizin”, University of Würzburg

Tutor of Advanced Methods Courses

2008 - 2009 “Assessing promoter activity by luciferase assays” within the Max-Planck International Research School MSc/PhD Program “Molecular Biology”

HONORS AND AWARDS

2005 - 2006 Stipend Max-Planck International Research School

2004 Student exchange scholarship at the University of New Mexico

2003 - 2006 Stiftung der deutschen Wirtschaft (Foundation of German Economy)

CONFERENCES

2009 First AACR International Conference on Frontiers in Basic Cancer Research, Boston, MA, USA (poster)

2007 PhD Symposium “Horizons in Molecular Biology” Göttingen, Germany (poster)

EXTRACURRICULAR ACTIVITIES

Student representative

2007 - 2009 Max-Planck International Research School MSc/PhD Program “Molecular Biology”, Georg August University Göttingen, Germany

PhD representative

2007 - 2009 Göttingen Center for Molecular Biosciences (GZMB), Georg August University Göttingen, Germany

THE ACCUMULATION OF MUTANT P53

IN HUMAN CANCER CELLS

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born in

Hanau

SEPTEMBER 2010

Affidavit

I hereby declare that this doctoral thesis has been written only by the undersigned and without any assistance from third parties.

Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Göttingen, 30.09.2010

Monika Bug

LIST OF PUBLICATIONS

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Bug M. and Dobbelstein M. **Anthracyclines induce the accumulation of mutant p53 through E2F1-dependent and -independent mechanisms.** (Submitted)

TABLE OF CONTENTS

ABSTRACT

The tumor suppressor p53 is mutated in more than 50% of all human solid tumors. This comprises mostly single residue missense point mutations that entail a loss of p53 tumor suppressor function. But at the same time, mutant p53 protein was shown to possess oncogenic activities, i.e. a gain of function, promoting invasion and chemoresistance. Mutant p53 specifically accumulates in advanced tumors, but not in normal tissues, engineered to contain a mutant p53 gene. This means that tumor specific changes evoke the accumulation of mutant p53 during tumor progression. Within this study we observed that mutant p53 accumulates even further, when tumor cells are exposed to some, but not all chemotherapeutic drugs. While the anthracyclines doxorubicin, daunorubicin and epirubicin led to the accumulation of mutant p53, the highly similar compound idarubicin did not. We found the expression of mutant p53 to be regulated at different levels: First, treatment with the topoisomerase II inhibitors daunorubicin, doxorubicin, epirubicin, idarubicin, and etoposide, evokes a DNA damage response that results in the activation of E2F1 and its target gene TAp73. Our data suggest that, upon these genotoxic treatments, E2F1 contributes to the transcriptional activation of mutant p53 pre-mRNA synthesis, both directly and through induction of TAp73. We further show for the first time that the transcription factor E2F1 associates with the promoter DNA of *TP53.* Second, among these chemotherapeutics that induce p53 transcription, we found two members to additionally induce a natural antisense transcript to p53, WRAP53. We further observed that the induction of WRAP53 coincides with impaired p53 mRNA maturation. We therefore hypothesize that the expressed antisense transcript interferes with p53 pre-mRNA stability or its nuclear export. Third, the accumulation that is inflicted on the cells during

carcinogenesis seems to be mostly regulated on the protein level. We performed a high-content siRNA screen, using single-cell based microscopy analysis, and thereby identified the ribosomal S6 kinases to be involved in mutant p53 expression regulation in advanced cancer cells. We believe that our findings should be considered for chemotherapy prescription, since we have shown that some topoisomerase II inhibitors augment mutant p53 expression and thus might favor unwanted tumor progression.

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ABBREVIATIONS

ABBREVIATION	FULL NAME
5'FU	5-fluorouracil
AA/ BAA	Acrylamide/ bisacrylamide
APS	Ammonium persulfate
AraC	Cyctosine arabinose
AraCTP	Cytidine triphosphate arabinose
ARF-BP1	ARF-binding protein 1
ATM	Ataxia-telengiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia-telengiectasia mutated and Rad3-related
Bax	Bcl2-associated X protein
BCLAF	B-cell lymphoma associated factor
BS	Binding site
BSA	Bovine serum albumin
°C	Degree Celsius
CBP	cAMP response element-binding protein binding protein
Cdc	Cell cycle division
Cdk	Cyclin dependent kinase
cDNA	complementary DNA
ChIP	Chromatin immunoprecipitation
Chk	Checkpoint kinase
CK1	Casein kinase 1
COP1	Constitutive photomorphogenesis protein 1
CPT	Camptothecin
Da	Dalton
Dauno	Daunorubicin
dCTP	Deoxycytidine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxynucleoside triphosphate
Dox	Doxorubicin
dTMP	Deoxythimidine monophosphate
dUMP	Deoxyuridine monophosphate
DTT	1,4-Dithiothreitol
DYRK	Dual specificity tyrosine-phosphorylation-regulated kinase
ECL	Enhanced chemoluminescence
Epi	Epirubicin
et al.	et altera
Etop	Etoposide
FCS	Fetal calf serum
g	Gram; Gravitational acceleration
GC rich	Guanine and Cytosine rich
h	Hour
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIPK2	Homeodomain-interacting protein kinase 2
Ida	Idarubicin
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
l	Liter
M	Molar
m	meter
Mdm	Mouse double-minute
min	Minute
MK2	Mitogen-activated protein kinase-activated protein kinase
M-MuLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
µ	Micro
NHEJ	Non-homologous end joining
NP-40	Nonidet P40
P-	Phospho-

PAGE	Polyacrylamide gel elektrophoresis
PBS	Phosphate buffered saline
PCAF	p300/ CBP associated factor
PCR	polymerase chain reaction
PDK	Phosphoinositide-dependent protein kinase
pH	Negative logarithm (base 10) of the molar concentration of dissolved protons
Pirh2	p53-induced protein, RING-H2 domain-containing
PUMA	p53-upregulated modulator of apoptosis
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNA Pol II	RNA polymerase II
RNAse	Ribonuclease
RPL27	Ribocomal protein L26
RPMI	Roswell Park Memorial Institute medium
RPS6K	Ribosomal protein S6 kinase
RREB	Ras-responsive element binding protein
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
sec, s	Second
SDS	Sodium dodecyl sulfate
siRNA	small interfering ribonucleic acid
Taq	*Thermus aquaticus*
TEMED	N,N,N´,N´- Tetramethylethylenediamine
TIP60	TAT-interacting protein, 60-kDa
Tris	tris(hydroxymethyl)aminomethane
TSS	Transcriptional start site
U	Unit of enzyme activity
UV	Ultra violet
V	Volt
w/v	weight per volume
WB	Westernblot = immunoblot
WRAP53	WD repeat-containing antisense to p53
wt	wild type

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Most cells of our body have the potential to divide, an essential process in the renewal of tissues and the scope of our immune system. Some cells proliferate rapidly, others rarely, but proliferation happens in a controlled fashion, with a large set of check points and back-up mechanisms (Pagano and Draetta, 1991). Due to exogenous stimuli like irradiation, exposure to toxins or other forms of stress, key players of this regulation cascade can be mutated and become dysfunctional. If the affected cell thereby gains a certain growth advantage, but is not recognized by the immune system and eliminated, it starts to proliferate in an uncontrolled fashion. Over time, more and more mutations accumulate in these rapidly dividing cells and, depending on the genes that were hit by mutations; this can lead to the development of malignancies.

When a tumor is diagnosed, it is often already in an advanced stage and needs to be treated by chemotherapy. Nowadays a number of chemotherapeutic agents are available; their mechanisms of action are diverse and often not completely understood yet. Depending on the cell type and the mutational spectrum, tumors are treated with different chemotherapeutic agents. Nevertheless, the mechanistic details and the cause of some side effects are widely unknown and a matter of current research.

2.1. CHEMOTHERAPEUTICS

The idea behind most chemotherapeutic drugs is that they trigger a DNA damage response in proliferating cells and subsequently lead to apoptosis (Johnstone *et al.*, 2002). Since tumor cells are normally proliferating faster than most other cells of our body, they are preferentially targeted. Nevertheless,

hematopoetic cells, gastrointestinal mucosal cells and hair are examples of rapidly dividing cells that often get affected by these drugs although their fast proliferation happens in a controlled fashion and is important for their proper function (Tannock, 1986).

Generally, chemotherapeutic drugs can be clustered in three groups according to their mechanism of action: nucleoside analogs, inhibitors of enzymes involved in replication and transcription, and drugs that directly damage the DNA (Pommier and Diasio, 2006).

2.1.1. NUCLEOSIDE ANALOGS

This group of drugs is also called 'antimetabolites' and either inhibits the formation of functional nucleotide triphosphates, or interferes with replication elongation (Daher *et al.*, 1990). All agents that belong to this class prevent efficient DNA synthesis and mostly affect the cells in S phase of the cell cycle. Examples are on the one hand 5-fluorouracil (5'FU), which inhibits the conversion of dUMP to dTMP and causes due to depletion of dTMP defects in DNA synthesis and cell division (Daher *et al.*, 1990). On the other hand, agents like Cytosine arabinose (AraC) affect replication elongation; AraC is recognized by DNA polymerase α as deoxycytosine, but the incorporation of AraCTP in the elongating DNA strand fails due to sterical hindrance resulting in the termination of DNA replication (Chrencik *et al.*, 2003).

2.1.2. ENZYME INHIBITORS

Enzymes with specific functions during replication are the polymerases, topoisomerases and helicases; these also reassemble the most common drug targets of this class. Polymerases are for example targeted by aphidicolin and foscarnet that block dCTP incorporation or pyrophosphate cleavage, respectively (Crumpacker, 1992; Sheaff *et al.*, 1991).

Topoisomerases are enzymes that relax supercoiled DNA by cleavage and religation (D'Arpa and Liu, 1989). The chemotherapeutic drugs camptothecin, etoposide and the anthracyclines daunorubicin, doxorubicin, epirubicin and idarubicin are well known representatives of this class. While camptothecin specifically acts on topoisomerase I, an enzyme that functions through single strand cleavage, and traps the cleavage intermediates (Pommier *et al.*, 2003), the other mentioned drugs are mainly known to act on topoisomerase II (Fortune and Osheroff, 2000). Even though all of these agents in the end lead to double strand breaks that trigger a DNA damage response and induce apoptosis. The topoisomerase II inhibitors additionally can interfere with other metabolic processes of the DNA, like transcription, DNA repair, and chromatin remodeling (Fortune and Osheroff, 2000). In contrast to camptothecin these drugs trap the cleavage intermediate, with the two enzyme subunits of 170 and 180 kDa covalently linked to the DNA. Thereby large protein-DNA adducts are produced that form steric blocks on the template DNA (Fortune and Osheroff, 2000).

The planar structure of anthracyclines additionally allows them to intercalate into DNA, preferentially in GC rich regions. This was shown to stabilize the duplex DNA and to prevent helicases from separating the strands (Bachur *et al.*, 1992).

Inhibitors that act independently of these enzymes, directly involved in replication, but still inhibit cell cycle progression interfere with cyclin-dependent kinases (Cdk) or the checkpoints. The Cdk inhibitors flavopiridol and roscovitine are competitive inhibitors of ATP binding and interfere at various steps in the cell cycle: G1/S transition (restriction point) through Cdk4/6, the activation of replication origins (S-phase) through Cdk2, and the inactivation of these replication origins by Cdk1-cyclin B complexes (De Falco and De Luca, 2010). Additionally, it was shown that these Cdk inhibitors inhibit RNA polymerase II and thereby transcription (Wesierska-Gadek and Krystof, 2009). Nevertheless,

currently most of these kinase inhibitors lack specificity. This also holds true for the checkpoint inhibitor 7-hydroxystaurosporine, a checkpoint kinase 1 (Chk1) inhibitor that was found to additionally inhibit Chk2 and phosphoinositide-dependent protein kinase 1 (PDK1) (Sato *et al.*, 2002; Yu *et al.*, 2002), as well as caffeine, the first drug identified to abrogate a cell cycle checkpoint by inhibiting Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and RAD3-related (ATR), but also a number of additional kinases (Sabisz and Skladanowski, 2008; Sarkaria *et al.*, 1999).

2.1.3. DNA DAMAGING DRUGS

Additionally to radiotherapy there are chemotherapeutic drugs that block the replication fork by inducing DNA template lesions, like DNA adducts, DNA strand breaks, and DNA protein crosslinks. The alkylating agents modify bases within the DNA, either through methylation of Guanine (methylmethanesulfonate), DNA-DNA crosslinks and DNA-protein crosslinks (cisplatin), or interstrand DNA crosslinks (cyclophosphamide) (DeNeve *et al.*, 1990; Hausheer *et al.*, 1989; Mirzayans *et al.*, 1988). In contrast to that, radiomimetic DNA cleaving agents like bleomycin and neocarcinostatin induce single- as well as double-strand breaks of the DNA (Goldberg, 1987; Huang *et al.*, 1981). As for most of the mentioned drugs, the induction of such DNA lesions triggers a DNA damage response, which signals from ATM or ATR down to the effectors p53, E2F1, cell division cycle 25 (CDC25) and others (Darzynkiewicz *et al.*, 2009).

2.2. THE DNA DAMAGE RESPONSE

Originally two different DNA damage pathways were identified. Their activation was observed depending on the kind of damage that was inflicted on the DNA. In response to double strand breaks ATM is recruited to the sites of DNA damage and gets activated, the signal is transduced by Chk2 which in turn

leads to the accumulation and activation of p53, E2F1 and other effectors (Lavin and Khanna, 1999). In contrast to that, ATR is activated by single strand breaks, the signal transduced by Chk1 and finally effectors like p53, Cdc25 and others get activated (Paulsen and Cimprich, 2007). Nowadays, there is a lot more crosstalk between the two pathways known and additional kinases at the levels of ATM, ATR as well as Chk1 and Chk2 were identified.

2.2.1. KINASES IN THE DNA DAMAGE RESPONSE

Not only the role of DNA-dependent protein kinase (DNA-PK), sensing DNA double strand breaks and lesions of non homologous end joining (NHEJ), amends the network of kinases activated in response to DNA damage (Danska and Guidos, 1997; Rathmell *et al.*, 1997), also p38 and its activation of mitogen-activated protein kinase-activated protein kinase 2 (MK2), as well as the recently identified cross talks between the pathways (Reinhardt *et al.*, 2007). The impact of phosphorylations involved in this network is immense and our knowledge about these is most probably far from being complete. The current view on central players within this network and their most prominent targets is summarized in Figure 1.

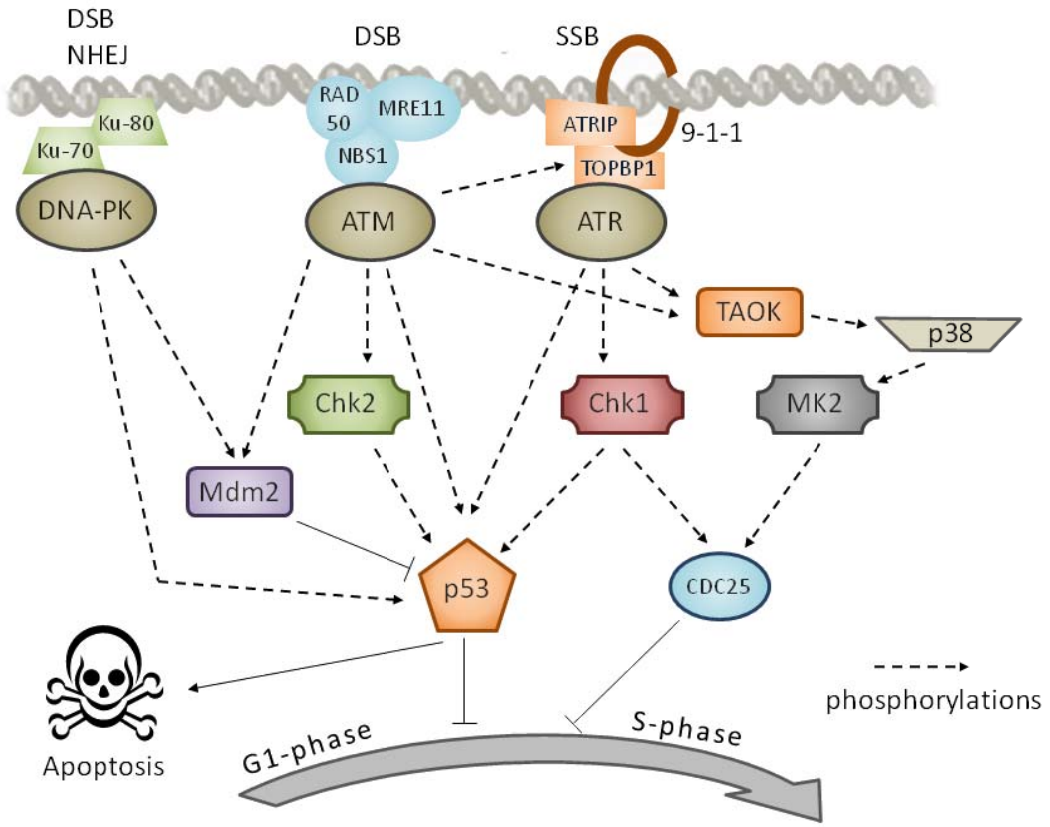


Figure 1: The role of kinases in the DNA damage response pathways.
Due to different stimuli sensor proteins get recruited to the site of DNA damage and trigger a DNA damage response. Double strand breaks (DSB) or lesions of non-homologous end joining (NHEJ) are recognized by the heterodimer Ku70/ 80 and lead to the recruitment and activation of DNA-dependent protein kinase (DNA-PK). This in turn activates p53, on the one hand through direct phosphorylation and on the other hand through phosphorylation of Mouse double-minute 2 (Mdm2). Additionally DSB can be sensed by the MRN complex (Meiotic recombination 11 (MRE11), Rad 50, and Nijmegen breakage syndrome 1 (NBS1)) that activates the transducer kinase Ataxia-telangiectasia mutated (ATM). ATM has a number of different phosphorylation targets that in turn all lead to the activation of p53: Mdm2, checkpoint kinase 2 (Chk2), p53 itself, TAO kinase (TAOK), and also DNA topoisomerase II-binding protein 1 (TOPBP1). The latter itself is part of the DNA single strand break (SSB) sensor complex and involved in the activation of Ataxia-telangiectasia and RAD3-related (ATR). Besides TOPBP1 also the Rad9-Hus1-Rad1 clamp complex (9-1-1) and ATR-interacting protein (ATRIP) contribute to the sensing of DNA single strand breaks and the activation of ATR. Similar to ATM, also ATR has a number of different phosphorylation targets that activate p53 and mark the cell cycle regulatory protein cell division cycle 25 (CDC25) for degradation: p53 itself, checkpoint kinase 1 (Chk1) and TAOK. The latter was found to phosphorylate and activate p38, which in turn phosphorylates and activates mitogen-activated protein kinase-activated protein kinase 2 (MK2), a kinases that was shown to directly phosphorylate CDC25A, leading to its destabilization.

2.2.2. E2F ACTIVITIES IN RESPONSE TO DNA DAMAGE

Since E2F1 was identified in 1987 by Kovesdi *et al.* the number of known E2F family members increased and currently comprises eight genes (E2F1 to 8), which give rise to nine distinct proteins (DeGregori and Johnson, 2006). The transcription factors can be categorized into three groups: E2F1 – 3A are mostly found as activating transcription factors that can get inactivated through their binding to the retinoblastoma protein (Rb). E2F4 and -5 are frequently detected in their inactive state, bound to one of the three pocket proteins (Rb, p107, or p130), but are generally categorized as weak activators. Finally, E2F6 – 8 are classified as transcriptional repressors, which do not interact with any of the pocket proteins (Trimarchi and Lees, 2002). The E2F proteins transactivate several Cdks, as well as cyclins and thereby contribute positively to cell cycle progression and cell proliferation.

Additionally to its cell cycle related functions, E2F1 was found to be an activator of the DNA damage response pathway. It was shown that over-expression of E2F1 leads to increased Chk2 mRNA, as well as protein levels (Rogoff *et al.*, 2004). Over and above, Stevens *et al.* (2003) reported that Chk2 phosphorylates E2F1 and thereby alters the DNA binding specificity of E2F1 from S-phase genes to the pro-apoptotic gene p73. These findings underscore the controversial activities that were implied to E2F1 in the literature. The transcription factor was originally identified as an oncogene, whose hyperactivation leads to uncontrolled cell proliferation, and was later on found to actively suppress tumorigenesis by inducing pro-apoptotic genes in response to DNA damage.

2.2.3. P53 IN THE DNA DAMAGE CASCADE

The tumor suppressor p53, as well as the two E3 ubiquitin ligases Mdm2 and Mdm4 are common phosphorylation targets of Chk1, Chk2, but also the

upstream components of the DNA damage signaling pathways ATM, ATR and DNA-PK (Figure 2) (Meek, 2009).

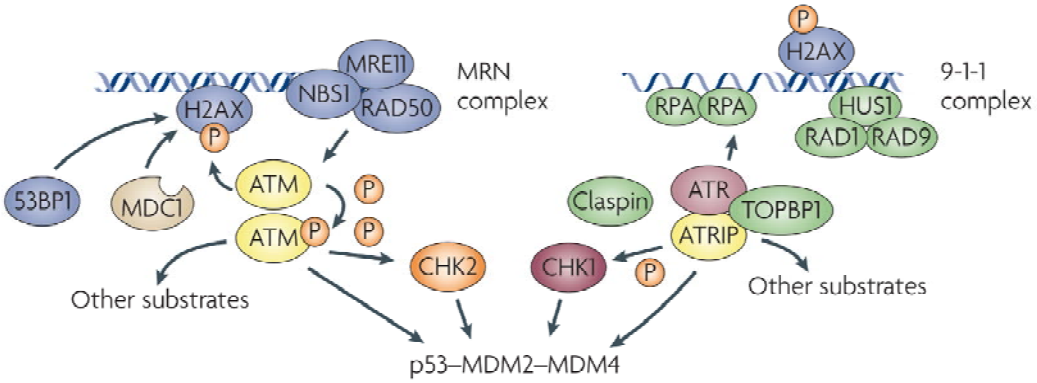


Figure 2: All DNA damage pathways converge at the point of p53 phosphorylation [taken from Meek (2009)].
The activation of Ataxia-telangiectasia mutated (ATM) and checkpoint kinase 2 (Chk2) in response to double strand breaks, sensed by proteins of the MRN complex (Meiotic recombination 11 (MRE11), Rad 50, and Nijmegen breakage syndrome 1 (NBS1)) results in the phosphorylation of p53 and its two antagonists Mouse double-minute 2 (Mdm2) and Mouse double-minute 4 (Mdm4). The same is achieved in response to single strand breaks, which are sensed by the 9-1-1 complex (RAD 9, RAD 1, and HUS 1) and transduced through the activation of the Ataxia-telangiectasia and RAD3-related (ATR) and checkpoint kinase 1 (Chk1) kinases.
H2AX: Histone variant; MDC1: Mediator of DNA damage checkpoint protein 1; 53BP1: p53-binding protein 1; RPA: Replication protein A; TOPBP1: DNA topoisomerase II-binding protein 1; ATRIP: ATR-interacting protein.

While p53 is activated and stabilized through these phosphorylations (Canman *et al.*, 1998), it was shown that its antagonists Mdm2 and Mdm4 get destructed (Maya *et al.*, 2001). Following from the above, p53 is stabilized in two ways in response to DNA damage, since Mdm2 in complex with Mdm4 is known to be the most prominent negative regulator of p53 (Meulmeester *et al.*, 2005; Montes de Oca Luna *et al.*, 1995). These ATM and ATR mediated phosphorylations trigger a cascade of additional posttranslational modifications of p53 that can tailor its response in an appropriate and proportionate manner according to the nature and intensity of the damage (Murray-Zmijewski *et al.*, 2008).

2.3. THE TUMOR SUPPRESSOR P53

Already in the early 90s the human p53 protein was identified to bind to the palindromic DNA sequence Pu-Pu-Pu-C-$^{A}/_{T}$-$^{T}/_{A}$-G-Py-Py-Py and its biological function as transcription factor was proposed (el-Deiry *et al.*, 1992; Kern *et al.*, 1991). The C-terminal oligomerization domain of the protein facilitates its tetramerization, which is essential for DNA binding as well as transcriptional activation of target genes (McLure and Lee, 1998). Nowadays, hundreds of genes regulated by p53 are known that can generally be classified upon their functions in cell cycle arrest, apoptosis, DNA repair, angiogenesis, and senescence (el-Deiry, 1998). The fine tuning of transcriptional activation through p53 mostly happens on the level of posttranslational modifications.

2.3.1. POSTTRANSLATIONAL MODIFICATIONS

The tumor suppressor p53 is known to be modified by all kinds of posttranslational modifications like phosphorylation, acetylation, methylation, neddylation, ubiquitination, and sumoylation. Most sites of modification as well as a number of modifying and demodifying enzymes are known so far (Olsson *et al.*, 2007). Nevertheless, the causes and consequences of the different modification patterns are not completely understood yet and a matter of current research.

PHOSPHORYLATION

Numerous Threonine and Serine residues, mainly within the transactivation domain of p53, have been identified as targets of phosphorylation by kinases like ATM, ATR, DNA-PK, Chk1, Chk2, CK1, JNK, HIPK2 and DYRK2 (Bode and Dong, 2004). These modifications often lead to the stabilization of the protein and hence to its activation in response to genotoxic and other forms of stress. Data from in vitro or over-expression studies indicate that phosphorylation at Serine 15 stimulates p53-dependent transactivation, growth arrest and apoptosis in response to DNA damage, whereas it is still under

debate whether phosphorylation of this site affects Mdm2 binding (Dumaz and Meek, 1999). Two groups established mouse models that express a mutant version of p53 where Serine 18 (corresponding to Serine 15 in humans) is replaced by Alanine and can therefore not be phosphorylated any longer. Thymocytes of these mice displayed a reduced induction of DNA damage mediated apoptosis, indicating that this phosphorylation in vivo contributes to the specific activation of target genes (Chao *et al.*, 2003; Sluss *et al.*, 2004).

ACETYLATION

The histone acetyltransferase (HAT) heterodimers CBP/p300 were found to acetylate p53 at Lysines 370, 372, 373, 381, and 382 (Gu and Roeder, 1997). In contrast, Lys320 and Lys305 in the nuclear localization domain of p53 are acetylated by PCAF and p300 respectively (Liu *et al.*, 1999). Some studies reported an enhancement of sequence-specific DNA-binding activity of acetylated p53, as well as more potent transcriptional activation of target genes (Barlev *et al.*, 2001; Sakaguchi *et al.*, 1998). Along that line, it was shown by two independent groups that acetylation of Lys120 of p53, by the MYST acetyltransferases MOF and TIP60, leads to the preferential induction of pro-apoptotic target genes such as PUMA and Bax, whereas the expression of other target genes like p21 and Mdm2 remains unaffected (Sykes *et al.*, 2006; Tang *et al.*, 2006). As the lysine residues within the C-terminal domain of p53 are also targets for ubiquitination, it was proposed that acetylation of these residues may promote the stabilization of p53 by interfering with proteasomal degradation (Brooks and Gu, 2003; Ito *et al.*, 2002).

To elucidate the impact of these acetylations in vivo, different mouse models were generated harboring up to 7 Lysine to Arginine mutations. Unfortunately these studies were not conclusive, since the phenotypes of these mice were very mild. The fact that various posttranslational modifications are conjugated to the same set of Lysines implies that the biological consequences, caused by

these, cancel each other out and therefore burrow the actual activities (Olsson *et al.*, 2007).

UBIQUITINATION

In contrast to the above it appeared to be very conclusive when mouse models were used to unravel the biological consequences of p53 ubiquitination. Montes de Oca Luna *et al.* (1995) generated a knock-out mouse line for Mdm2, the most prominent E3 ubiquitin ligase of p53. This resulted in embryonic lethality of the mice, a strong phenotype that was rescued by the additional knock-out of p53. These observations indicate that the lack of Mdm2-mediated p53 degradation leads to massive apoptosis and therefore to embryonic lethality of the mice. The E3 ubiquitin ligases COP-1, Pirh2, and ARF-BP1 were as well described to ubiquitinate p53 and to induce its proteasomal degradation (Chen *et al.*, 2005a; Dornan *et al.*, 2004; Leng *et al.*, 2003). Nevertheless, the above mentioned Mdm2 knock-out study suggests that in unstressed cells no additional E3 ubiquitin ligase is able to prevent the accumulation of p53 and its induction of apoptosis.

2.3.2. REGULATION OF P53 EXPRESSION

The expression levels of p53 are mainly regulated on the protein level. Mdm2, the above described essential p53 ubiquitin ligase, is itself one of the p53 target genes and thereby forms an autoregulatory feedback loop with the tumor suppressor (Freedman *et al.*, 1999). Mdm2 binds to the N-terminus of p53 and ubiquitinates it, either at C-terminal Lysines, or at Lysines within the DNA binding domain, this subsequently leads to the nuclear export or proteasomal degradation of the protein (Li *et al.*, 2003). Even though it seems to be an energetically unfavorable mechanism, the constant transcription, translation and proteasomal degradation of p53 allows the cell to rapidly react to various stress conditions, like DNA damage, oncogene activation, hypoxia and other inducers of the p53 network (Figure 3).

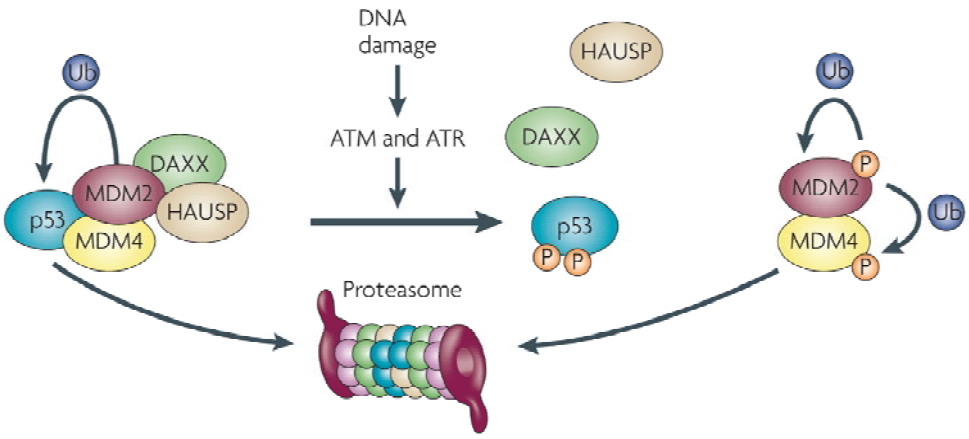


Figure 3: The fate of p53: between proteasomal degradation and DNA damage induced phosphorylation
[taken from Meek (2009)].
In unstressed cells the expression of p53 is kept at low levels. The E3 ubiquitin ligases Mouse double-minute 2 (Mdm2) and Mouse double-minute 4 (Mdm4), as well as the deubiquitinating enzyme Ubiquitin-specific protease (HAUSP) regulate its nuclear export and proteasomal degradation. In response to DNA damage, signaling cascades via Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and RAD3-related (ATR) lead to the phosphorylation of p53 and the E3 ligases Mdm2 and Mdm4. While p53 gets stabilized through these modifications, its antagonists get ubiquitinated (Ub) and subjected to proteasomal degradation.

Other kinds of p53 regulation received less attention. But, meanwhile it became evident that there are a few regulators that induce p53 expression through transcriptional activation. First, HOXA5 was found as a positive regulator of p53 transcription in response to DNA damage with the additional observation that its loss of mRNA expression in tumor samples is positively correlated with a loss of p53 mRNA expression (Raman *et al.*, 2000). Liu *et al.* (2007) reported that upon exposure to genotoxic stress, PKCdelta gets activated and interacts with the death-promoting transcription factor Btf (alias BCLAF) to co-occupy promoter elements within *TP53*. They furthermore reported that siRNA mediated knock-down of Btf suppresses p53-mediated apoptosis in response to DNA damage. Wang and el-Deiry (2006) found that p53 itself and, its structurally und functionally related family member, TAp73 are capable of regulating the expression of p53 on the mRNA level. They described three potential p53/ TAp73 responsive elements in the promoter region of p53, further identifying one of them to be essential using luciferase assays. Recently also

Ras-responsive element binding protein 1 (RREB1) was identified as potential transcriptional activator of p53 expression in response to DNA damage (Liu *et al.*, 2009).

In contrast to the forecited transcription factors, Mahmoudi *et al.* (2009) discovered an additional mechanism of p53 mRNA expression regulation. The natural antisense transcript to p53 (WRAP53) was found to mediate p53 mRNA stability in response to DNA damage. It was identified as a predicted gene within the *TP53* genomic locus on chromosome 17, encoded on the opposite strand of the tumor suppressor. The biological role of WRAP53 protein is completely unknown, whereas the specific over expression of certain transcripts was shown to increase p53 mRNA expression.

2.3.3. THE INTERPLAY OF P53/ P73 AND E2F1

In response to DNA damage p53 and E2F1 both get stabilized through phosphorylation by the same set of kinases: ATM, Chk1, and Chk2. The phosphorylation of E2F1 through Chk1 and Chk2 then leads to the induction of pro-apoptotic target genes like TAp73 (Stevens *et al.*, 2003). This is proposed to be a backup mechanism, when p53 is defective, since TAp73, a paralog of p53, is known to transactivate the same pro-apoptotic target genes as p53 (McKeon, 2004).

But, there is also direct cross-talk between the two transcription-factors p53 and E2F1 reported. On the one hand, deregulated E2F was found to directly transactivate the expression of $p14^{ARF}$, which inhibits Mdm2 and thereby leads to the stabilization and activation of p53 (Bates *et al.*, 1998). While in the absence of $p14^{ARF}$, E2F1 was found to stimulate p53 phosphorylation. Within the same study, it was claimed that this, most probably ATM or ATR dependent, posttranslational modification of p53 is crucial for E2F1-mediated apoptosis (Rogoff *et al.*, 2002).

2.3.4. MUTATIONS OF P53

In 1979 p53 was identified as a protein accumulated in the majority of the analyzed tumors and therefore characterized as tumor antigen (Crawford *et al.*, 1981; DeLeo *et al.*, 1979; Rotter *et al.*, 1980). Almost 10 years later Finlay *et al.* (1988) among others discovered that for all the work that was performed meanwhile a mutant p53 clone was used and that p53 in fact acts as a tumor suppressor. The mutations found in p53 do not reflect the classical spectrum known from other tumor suppressors, where frame shifts or large deletions mainly cause the loss of tumor suppressor activity. On the contrary, point mutations of single amino acids, as they are found in p53, are characteristic for oncogenes. Nevertheless, point mutations in oncogenes normally affect a small number of codons, encoding residues involved in their enzymatic activity, whereas the mutational spectrum of p53 ranges throughout the whole DNA binding domain of the protein, with a number of hotspot mutations that occur more frequently than others (Soussi and Lozano, 2005) (Figure 4).

A comprehensive list of published studies where p53 mutations have been analyzed by gene sequencing is provided on the IARC *TP53* database (http://www.iarc.fr/p53/). Evaluation of these data revealed that in about 70% of the reported studies the presence of a *TP53* mutation is significantly associated with bad prognosis, whereas only 5% of the studies reported a significantly good prognosis upon *TP53* mutation (Olivier *et al.*, 2005).

These observations indicate that cancer-associated mutant p53 isoforms are more than just relics of wt p53 inactivation and possess distinctive roles in tumor cells. Firstly, this can be achieved through dominant-negative effects over co-expressed wild type p53 proteins, forming mixed tetramers that are incapable of DNA binding and transactivation. Secondly, the generated mutant p53 protein might possess activities of its own, which could actively contribute to tumor progression.

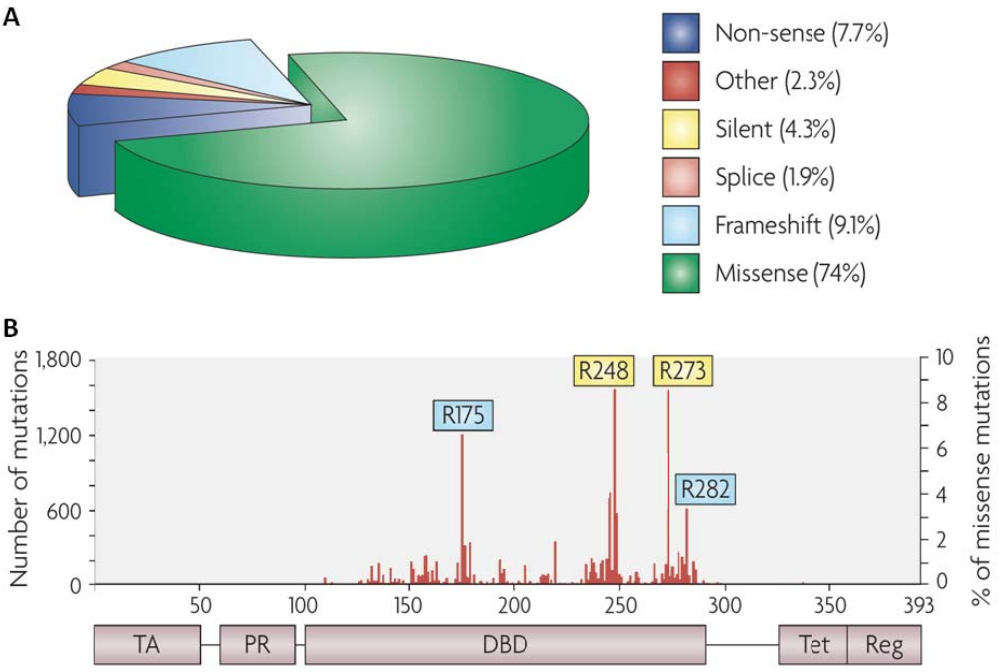


Figure 4: The tumor suppressor p53 is most frequently inactivated through a missense mutation within the DNA binding domain
[adopted from Brosh and Rotter (2009)].
A: Pie chart representing the different tumor-derived mutation types reported in the IARC *TP53* Mutation Database. **B:** Almost every amino acid of the central DNA binding domain of p53 is hit by a point mutation leading to p53 loss of function. Four amino acids at the positions 175, 248, 273, and 282 are more frequently hit and represent the so called hotspot mutations of p53.
TA: transactivation domain; PR: proline-rich domain; DBD: DNA binding domain; Tet: tetramerization domain; Reg: carboxy-terminal regulatory domain. Data derived from the IARC *TP53* Mutation Database version R13 (November 2008).

2.3.5. P53 GAIN OF FUNCTION

Mutant p53 gain of function (GOF) was described first by Dittmer *et al.* (1993). They showed that ectopic mutant p53 expression can transform p53-null cells, leading to increased colony formation in culture and raised numbers of tumors in mice. Meanwhile three hallmarks of transformation were described to promote mutant p53 gain of function:

GENOMIC INSTABILITY

Mutant p53 expression was shown to disrupt normal spindle checkpoint control leading to polyploid cells (Gualberto *et al.*, 1998). Additionally, mice with over-expressed mutant p53 were described to exhibit a high degree of genomic

instability with aberrant centrosome amplification, as well as chromosome translocations (Caulin *et al.*, 2007).

ANTIAPOPTOTIC SIGNALING

Mutant p53 can suppress c-myc induced apoptosis in leukemic cells and thereby allows the cell to benefit from the pro-proliferative effects of the oncogene, without inducing apoptosis at the same time (Lotem and Sachs, 1995). Additionally, mutant p53 expression decreases the induction of apoptosis in response to chemotherapeutics, as well as other kinds of DNA damage, thereby conferring chemoresistance on the tumor cells (Blandino *et al.*, 1999; Li *et al.*, 1998).

CELL MIGRATION AND INVASION

In vitro studies by Adorno *et al.* (2009) and Wang *et al.* (2009) indicated that mutant p53 can augment cell migration and invasion. Nevertheless, it is believed that this process is highly cell-context dependent and in many cases additional signals like oncogenic Ras or TGF-β are needed to unleash this gain of function activity. To estimate the biological relevance of these observations, data from different mouse models were used. Both, mutant p53 over-expressing cells intravenously inoculated into syngeneic mice and knock-in studies, where the endogenous wt p53 was replaced by its mutant variant, revealed that mutant p53 expression leads to the development of more aggressive, metastatic tumors. This supports the concept that mutant p53 gain of function actively contributes to tumor progression (Heinlein *et al.*, 2008; Pohl *et al.*, 1988).

The mechanistic understanding of the role of mutant p53 in tumor cells is still not complete, but the available reports offer some insights. Microarray analysis yielded a large list of genes regulated in their expression by mutant p53. Nevertheless, it was also shown that most mutant p53 variants cannot directly bind to the p53 responsive elements, since either the amino acids involved in direct DNA binding are mutated or the gained mutations lead to overall changes

in the conformation of the protein. Therefore, the effects of mutant p53 on the transcriptional regulation of other genes have to occur indirectly (Figure 5) (Oren and Rotter, 2010). First, mutant p53 was found in complex with its two family members p63 and p73, thereby inhibiting their transcription factor activities (Gaiddon *et al.*, 2001; Strano *et al.*, 2002). Second, mutant p53 was shown to bind to a number of other transcription factors, either leading to the repression of their activity, or recruiting transcriptional activators that facilitate the transcription of the downstream genes (Di Agostino *et al.*, 2006; Stambolsky *et al.*, 2010; Weisz *et al.*, 2007). Last but not least, it was shown that mutant p53 can bind specific DNA elements, such as matrix attachment regions, in a conformation dependent manner. This is proposed to block the binding of other transcription factors to adjacent binding sites, resulting in transcriptional inhibition (Gohler *et al.*, 2005).

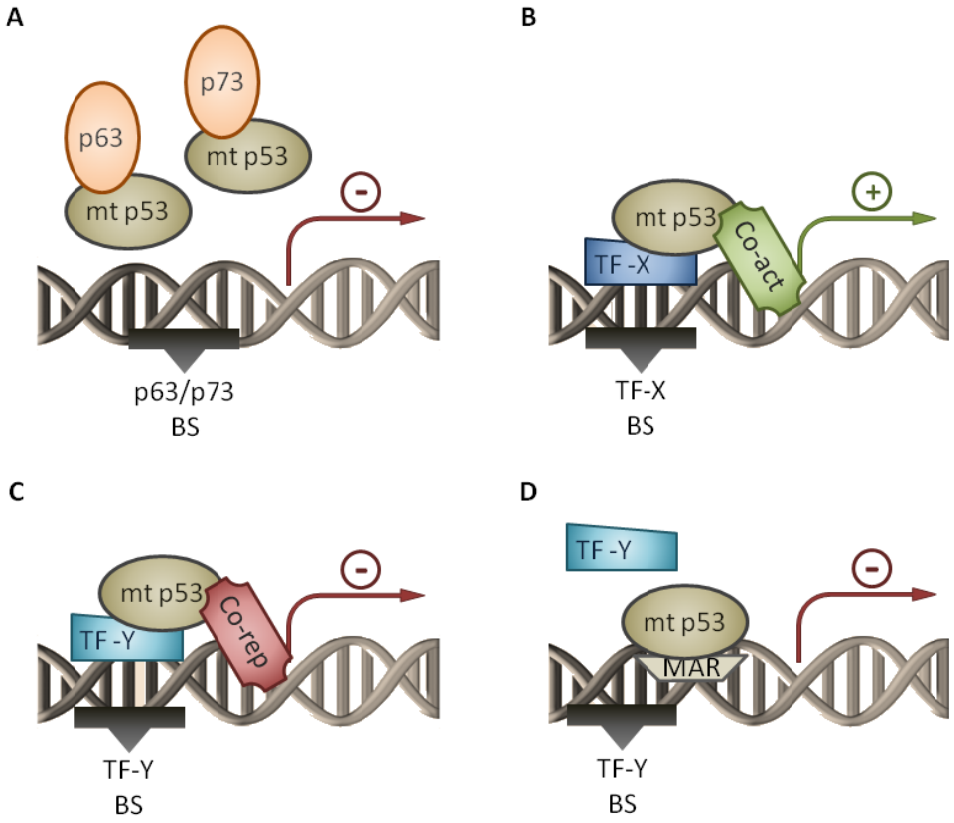


Figure 5: Transcriptional regulation by mutant p53 independently of its DNA binding activity.
A: Transcriptional inhibition through direct interaction and inactivation of its family members p63 and p73. **B:** Transcriptional activation of target genes through interaction with other sequence specific transcription factors (TF-X) and the recruitment of co-activators. **C:** Transcriptional repression of target genes through interaction with other sequence specific transcription factors (TF-Y) and the recruitment of co-repressors. **D:** Association with matrix-attachment regions (MAR) that partially overlap with other transcription factor binding sites and thereby prevent the binding and transactivation of the down-stream genes.
[Adopted from Oren and Rotter (2010)]

2.3.6. MUTANT P53 ACCUMULATION

Mutant p53 protein often accumulates in tumor cells and reaches steady-state levels that greatly exceed those of wt p53. Additionally, it was reported that mutant p53 is not intrinsically stable. This was on the one hand observed in primary cells derived from Li-Fraumeni syndrome patients, who carry germ line p53 mutations (Yin *et al.*, 1992), and on the other hand in the knock-in studies performed by Lang *et al.* (2004) and Olive *et al.* (2004), where mutant p53 protein was shown to only accumulate in tumors, but not in the surrounding tissue.

2.4. THE AIM OF THIS WORK:

THE MECHANISMS OF MUTANT P53 ACCUMULATION

During the last 30 years of p53 research, it was repeatedly shown that the tumor suppressor p53 strongly accumulates in response to chemotherapeutic treatment, going along with posttranslational modifications of the protein at various sites. A similar or even stronger accumulation of p53 is observed in tumor cells that express a mutant variant of the protein. Missense mutations of the protein were shown to not only abrogate its tumor suppressive activities, but also to actively promote oncogenic functions, ranging from genomic instability, over antiapoptotic signaling to increased metastasis and proliferation.

The question that arises from the above is whether the mechanisms leading to p53 accumulation in response to chemotherapeutic treatment, act synergistically with the generally observed augmentation of p53 expression in cancer cells harboring a p53 point mutation. Within this study we observed that such a synergism can lead to the further accumulation of mutant p53 in cancer cells upon treatment with some but not all chemotherapeutic drugs. Therefore we investigated the mechanistic details of mutant p53 accumulation on the one hand gained due to cellular transformation and on the other hand through chemotherapeutic treatment. We found that mutant p53 expression is regulated differently during these two processes causing its accumulation. To achieve the benefits of chemotherapeutic treatment and at the same time circumvent the undesired side effects of mutant p53 accumulation, it would be advantageous to use the obtained information for the development of new therapeutics that could be used in combination with classical chemotherapeutics.

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. TECHNICAL DEVICES

EQUIPMENT	COMPANY
Agitator, magnetic, heated (MR 3001)	Heidolph
Bioruptor (UCD-200TM-EX)	Diagenode
Blotting-chamber (EasyPhor Wet-Blotter)	BioZym
Centrifuge, mini (GMC-060 LMS)	Eppendorf
Centrifuge (5415R)	Eppendorf
Centrifuge (Megafuge 1.0 R)	Heraeus Instruments
ChemoCam Imager (ECL detection)	Intas
Countess	Invitrogen
Electrophoresis-System, for SDS-PAGE	Amersham Biosciences
Foil swelding machine	KRUPS
Freezer -20°C	Liebherr
Freezer -80°C	Heraeus Instruments
Heating block (HTB-1-131 HLC)	Haep Labor Consult
Thermomixer comfort	Eppendorf
Ice-machine (B100)	Ziegra
Incubator for cell cultures (Hera Cell 150)	Heraeus Instruments
Laminar flow cabinet (Hera Safe)	Heraeus Instruments
Light microscope (Axovert 40C)	Zeiss
Liquid Nitrogen Tank (LS 4800)	Taylor-Wharton
Microscope, fluorescent (AxioImager.Z1)	Zeiss
Microscope, automated (Pathway 855)	Becton, Dickinson and Company
PCR machine Thermocycler (T personal)	Biometra
pH-Meter (WTW-720)	WTW, Weilheim, DE
Pipet, electric (Portable-XP)	Drummond
Pipets 2.5, 20, 200, 1000 µl	Eppendorf
Power supply unit (Powerpack P25T)	Biometra

Power supply unit (Power-Pac Basic)	Biorad
Real-time PCR machine (Chromo4™)	Bio-Rad Laboratories
Real-time PCR machine (CFX96; C1000)	Bio-Rad Laboratories
Refrigerator 4°C	Liebherr
Rotator (PTR 300)	Grant Bio
Scales (Acculab ALC-6100.1, LE623S)	Sartorius
Shaker (DRS-12)	neo Lab
Shaker (Promax 2020)	Heidolph
Shaker (Rocky)	Schütt Labortechnik
Spectrophotometer NanoDrop (ND-1000)	PeqLab
UV-transilluminator (Intas UV system)	Intas
Vacuum pump	IBS Integra Biosciences
Vortex (Genie 2)	Scientific Industries
Water bath (TW 20)	Julabo Labortechnik

3.1.2. CONSUMABLE MATERIALS

CONSUMABLE	**COMPANY**
6- and 12- well cell culture plates (Cellstar)	Greiner-bio-one
96 well imaging plates (black)	BD-Falcon
96 well PCR plate (duo plate, skirted)	Sarstedt
Adhesive aluminum foil	Sarstedt
Cell scraper (16mm, 25mm)	Sarstedt
Centrifuge tubes (15ml, 50ml)	Sarstedt
Cryo Tube Vials (1.8ml)	Nunc
Gloves (Latex Safe Skin PFE)	Kimberly Clark
Micro tubes (0.5ml, 1.5ml, 2ml)	Sarstedt
Nitrocellulose, poresize: 0.2µM (Protran BA83)	Omnilab
Pasteur pipets, glass (230mm)	VWR international
Parafilm	Pechiney
Pipet tips with or without filter (20 µl, 200 µl, 1000 µl)	Sarstedt
Sealing tape (optically clear for 96 well PCR Duo plates)	Sarstedt
Syringe (1 ml)	BD Plastipak
Syringe needles (0,6 x 25mm)	BD Microlance
Tissue culture dish (CELLSTAR 60x15 mm)	Greiner-bio-one

Tissue culture dish (CELLSTAR 100x20 mm)	Greiner-bio-one
Tissue culture dish (CELLSTAR 145x20 mm)	Greiner-bio-one
Whatman paper (GB002)	Schleicher & Schuell

3.1.3. CHEMICALS

NAME	COMPANY
10 x Taq buffer with KCl (B38)	Fermentas
2-mercaptoethanol	Roth
2-propanol	Roth
25 mM $MgCl_2$ (R0971)	Fermentas
β-glycerol phosphate disodium salt pentahydrate (β-glycerophosphate)	Fluka
Ammonium persulfate (APS)	Roth
Bromphenol blue	Sigma-Aldrich
BSA	Roth
Calcium chloride ($CaCl_2$)	Roth
Camptothecin	Sigma-Aldrich
Chelex 100	Bio-Rad
Chloroform	Roth
Ciprobay 200	Bayer
Cycloheximide	Sigma-Aldrich
DAPI dilactate	Sigma-Aldrich
Di-sodiumhydrophosphate dihydrate (Na_2HPO_4 x H_2O)	Roth
Dithiothreitol (DTT)	Roth
dNTPs, 25 µM each (U1420)	Promega
Dulbecco's Modified Eagle's Medium (31600-091)	GIBCO/ Invitrogen
EDTA	Roth
Ethanol, >99.9%	Merck
Ethanol denatured, 99.8%	Roth
Ethidium bromide	Roth
Fetal Calf Serum (FCS)	GIBCO / Invitrogen
Formaldehyde, 37%	Roth
Glycerol	Roth
Glycine	Roth

GlycoBlue (AM9516)	Ambion
HEPES	Roth
Hydrochloric acid (HCl)	Roth
Immobilon western chemiluminescent HRP substrate	Millipore
Iodacetamide	AppliChem
L-glutamine	GIBCO / Invitrogen
Lipofectamine 2000	Invitrogen
Magnesium chloride ($MgCl_2$)	Roth
McCoy's Medium 5A	GIBCO / Invitrogen
Methanol	Roth
Milk powder, non fat	Roth
NEBuffer for M-MuLV reverse transcriptase (B0253)	NEB
N-ethylmaleimide	Sigma
N,N,N´,N´-Tetramethylendiamin (TEMED)	Roth
NP-40	USB
PBS tablets (18912-014)	GIBCO/ Invitrogen
Penicillin/Streptomycin	GIBCO / Invitrogen
pH-Solution 10,01	Roth
pH-Solution 4,01	Roth
pH-Solution 7,01	Roth
Ponceau S	Roth
Proteinase K (EO 0491)	Fermentas
Protein A sepharose CL-4B (17-0780-01)	GE Healthcare
Rotiphorese Gel 30 (30% acrylamide bisacrylamide solution; ratio 37.5:1)	Roth
Potassium chloride (KCl)	Roth
Potassium dihydrogen phosphate (KH_2PO_4)	Roth
RNase Inhibitor (M0307)	NEB
RPMI Medium 1640	GIBCO / Invitrogen
Sepharose CL-4B (17-0150-01)	Amersham
Sodium acetate	Roth
Sodium deoxycholate	AppliChem
Sodium dodecyl sulfate (SDS)	Roth
Sodium chloride (NaCl)	AppliChem
Sodium hydrogen carbonate ($NaHCO_3$)	Roth

Sodium hydroxide (NaOH)	Roth
Sonicated salmon sperm DNA (201190-81)	Stratagene
SuperSignal west femto maximum Sensitivity (34095)	Pierce
Tetracycline	Sigma
Trasylol (aprotinin 500.000 KIE)	Bayer
Trichostatin A	Sigma-Aldrich
Tris	Roth
Triton X-100	AppliChem
Trizol (15596-018)	Invitrogen
Trypanblue	Invitrogen
Trypsin-EDTA	GIBCO / Invitrogen

3.1.4. ENZYMES

NAME	COMPANY
M-MuLV Reverse Transcriptase (M0253)	NEB
Taq DNA polymerase	PrimeTec

3.1.5. CHEMOTHERAPEUTICS

NAME	COMPANY
5-Fluorouracil	SIGMA
Camptothecin	SIGMA
Daunorubicin	SIGMA
Doxorubicin	Santa Cruz
Epirubicin	SIGMA
Etoposide	SIGMA
Idarubicin	SIGMA

3.1.6. BUFFERS

BLOCKING SOLUTION	
PBS	
FCS	10%

CHIP BUFFER	
Tris-HCl pH 8.0	50 mM

NaCl	150 mM
EDTA pH 8.0	5 mM
NP40	0.5% (v/v)
Triton X-100	1% (v/v)

CHIP++ BUFFER

ChIP buffer	
Aprotenin/ Leupeptin	1 µg/ ml each
Pepstatin A	1 µg/ ml
Pefablock	1 mM

LAEMMLI BUFFER 6X

Tris-HCl pH 6.8	0.35 mM
Glycerol	30% (v/v)
SDS	10% (w/v)
DTT	9.3% (w/v)
Bromphenol blue	0.012% (w/v)

PBST

PBS	
Tween-20	0.1%(v/v)

RIPA-BUFFER

Tris-HCl pH 7.5	20 mM
NaCl	150 mM
Sodium deoxycholate	1% (w/v)
SDS	0.1% (w/v)
Triton-X 100	1% (v/v)
EDTA	10 mM
Trasylol	5 % (v/v) (equals 100,000 KIE)
pH was adjusted to 7.5 with 1M NaOH	

SDS RUNNING BUFFER (FOR SDS-PAGE)

Tris	25 mM
Glycine	192 mM
SDS	0.1% (w/v)

WESTERN SALTS

Tris	25 mM
Glycine	192 mM
SDS	0.02% (w/v)
Methanol	15%
pH was adjusted to 8.3 with HCl	

3.1.7. OLIGONUCLEOTIDES

siRNAs

NAME	ID	SEQUENCE (5' → 3')
N.C. 1		UNKNOWN
N.C. 2		UNKNOWN
BCLAF1	s18874	CAUUGAUCGCCGUAGAAAAtt UUUUCUACGGCGAUCAAUGtc
BCLAF1_2	s18875	CGCGAUUACAGAAAUAAUAtt UAUUAUUUCUGUAAUCGCGac
CBP	s3495	GAAUCUUUCCCAUAUCGAAtt UUCGAUAUGGGAAAGAUUCag
CBP_2	s3496	GGAUAUUGCUGUGGACGCAtt UGCGUCCACAGCAAUAUCCaa
HOXA5	s6765	GACUACCAGUUGCAUAAUUtt AAUUAUGCAACUGGUAGUCcg
HOXA5_2	s6766	CCAGUUGCAUAAUUAUGGAtt UCCAUAAUUAUGCAACUGGta
p300	s4696	CCACUACUGGAAUUCGGAAtt UUCCGAAUUCCAGUAGUGGat
p300_2	s4697	GCCUGGUUAUAUAACCGGAtt UCCGGUUAUAUAACCAGGCat
p53	s605	GUAAUCUACUGGGACGGAAtt UUCCGUCCCAGUAGAUUACca
PCAF	s16895	GGUACUACGUGUCUAAGAAtt UUCUUAGACACGUAGUACCta
PCAF_2	s16896	GGAGUUCGACAGAUUCCUAtt UAGGAAUCUGUCGAACUCCat
RPL26	s12203	GAAAUAUGUUAUCUACAUUtt AAUGUAGAUAACAUAUUUCtt
RPS6KA1	s12273	CACUGAUUCUGAAGGCGAAtt UUCGCCUUCAGAAUCAGUGtc
RPS6KA1_2	s12275	CCAUUGACUGGAAUAAGCUAtt UAGCUUAUUCCAGUCAAUGgt
RPS6KB1	s12283	GGUUUUUCAAGUACGAAAAtt UUUUCGUACUUGAAAAACCtt
RPS6KB1_2	s12284	GGACUAUGCAAAGAAUCUAtt UAGAUUCUUUGCAUAGUCCaa
RPS6KB2	s12286	CCCUUUUUCCGGCACAUGAtt UCAUGUGCCGGAAAAAGGGat

RPS6KB2_2	s12285	ACAUCAAACUGACCGACUUtt AAGUCGGUCAGUUUGAUGUgg
RPS6KL1	s38111	GGUACUUUGUGAGCGAGGAtt UCCUCGCUCACAAAGUACCtg
RPS6KL1_2	s38110	CGAUGUUAGUGAGGACUAUtt AUAGUCCUCACUAACAUCGcg
RREB1	s12354	CCAUCUCCUCUGAAACGUAtt UACGUUUCAGAGGAGAUGGag
RREB1_2	s12356	GGAGUUUGUUUGCAAGUAUtt AUACUUGCAAACAAACUCCtt
TIP60	s20630	GCAAGCUGCUGAUCGAGUUtt AACUCGAUCAGCAGCUUGCcg
TIP60_2	s20631	GGACGGAAGCGAAAAUCGAtt UCGAUUUUCGCUUCCGUCCtg
TP73	s14319	GCAAUAAUCUCUCGCAGUAtt UACUGCGAGAGAUUAUUGCct
TP73_2	s14320	CCACCAUCCUGUACAACUUtt AAGUUGUACAGGAUGGUGGtg
WRAP53_1	s30251	CCUCUGCUUUCAUCCCGAUtt AUCGGGAUGAAAGCAGAGGtg
WRAP53_2	s30252	GAAGCAAACGGGAGCCUUUtt AAAGGCUCCCGUUUGCUUCtt

PCR

NAME	**SEQUENCE (5' → 3')**
36B4_for	GATTGGCTACCCAACTGTTG
36B4_rev	CAGGGGCAGCAGCCACAAA
p53_for	ATGGAGAGAGCCGCAGTCAGATC
p53_rev	GGGAGCAGCCTCTGGCATTCTG
p53-Int1_for	GCCGAGACGGGCCATTCGTG
p53-Int1_rev	TCTCACCGCTCACCTGCCCA
WRAP53-Exon1a_for	CGGAGCCCAGCAGCTACC
WRAP53-Exon1a_rev	TTGTGCCAGGAGCCTCGCA
WRAP53-Exon2_rev	GTCCTGGTCTGAAGGACAGC
WRAP53-Exon7_for	GACTGCGAGGTCCGAGCCACATTTG
WRAP53-Exon8_rev	GAGCCATCATCCCAGGCATACAGAC
E2F1_for	CGGTGTCGTCGACCTGAACT
E2F1_rev	AGGACGTTGGTGATGTCATAGATG
TAp73_Exon1_for	GGGCTGCGACGGCTGCAG
TAp73_Exon3_rev	GATGTAGTCATGCCCTCCAGG

NOXA_for	GGACTGTTCGTGTTCAGCTCGC
NOXA_rev	GCCGGAAGTTCAGTTTGTCTCC

ChIP

NAME	SEQUENCE (5' → 3')
ChIP_p53-E2F1 BS-I_for	TGCACCCTCCTCCCCAACTCC
ChIP_ p53-E2F1 BS-I_rev	GCTCCCTGGACGGTGGCTCT
ChIP_p53-E2F1 BS-II_for	CCCGGGAGGAGAGGCGAACA
ChIP_ p53-E2F1 BS-II_rev	TGGGTCGCCCGCGAAATCTG
ChIP_p53+19.5kb_for	GCCACGGCTGGCACAAGGTT
ChIP_p53+19.5kb_rev	GCTGCCCCCACTTTCCTGGG
ChIP_p107-E2F1 BS_for	AGGCAGACGGTGGATGACAACAC
ChIP_p107-E2F1 BS_rev	TCAGCGTGGGGCTTGTCCTCGAA
ChIP_TP73-E2F1 BS_for	GAGCGCCGGGAGGAGACCTT
ChIP_TP73-E2F1 BS_rev	GCGGGCGTTAGCGCCTTTTT

3.1.8. ANTIBODIES

PRIMARY ANTIBODIES

NAME	DILUTION	APPLICATION	SOURCE	COMPANY
E2F1 KH95 and KH129	1:500 each	WB	mouse monoclonal	Santa Cruz Biotechnology
E2F1 KH20 and KH95	1µg	ChIP	mouse monoclonal	Upstate
p53 D0-1 HPR-conjugated	1:8000	WB	mouse monoclonal	Santa Cruz Biotechnology
p53 FL-393	1:500	IF	rabbit polyclonal	Santa Cruz Biotechnology
p53 (pSer15)	1:1000	WB	mouse monoclonal	Cell Signaling
p53 (acLys382)	1:1000	WB	rabbit polyclonal	Cell Signaling
RNA pol II	1µg	ChIP	rabbit polyclonal	Santa Cruz
β-actin	1:10000	WB	mouse monoclonal	Abcam

SECONDARY ANTIBODIES

NAME	DILUTION	APPLICATION	COMPANY
Donkey α-mouse IgG	1:10000	WB	Jackson ImmunoResearch

(H+L) HPR-conjugated			
Donkey α-rabbit IgG (H+L) HPR-conjugated	1:10000	WB	Jackson ImmunoResearch
Alexa Fluor 488 anti-rabbit	1:500	IF	Molecular Probes, Invitrogen
Alexa Fluor 594 anti-rabbit	1:500	IF	Molecular Probes, Invitrogen

3.1.9. EUKARYOTIC CELL LINES

NAME	SOURCE	P53 STATUS
5637	human bladder carcinoma	R280T
A431	human squamous cell carcinoma	R273H
HCT116	human colon carcinoma; p21 wt or $p21^{-/-}$	wt
U251	human glioma cells	R273H
U2OS	human osteosarcoma	wt

3.1.10. CELL CULTURE WORKING SOLUTIONS

DULBECCO'S MODIFIED EAGLE'S MEDIUM (DMEM -)

Dulbecco's Modified Eagle's Medium	10g
NaHCO3	3.7 g/L
HEPES	5.96 g/L
H_2O	ad 1L

The medium was filtered and stored at +4°C

DULBECCO'S MODIFIED EAGLE'S MEDIUM WITH SUPPLEMENTS (DMEM + FCS)

DMEM -	450 ml
FCS	10%
Penicillin/Streptomycin	50 U/mL
Tetracycline	2 µg/mL
L-glutamine	200 µM
Ciprobay 200	10 µg/mL

DMEM+FCS was and warmed up to +37°C directly before use

RPMI MEDIUM 1640 WITH SUPPLEMENTS (RPMI + FCS)

RPMI -	450 ml
FCS	10%
Penicillin/Streptomycin	50 U/mL
Tetracycline	2 µg/mL

L-glutamine	200 µM
Ciprobay 200	10 µg/mL

DMEM+FCS was and warmed up to +37°C directly before use

MCCOY'S MEDIUM 5A WITH SUPPLEMENTS (MCCOY'S + FCS)

McCoy's 5A -	450 ml
FCS	10%
Penicillin/Streptomycin	50 U/mL
Tetracycline	2 µg/mL
L-glutamine	200 µM

DMEM+FCS was and warmed up to +37°C directly before use

PBS BUFFER

PBS tablets	
H2O	500 ml

PBS for cell culture was autoclaved and stored at +4°C

CELL FREEZING SOLUTION

DMSO	10 %
FCS	90 %

3.2. METHODS

3.2.1. CELL BIOLOGY

MAINTENANCE OF CELL CULTURES

All cell lines were cultivated at 37°C in a humidified incubator with 5% CO_2 either in RPMI medium 1640 (A431, U251, 5637) or in Dulbecco's Modified Eagle's medium (DMEM) (U2OS), or McCoy's medium (HCT116 wt, HCT116 $p21^{-/-}$), all supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 µg/ml streptomycin, 200 µM Glutamine, 2 µg/ml tetracycline, and 10 µg/ml Ciprobay 200 (not for the HCT116 cells). Sub cultivation was performed every 3-4 days, as soon as the cells reached 70-80% of confluence. For passaging, the medium was removed; the cells were rinsed once with PBS and incubated at 37°C for a few minutes with 0.05% Trypsin-EDTA solution, to induce detachment from the culture dish. Upon neutralization of the Trypsin with fresh medium the cells were carefully resuspended and diluted 1:8 - 1:10 in fresh

medium. For experiments, the cell number was determined using trypanblue staining of living cells that was subsequently quantified using the Countess system. The required amount of cells was seeded into the corresponding culture dishes/ well plates. For long term storage, the cells were frozen in liquid nitrogen.

CELL FREEZING PROCEDURE

The cells from a 10 cm culture dish at 70-80% confluency were frozen in 1 cryovial. After trypsinization and dilution with fresh medium+FCS as described above, the cell suspension was centrifuged 10 min at 800 rpm. The supernatant was aspirated; the cell pellet was resuspended in 1ml cold cell freezing solution (10% DMSO in FCS) and transferred into pre-cooled cryovials. The vials were stored in -80°C for 2 days and then kept in a liquid nitrogen tank for long term storage.

To take frozen cells in culture, the vials were quickly thawed by hand and immediately transferred into a 15cm culture dish with prewarmed medium. After one day of incubation at 37°C, the medium was changed to remove the residual DMSO.

REVERSE SIRNA TRANSFECTION

Pre-designed or validated siRNAs from Applied Biosystems were used for all siRNA transfection experiments. Both the siRNAs and the transfection reagent Lipofectamine 2000 were diluted in cell culture medium without supplements and incubated for 5 minutes. The solutions were combined in an empty well-plate and incubated for additional 20 minutes to allow the siRNA-lipid-micelles to form. The cells were counted and the appropriate number of cells was added to the transfection mix and diluted with medium+FCS to the final volume of the corresponding well plate.

Different amounts of oligonucleotides, transfection reagent, cells and medium were used depending on the well sizes:

	siRNA + MEDIUM	LIPOF. 2000 + MEDIUM	CELL NUMBER	TOTAL VOLUME
6-well	30 pmol	2.7 µl	$2.5 - 3 \cdot 10^5$	2 ml
12-well	15 pmol	1.35 µl	$1.5 - 1.8 \cdot 10^5$	1 ml
96-well	1 pmol	0.25 µl	8000	100 µl

After 48 hours the cells were fixed for immunofluorescence staining or harvested for immunoblot analysis or RNA isolation.

LONG-TERM SIRNA TRANSFECTION:

The cells were reverse transfected as described above. 48 hours later the samples were trypsinized in the well plate and 25 - 33% of the cells were used for a second reverse transfection following the same protocol as above. After an additional incubation for 72 hours the cells were harvested for RNA isolation or immunoblot analysis.

CHEMOTHERAPEUTIC TREATMENT

Most treatments were performed for 24 hours; therefore the cells were either seeded about 12 hours before treatment or siRNA transfected 24 hours before treatment. The medium was removed from the cells and fresh medium containing the chemotherapeutic drug at the desired final concentration was added to the cells. The mock sample was treated with the same volume of dissolvent only.

	STOCK CONC.	FINAL CONC.	DISSOLVENT
5-Fluorouracil	0.3 M	500 µM	DMSO
Camptothecin	2.87 mM	2.87 µM	DMSO
Daunorubicin	4 mM	500 nM	H_2O
Doxorubicin	4 mM	500 nM	H_2O
Epirubicin	4 mM	500 nM	H_2O
Etoposide	20 mM	100 µM	DMSO
Idarubicin	4 mM	500 nM	H_2O

3.2.2. MOLECULAR BIOLOGY

TOTAL RNA ISOLATION

For the preparation of total RNA 0.5 - $1 \cdot 10^6$ cells per sample are needed (corresponds to one 6-well). The medium was aspirated and 800 µl Trizol (Invitrogen) was added to the cell layer for lysis. After 5 minutes at room temperature the lysates were transferred into microtubes and supplemented with 180 µl chloroform. The mixture was vigorously shaken and further incubated for 3 min at room temperature, followed by a centrifugation step (4°C, 16000 g, 20 min). The upper aqueous phase, containing RNA, was carefully transferred into a new microtube and supplemented with the same amount of isopropyl alcohol. The samples were mixed vigorously by hand and incubated for 3 min at room temperature and for 2 - 24 hours at -20°C. RNA was precipitated by centrifugation (4°C, 16000 g, 20 min), the pellet was washed with 70% ethanol, centrifuged (4°C, 9000 g, 10 min), air dried for 10 minutes at 37°C and resuspended in 30 µl nuclease free water.

QUANTIFICATION OF RNA

The RNA concentration was measured, using a NanoDrop spectrophotometer (PeqLab). The absorbance at 260 nm was used to determine the concentration, whereas the ratios 260:230 and 260:280 were used as indicators for the purity of the isolated RNA. The ratios 260:230 around 1.9-2.0 and 260:280 in the range of 2.0-2.1 were considered as ‘pure’ RNA. In case these values were appreciably lower, RNA was additionally purified, as described in the following section.

PURIFICATION OF RNA

The RNA sample (30µl) was mixed with 20µl H_2O, 1µl 125 mM EDTA, 1µl 3M sodium acetate and 70µl 100% ethanol. The samples were vortexed and incubated for 5 min at room temperature. After shock-freezing in liquid N2 the samples were centrifuged at 4°C for 20 min at 16000 g. The pellet was washed with 70 µl 70% ethanol and centrifuged (4°C, 10 min, 9000 g). The Supernatant

was aspirated and the pellet was air dried for 10 minutes at 37°C. The precipitated RNA was resuspended in 30µl nuclease free water and the concentration and purity of the RNA was determined, again using the NanoDrop spectrophotometer as above.

REVERSE TRANSCRIPTION

Reverse transcription was performed using moloney murine leukemia virus (M-MuLV)-derived reverse transcriptase (NEB). The following stock solutions were prepared and aliquots were kept at -20°C:

- Combined primer stock: 15µM random nonamers (N9) and 50µM oligo dT23VN
- Deoxyribonucleoside triphosphates (dNTPs): 2.5mM of each (dCTP, dATP, dTTP, dGTP) (Promega)

1µg of total RNA was used for the reverse transcription and diluted with nuclease free water to a final volume of 10µl. 2µl of the combined primer stock and 4µl dNTP mix were added to the diluted RNA. The samples were incubated for 5 minutes at 70°C. Meanwhile a master mix of 2µl NEBuffer for M-MuLV reverse transcriptase (NEB), 0.25µl RNase inhibitor (10U, NEB), 0.125µl M-MuLV reverse transcriptase (25U, NEB), and 1.625µl nuclease free water per sample was prepared. The transcriptase master mix was added to the RNA samples and incubated for 1 hour at 42°C. The enzyme was inactivated at 95°C for 5 min and the cDNA was diluted with 30µl nuclease free water. It was either directly used for real-time PCR or stored at -20°C. To control for genomic DNA contamination each reaction was also performed as noRT control, omitting the reverse transcriptase in the master mix.

REAL-TIME PCR

Real-time PCR (qPCR) was used to obtain semi-quantitative measurements of gene expression or to quantify the recovered sheared DNA from chromatin

immunoprecipitation experiments. The following master mix was prepared on ice and aliquoted in microtubes:

	FINAL CONC.	FILTERED
Tris-HCl pH 8.8	75 mM	✓
$(NH_4)_2SO_4$	20mM	✓
Tween-20	0.01 %	✓
$MgCl_2$	3 mM	✓
Triton X-100	0,25%	✓
Trehalose	300 mM	✓
Sybr Green	1:80000	
dNTPs	0.2 mM	
Taq-Polymerase	20U/ ml	

The tubes were shock-frozen in liquid N2 and kept at -20°C for up to 3 month.

For the PCR reaction 14µl master mix were mixed with 0.075µl of each primer (stock concentration: 100µM) and 9.85µl H_2O. This was prepared as a master mix and aliquoted into a 96-well PCR plate. 1µl cDNA/ sheared DNA was added into the corresponding wells. The plate was sealed, centrifuged (30sec, 600rpm) and the following PCR program was used for the specific gene amplifications:

STEP	TEMPERATURE	TIME	FLUORESCENT READ STEP	
1 - DNA melting	95°C	3 min		
2 - DNA melting	95°C	15 sec		39x
3 - primer annealing and elongation	60°C	1 min	X	
4 - melting curve	60°C - 95°C		every 0.5°C	

For the semi-quantitative analysis of target mRNA expression the C_t-values of the genes of interest as well as the reference gene 36B4 were obtained. The ΔΔCt-method was used to determine the relative expression of the analyzed target genes:

$$\Delta\Delta C_t = \frac{C_t \text{ (36B4, untreated)} - C_t \text{ (target gene, untreated)}}{C_t \text{ (36B4, treated)} - C_t \text{ (target gene, treated)}}$$

(Livak and Schmittgen, 2001)

For ChIP analysis a serial dilution of the input DNA was used to determine the relative amounts of target DNA in the input samples, as well as the IP samples. The recovered DNA is diagramed relative to the input DNA.

$$\% \text{ of input DNA} = \frac{\text{rel. amount of target DNA (IP sample)}}{\text{rel. amount of target DNA (input sample)}}$$

3.2.3. BIOCHEMISTRY AND IMMUNOLOGICAL METHODS

IMMUNOBLOT ANALYSIS

CELL HARVESTING AND LYSIS

Adherent cells were grown, treated, or transfected in a 12-well plate for immunoblot analysis. For harvesting they were scraped in the growth medium, transferred to a microtube and centrifuged (5 min, 1000 rpm). The cell pellet was resuspended and the cells were lysed in 60µl of RIPA/ 6x Laemmli buffer (1:1 mixture). The samples were incubated for 5 min at 95°C for protein denaturation. The samples were centrifuged (1 min, 13000 rpm) and stored at -20°C or directly used for SDS-PAGE.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was developed 1967 by (Shapiro *et al.*, 1967) to determine the molecular weight of proteins. The detergent SDS coats the denatured proteins and translates their molecular weight into a negative charge, therefore a sample buffer developed by (Laemmli, 1970) is widely used. An electric field is applied to the gel and the negatively charged proteins migrate towards the anode. Within the stacking gel the pores are large and therefore the proteins form a concentrated stack between the leading chloride ions and the trailing ion Glycine. As soon as the sample migrates into the resolving gel, which obtains a pH that is 2 units higher than that of the stacking gel and pores that are

restrictively small for the proteins, the sample starts to resolve according to the molecular weight of the proteins. Depending on the molecular weight of the protein of interest different percentages of acrylamide/ bisacrylamide (AA/ BAA) are used within the resolving gel. All SDS-PAGE experiments within this study were performed using 10% AA/ BAA.

CHEMICAL	STACKING GEL (5%)	RESOLVING GEL (10%)
Acrylamide/ bisacrylamide	850 µl	4.15 ml
Tris-HCl pH 6.8	625 µl	-
1.5 M Tris-HCL pH 8.8	-	3.15 ml
H_2O	3.4 ml	5 ml
10% SDS	50 µl	125 µl
10% APS	50 µl	75 µl
TEMED	10 µl	7.5 µl

The resolving gel was casted between two glass plates, separated by spacers (1mm thick) and covered by a layer of 2-propanol to prevent air contact for polymerization. The solidified gel was rinsed with water to remove any residual 2-propanol and the stacking gel was casted on top of the resolving gel. A comb, either with 10 or 15 teeth was inserted into the stacking gel before polymerization in order to form separated slots for sample loading.

After gel polymerization, 10 to 20 µl of cell lysate were loaded into the pockets of the stacking gel. Electrophoresis was performed at 15mA per gel until the samples migrated into the resolving gel, then it was increased to 20 mA per gel.

WESTERN BLOT

For immunodetection of the proteins they were transferred onto a nitrocellulose membrane (pore size: 0.2µM) after the separation through SDS-PAGE using the tankblot technique (Bittner *et al.*, 1980). A stack of sponges, filter papers, the gel, the membrane, filter papers, and sponges was prepared, all soaked in transfer buffer. This was then placed within the vertical blotting chamber, filled up with transfer buffer and again an electric field was applied. After blotting for 1 hour at 100V all proteins were bound to the nitrocellulose membrane. The

quality of the transfer was controlled through the reversible protein staining with Ponceau S solution.

IMMUNOSTAINING

For specific protein visualization after western blotting, membranes were subjected to immunostaining. First, membranes were blocked with a 5% non-fat milk solution in PBST (milk) for 1 hour followed by the incubation with primary antibody, diluted in milk for 2 hours at room temperature or overnight at 4°C. Subsequently, membranes were washed twice according to the following protocol: 3 times in PBST followed by 15 min in milk. To visualize the specifically bound primary antibodies the membranes were incubated with HPR-conjugated secondary antibodies for one hour. Washing was repeated the same way as described above. All washing and incubation steps were fulfilled with gentle shaking at room temperature, if not specified otherwise. For protein detection enhanced chemiluminescence solutions (ECL) were used and the signal was measured using the ChemoCam Imager (Intas). For quantification the LabImage 1D software (Intas) was used.

CHROMATIN IMMUNOPRECIPITATION

$1 \cdot 10^6$ U251 cells were seeded per 10 cm culture dish and treated 18 hours later with 500nM doxorubicin. 24 hours after treatment protein-DNA crosslinking was performed using 1.42% (v/v) formaldehyde in PBS for 15 min and stopped by the addition of Glycine to a final concentration of 138 mM for 5 min. After washing with PBS twice, the cells were scraped in 1ml ChIP++ buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 0,5% NP-40, 1% Triton X-100, Leupeptin (1µg/ml), Aprotenin (1µg/ml), Pepstatin A (1µg/ml), Pefabloc (1mM)), transferred into a microtube and centrifuged at 1000 rpm for 5 min. The Pellets were washed once with 1 ml ChIP++ buffer and resuspended in 300 µl of the same buffer. The lysates were sonicated in an icewater bath sonicator (Bioruptor) to shear the chromatin to a length of 500 – 1000 base pairs (3 times 10 minutes using 10 sec on/ off cycles at maximum power). The lysates were

diluted in ChIP++ buffer before pre-clearing for 1 hour at 4°C with 100µl sepharose, washed 3 times in ChIP buffer and finally resupended in ChIP++ buffer to achieve a 50% slurry. The samples were centrifuged at 12000 rpm, 10 min, at 4°C, and the supernatants were transferred to new microtubes. The pre-cleared chromatin was diluted with ChIP++ buffer according to the number of immunoprecipitations that were performed. 1 µg of antibody per 50 µl of lysate was used for the immunoprecipitation (IP), additionally 50µl of the precleared DNA were used as input control. The IP samples were further diluted with ChIP++ buffer up to 500 µl and incubated overnight at 4°C with rotation. The input samples (50 µl) were mixed with 1µl glycogen (Glycoblue) and 100µl 100% ethanol and placed at - 20°C overnight for DNA precipitation. Protein A sepharose (GE Healthcare) beads were incubated over night in a 15ml tube filled up with ChIP buffer to allow the beads to swell. At the same time 0.5g BSA and 100µl sheared salmon sperm DNA were added to block the beads and avoid unspecific precipitation. Blocked protein A sepharose was washed three times with ChIP buffer (centrifuged at 2000 rpm, 2 min, 4°C) and finally resuspended in ChIP++ buffer to get a 50% sepharose slurry. 30µl of this slurry were added to each immunoprecipitation reaction and samples were incubated for 2 hours at 4°C with rotation. Meanwhile the input samples were centrifuged (13000 rpm, 20 min, 4°C), and the DNA pellets were washed once with 500 µl of 70% ethanol before they were air dried for 10 min at 37°C. The immune-sepharose complexes were washed 8 times with 1 ml cold ChIP buffer, centrifuged for 2 min at 2000 rpm and 4°C. 100 µl 10% (w/v) Chelex 100 slurry was added to the washed beads and to the input DNA pellet. After brief vortexing the samples were heated to 95°C for 10 min. 30µg Proteinase K was added to each sample and incubated at 55°C for 30 min with shaking at 1000 rpm. For the inactivation of Proteinase K the samples were heated to 95°C for 10 min. All beads were precipitated by centrifugation (12000 rpm, 1min, 4°C) and the supernatants were carefully transferred into new tubes. For

quantification of the precipitated/ recovered DNA 1µl of the supernatant was used for qPCR analysis.

IMMUNOFLUORESCENCE

Cells were grown in 96-well imaging plates (BD Falcon). Prior to immunofluorescence staining the cells were fixed using 3.7% formaldehyde in PBS for 20 minutes. After fixation the cells were washed with PBS containing 50mM Glycine in order to inactivate residual free formaldehyde that could otherwise unspecifically cross-link the primary antibodies to proteins of the cells. Permeabilization was achieved through 10 minutes incubation with PBS containing 0.5% Triton X-100. All buffers that were used from this step on contained 0.2% Triton X-100 in order to keep the cells in a permeabilized state. Incubation for 10 minutes in blocking solution (10% FCS in PBS + 0.2% TX100) was performed to block all unspecific binding sites in the cells before they were incubated for 1 hour with the primary antibodies (for dilutions see 3.1.8). The remaining primary antibodies were washed away with blocking solution 3 times for 5 minutes. The secondary antibodies coupled to the fluorophores Alexa488 or Alexa546 were incubated in a 1:500 dilution in blocking solution for 45 minutes in the dark. A nuclear stain (Hoechst 33342 or Doxorubicin) was additionally used during this incubation. We observed that the previous treatment of the cells with red fluorescent chemotherapeutic drugs (Daunorubicin, Doxorubicin, Epirubicin, or Idarubicin) has an impact on the fluorescent signal of Hoechst 33342; therefore we used in these cases a high dose of doxorubicin (10µM) for nuclear stain. The free secondary antibodies were washed away with blocking solution for 5 minutes, PBS +0.2% TX100 for 5 minutes and with PBS for additional 5 minutes; all incubations were performed in the dark. Finally the cells were kept in 100µl PBS and the plate was sealed with aluminum foil.

The fluorescent pictures were taken, using the BD Pathways system. In each well at least 9 pictures were taken using a 10x or 20x magnification. On the

basis of the nuclear stain the single nuclei within each well were defined and the average fluorescent intensity of the immunodetected proteins in each of these nuclei was measured. The results are either presented as average intensity per well, or the single nuclei intensities in each well are plotted in histograms.

3.2.4. THE SCREEN

THE LIBRARY

For the kinase screen a siRNA library (Silencer Human Kinase siRNA Library V3) was obtained from Applied Biosystems containing 3 different siRNAs against each of the 719 kinases included in the library. The siRNAs were obtained lyophilized in 96 well plates, containing 8 empty wells that were used for internal controls. The 3 siRNAs targeting the same gene were always localized on different plates. Before transfection all siRNAs were dissolved in nuclease free water at a final concentration of 50µM and dilution plates containing 5µM of the siRNAs were prepared. All pipetting steps were performed by the Biomek 2000 (Beckmann Coulter).

TRANSFECTION

For the siRNA transfections in a 96 well format the Biomek 2000 was used. The robot was programmed according to the siRNA transfection protocol as it was described in section 3.2.1 (REVERSE SIRNA TRANSFECTION), but using 4.5 times more siRNA. The reason for this is that the library consists of Silencer siRNAs that are less efficient compared to the Silencer Select siRNAs that were used throughout the other experiments.

IMMUNOFLUORESCENCE STAINING AND ANALYSIS

48 hours after reverse siRNA transfection of the cells they were fixed and stained for immunofluorescence analysis as described in 3.2.3 (IMMUNOFLUORESCENCE). The polyclonal p53 antibody (FL-393) was used for the immunostaining at a dilution of 1:500 in combination with an anti-rabbit

secondary antibody coupled to the fluorophore Alexa488 at a dilution of 1:500. Hoechst 33342 was used as nuclear stain to identify the regions of interest during the analysis. In each well 12 pictures were taken at a 10x magnification covering different positions. For the analysis around 10000 nuclei per well were used.

DATA MINING

To identify the HITs statistical methods were applied to the average expression per well data. Therefore the fluorescent signals were normalized to the average signal of the plate, this compensates for differences within the staining procedure or the microscopy. To finally evaluate the impact of the single knock-downs on the expression of p53 Z-scores were determined:

$$z\text{-}score = \frac{x - \mu}{\sigma}$$

x = average intensity in the well; μ = average intensity of all wells;
σ = standard deviation of the intensities of all wells

The relative p53 expression intensities of the individual siRNAs as well as the sum of the three siRNAs targeting the same kinase were used for the z-score analysis. On the basis of these results the kinases that revealed the strongest down-regulation of mutant p53 expression were further analyzed on the basis of the following three criteria:

1) How many of the siRNAs revealed this down-regulation?
2) Do we see a peak-shift in the histograms of the p53 staining intensities, when comparing the three siRNAs to the negative control siRNAs?
3) Visual inspection of the microscopic raw data. Do we observe morphological changes or increased cytoplasmic staining upon knock-down?

RESULTS

Most chemotherapeutic agents induce a DNA damage response in the cells subsequently leading to apoptosis. This comprises the activation and stabilization of the tumor suppressor p53, mainly through posttranslational modifications (Murray-Zmijewski *et al.*, 2008). It is estimated that 50% of all human tumors carry a p53 mutation, accompanied by a strong accumulation of the mutant p53 protein. Since most of these mutations are substitutions of single amino acids, we expect that at least some of the enzymes that were found to be responsible for the modification of wild type p53 also affect the mutant variants of the protein in response to DNA damage. Therefore, we first investigated whether the evoked DNA damage response upon chemotherapeutic treatment influences the modification of mutant p53 and whether this goes along with a further stabilization of the protein.

4.1. THE ACCUMULATION OF MUTANT P53 UPON DOXORUBICIN TREATMENT

4.1.1. THE EXPRESSION LEVELS OF MUTANT P53 PROTEIN ARE ELEVATED IN RESPONSE TO DOXORUBICIN

U251 cells are derived from a glioma and harbor the hotspot p53 point mutation R273H. As most tumor cell lines, that express a mutated form of p53, these cells accumulate high levels of the protein. Nevertheless, we observed by immunoblotting that the expression levels of p53 in these cells get elevated even further, when treated with the chemotherapeutic drug doxorubicin (Figure 6A).

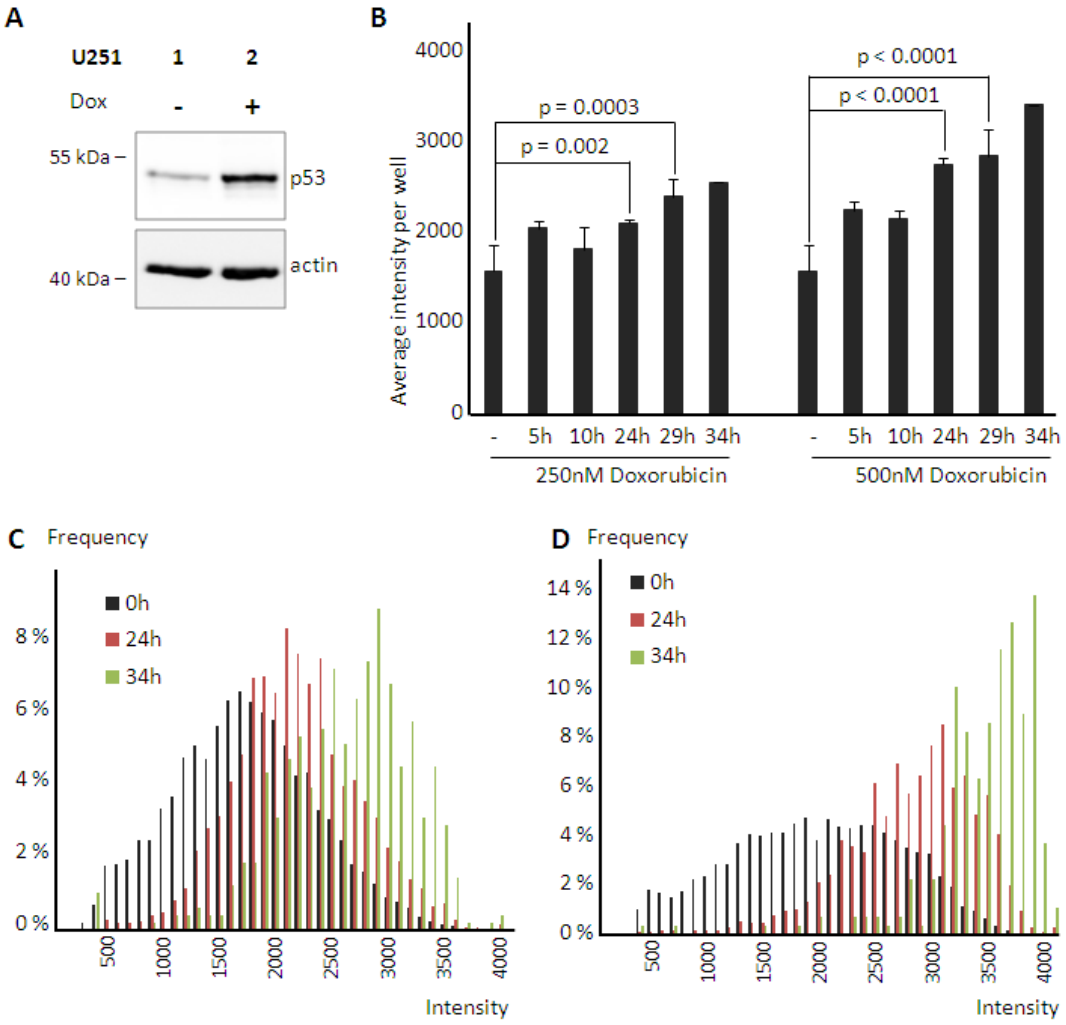


Figure 6: The accumulation of mutant p53 in response to doxorubicin treatment.
U251 cells (p53 R273H) were treated with 500nM doxorubicin for 24h. **A:** Total cell lysates were subjected to immunoblot analysis using antibodies against p53. Actin staining was used as loading control. **B-D:** Cells were fixed and stained for immunofluorescence analysis using antibodies against p53. Single nuclei were identified using Hoechst 33342 staining. The average p53-staining intensity was determined per nucleus. The experiment was performed in triplicate **B:** Means and standard deviations of the average p53-intensities per well are depicted. A Student's t-test (α=0.05) was performed for statistical analysis. **C, D:** Histograms of the p53-intensities per nucleus were generated for three different time-points at 250nM (**C**) and 500nM (**D**) final concentration of the drug.

This result was confirmed by quantitative immunofluorescence microscopy analyzing the p53 expression levels upon doxorubicin treatment in a time dependent manner at two different concentrations. The average expression of p53 was measured for each nucleus. In Figure 6B the mean intensities per well are diagramed, as they were determined in triplicate. The levels increased significantly after 29 hours of treatment using 250nM doxorubicin, as well as

after 24 hours of treatment with a final drug concentration of 500nM. The representation of the data in histograms (Figure 6C and D) shows that with both concentrations the majority of the cells accumulate their mutant p53 protein over time.

The mechanisms by which mutant p53 is generally stabilized in tumor cells are poorly understood. About the further accumulation of the protein upon chemotherapeutic treatment even less is known.

4.1.2. POSTTRANSLATIONAL MODIFICATIONS OF MUTANT P53 ARE INDUCED IN RESPONSE TO DOXORUBICIN TREATMENT, EVEN THOUGH THEY DO NOT DIRECTLY INFLUENCE ITS STABILITY

Kurz *et al.* (2004) showed that doxorubicin acts through the activation of the transducer kinase ATM. Additionally, it is known that ATM phosphorylates p53 at Serine 15, which in turn leads to the acetylation of Lysine 382 of p53 (Dumaz and Meek, 1999; Sakaguchi *et al.*, 1998). This suggests itself that also mutant p53 might get posttranslationally modified at these sites in response to doxorubicin treatment. We therefore analyzed the response of U251 cells to chemotherapeutic drug exposure by immunoblotting using antibodies against Serine 15 phosphorylated and Lysine 382 acetylated p53. We did not detect any modified p53 in untreated cells, but after incubation with doxorubicin for 24h the levels were dramatically increased (Figure 7A).

This result was confirmed using immunofluorescence analysis, quantifying the expression of Serine 15 phosphorylated p53 in individual cells treated with 500nM doxorubicin for 24 hours. The histogram of the obtained data clearly shows a peak shift towards higher intensities upon treatment (Figure 7B).

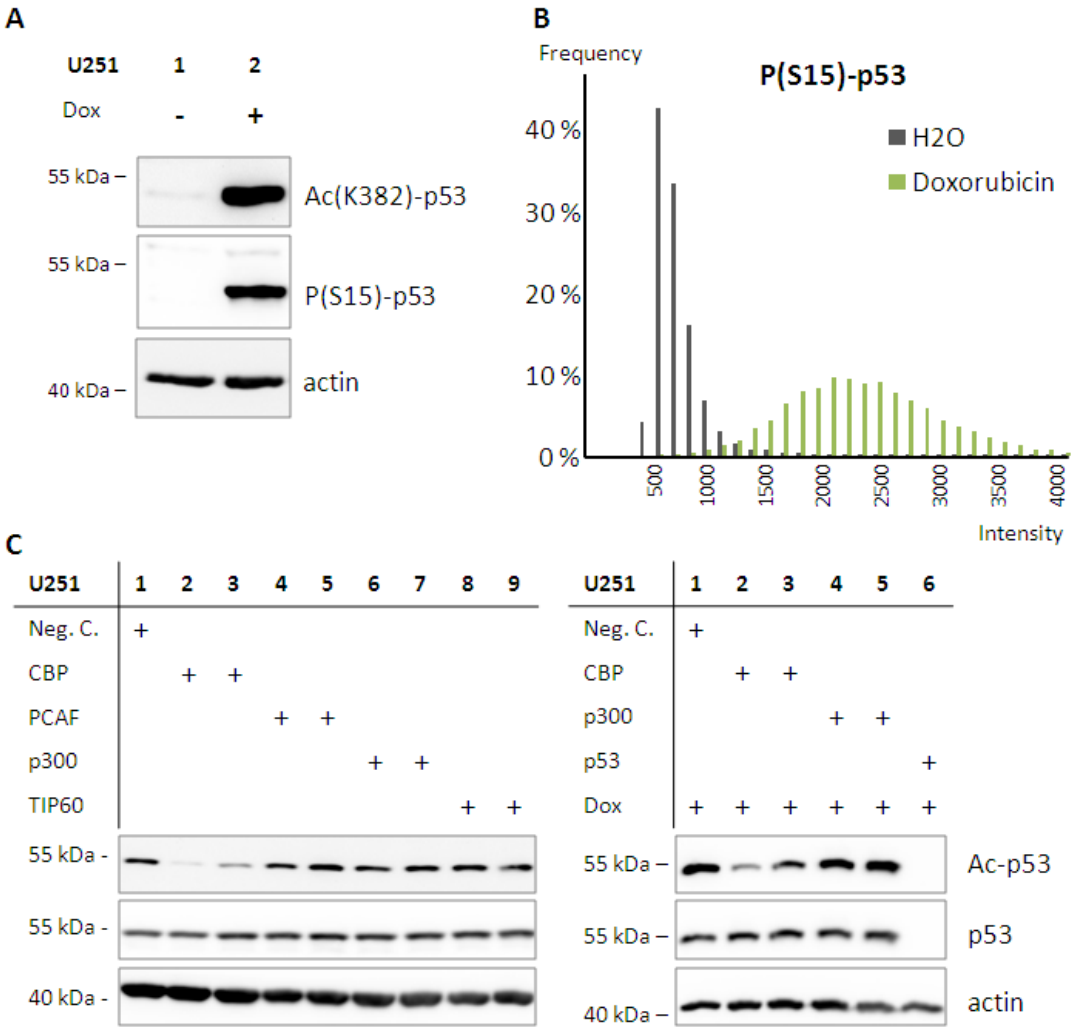


Figure 7: Mutant p53 gets posttranslationally modified upon doxorubicin treatment.
A: U251 cells were treated with 500nM doxorubicin for 24h. Total cell lysates were subjected to immunoblot analysis using antibodies against acetyl(Lys382)-p53 or phospho(Ser15)-p53. Actin staining was used as loading control. **B:** U251 cells were treated for 24 hours with 500nM doxorubicin. The cells were fixed and stained for immunofluorescence analysis using antibodies against phospho(Ser15)-p53. Single nuclei were identified using 10µM doxorubicin staining. The average p53-staining intensity per nucleus is diagramed in a histogram. **C:** U251 cells were reverse transfected with two different siRNAs per gene for 48 hours. As indicated, the samples in the right panel were additionally treated with 500nM doxorubicin for the last 24 hours. Whole cell lysates were subjected to immunoblot analysis using antibodies against acetyl(Lys382)-p53 and total p53. Actin staining served as loading control.

The impact of these modifications is unknown, but we cannot exclude that, as for wt p53, the protein gets stabilized through these modifications. To explore their functional significance with respect to the stabilization of the protein, we used siRNA mediated knock-down of known p53 acetyltransferases, as Ito *et al.* (2002) described their role in the regulation of wild-type p53 stability. 48

hours post transfection of U251 cells with siRNAs, targeting the 4 histone acetyl transferases CREB binding protein (CBP), p300/CBP-associated factor (PCAF), p300, and Lysine acetyl transferase 5 (KAT5 alias TIP60) total cell lysates were subjected to immunoblot analysis. Surprisingly, only the knock-down of CBP led to a clear reduction of Lys382-acetylated p53 in these cells. Nevertheless, this did not have any impact on the expression levels of total p53 protein (Figure 7C, left panel).

Since we have shown that the levels of Lys382-acetylated p53 are increased dramatically in response to doxorubicin (Figure 7A), we additionally investigated whether the knockdown of CBP and p300 impairs the further accumulation of mutant p53 protein levels upon doxorubicin treatment. But, we again did not detect any changes in the expression levels of total p53 protein (Figure 7, right panel). Therefore we conclude that the posttranslational modification of Lysine 382 of mutant p53 is not the primary regulator of its stability, neither in the default state of the cells, nor in response to chemotherapeutic treatment.

4.1.3. U251 CELLS DISPLAY AUGMENTED MRNA LEVELS OF P53 IN RESPONSE TO DOXORUBICIN TREATMENT

Apart from protein stability, many proteins are regulated in their expression on the transcriptional level. Even though there are only a few reports claiming that p53 gets differentially expressed due to transcriptional activation, it appears to be logic that if the general accumulation of mutant p53 is associated with increased half life of the protein the response to chemotherapeutic treatment happens to be regulated by other means. Therefore, we next aimed to test whether doxorubicin mediated accumulation of mutant p53 is caused by transcriptional activation of the gene. To investigate this, we isolated total RNA from U251 cells treated for 24 hours with 500nM doxorubicin. Strikingly,

quantitative real-time PCR analysis revealed that there was about 5 times more p53 mRNA upon doxorubicin treatment (Figure 8).

The activation of E2F1 through ATM, ATR and the checkpoint kinases Chk1 and -2 upon DNA damage leads to its stabilization and preferential transactivation of apoptotic target genes like TAp73 and NOXA (Hershko and Ginsberg, 2004). In our experiments the up-regulation of TAp73 mRNA is always used as a positive control for proper induction of the DNA damage response. It should be noted that the transcription of p53 mRNA in response to doxorubicin is surged as strong, as the well known E2F1 target gene TAp73 (Figure 8).

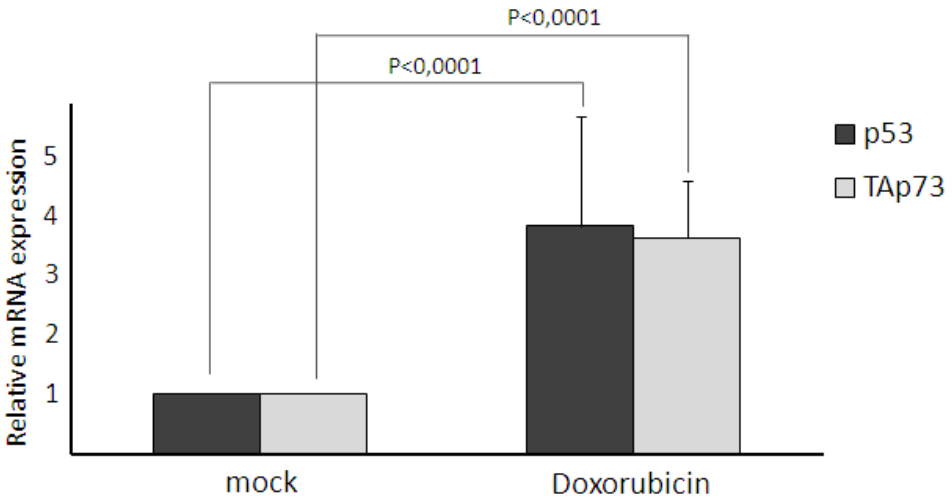


Figure 8: Doxorubicin induces the transcription of p53.
U251 cells were treated with 500nM doxorubicin for 24 hours. Total RNA was isolated and reverse transcribed into cDNA. Relative mRNA expression levels were determined using 36B4 as control gene. The mean and standard deviation of 8 independent replicates were plotted. For statistical analysis a Student's t-test was performed (α=0.05).

4.2. THE MECHANISMS OF P53 TRANSCRIPTIONAL REGULATION

It was previously reported that the two transcription factors HOXA5 and RREB1 contribute to the transcriptional regulation of p53. We confirmed that in our system HOXA5, as well as RREB1 contribute to the up-regulation of p53 mRNA in response to doxorubicin, as it was shown by Raman *et al.* (2000) and Liu *et al.* (2009) respectively (data not shown).

4.2.1. THE TRANSCRIPTION FACTORS E2F1 AND TAP73 ARE NECESSARY FOR THE INDUCTION OF P53 IN RESPONSE TO DOXORUBICIN

In addition to the above, Ren *et al.* (2002) published a ChIP-on-chip study where the promoter of p53 was found occupied by the transcription factor E2F4 four fold over average. Nevertheless, E2F4 is thought to be primarily involved in the repression of E2F-responsive genes (Dyson, 1998), whereas its homolog E2F1, which is known to be stabilized and activated in response to doxorubicin, is a potent transcriptional activator of its target genes (Dyson, 1998).

As a first step to determine whether E2F1 regulates the expression of p53, the messenger RNA levels of p53 were analyzed in response to doxorubicin treatment upon the previous knock-down of E2F1. The up-regulation of TAp73 transcription in this experiment occurred mainly through the activation of E2F1, as the increased transcription of TAp73 is abolished completely after knock-down of E2F1 with two different siRNAs (Figure 9, light grey bars). At the same time we observed that the knock-down of E2F1 diminishes the accumulation of p53 mRNA (Figure 9, dark grey bars) upon doxorubicin to a large extent even though the effect is not as strong, as it was observed for TAp73. The knock-down efficiencies for both siRNAs were very high as determined by qRT-PCR (Figure 9, upper chart) and immunoblot analysis (Figure 10C).

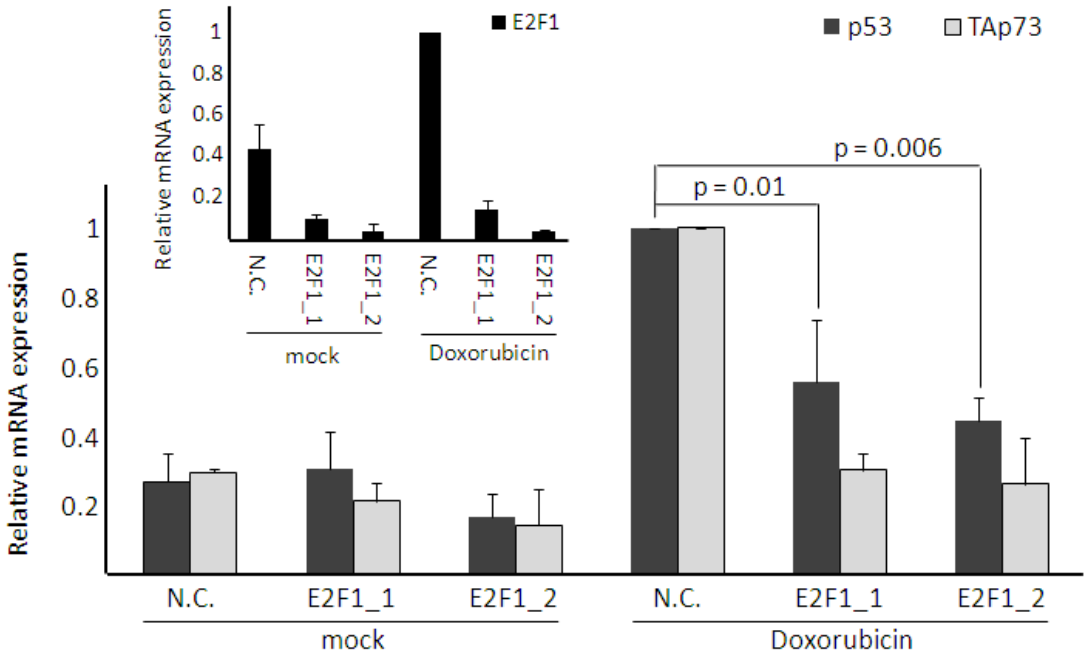


Figure 9: The knock-down of E2F1 alleviates the effects of doxorubicin on p53 transcription
U251 cells were transfected with siRNAs targeting E2F1 for 24 hours, before they were treated with 500nM doxorubicin for additional 24 hours. Total RNAs were reverse transcribed and quantified, relative to 36B4, by real-time PCR. The means and standard deviations of four biological replicates are depicted. For statistical analysis a Student's t-test was performed (α=0.05).

To further support the theory that the augmented transcription of p53 upon doxorubicin treatment is dependent on the transcription factor E2F1, we performed quantitative immunofluorescence microscopy, as well as immunoblotting. In both cases, we observed that also on the protein level the induction of p53 through doxorubicin treatment gets diminished by the knock-down of E2F1 (Figure 10).

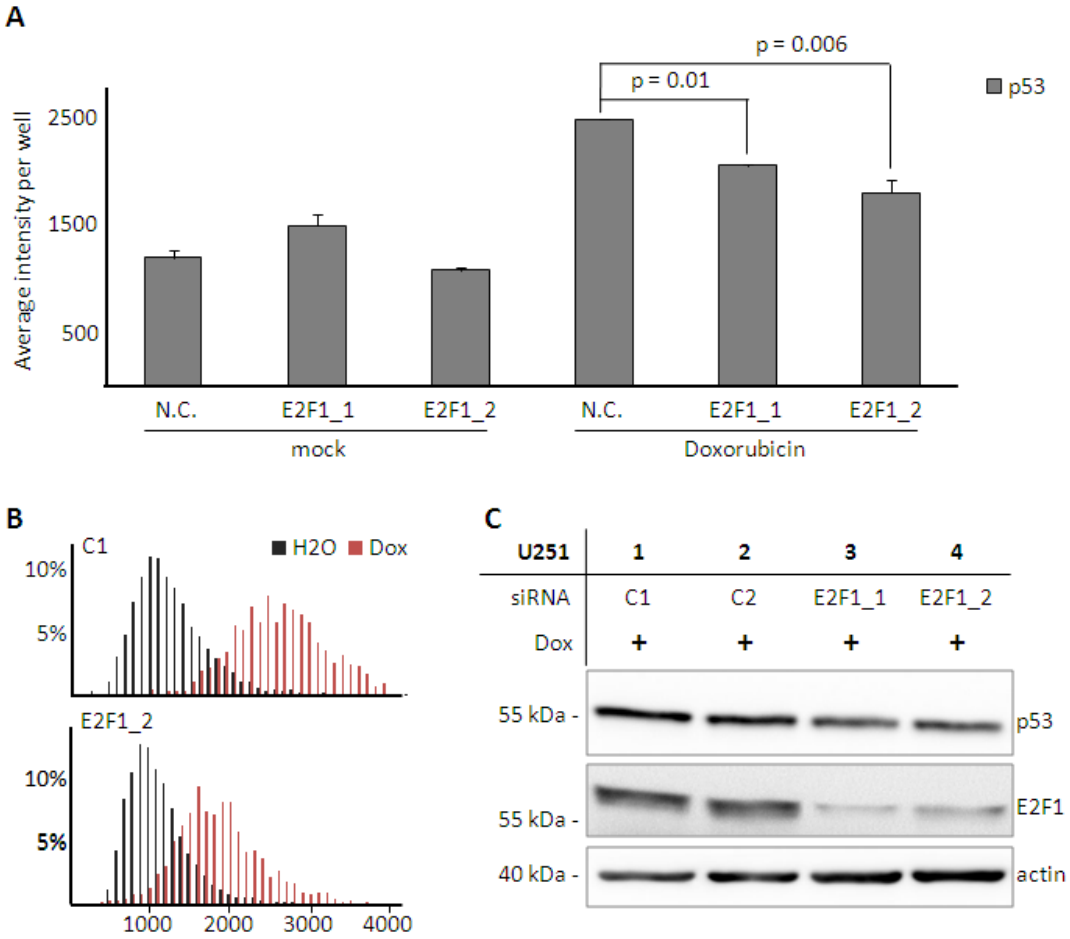


Figure 10: E2F1 contributes to the enhanced protein levels of mutant p53 upon doxorubicin.
U251 cells were transfected with two different siRNAs targeting E2F1 for 24 hours, and then they were treated for additional 24 hours with 500nM doxorubicin. **A, B:** The cells were fixed and stained for immunofluorescence microscopy. Single nuclei were identified using 10µM doxorubicin. The p53 staining was quantified per nucleus. **A:** The average intensity per well was determined in triplicate, means and standard deviations are depicted. For statistical analysis a Student's t-test was performed (α=0.05). **B:** Representation of the data in histograms. **C:** The cells were harvested for immunoblot analysis and stained for p53 as well as E2F1. Actin was used as a loading control.

Bearing in mind the observations of Wang and el-Deiry (2006) that p53 and TAp73 are capable of directly regulating the transcription of p53, the observed may in part be due to the E2F1 dependent induction of TAp73.

To investigate whether E2F1 is only indirectly acting on p53 through the co-regulation of its target gene TAp73 we used siRNA mediated knock-down of the p53 paralog. And indeed, TAp73 is as well necessary for the induction of p53 mRNA (Figure 11), even though it should be noted that the knock-down of

E2F1 had a stronger effect on the expression of p53 mRNA compared to the knock-down of p73, whereas the expression levels of TAp73 were unequivocally lower in the latter case. Therefore, we believe that E2F1 is acting on the transcriptional regulation of p53 not exclusively through TAp73.

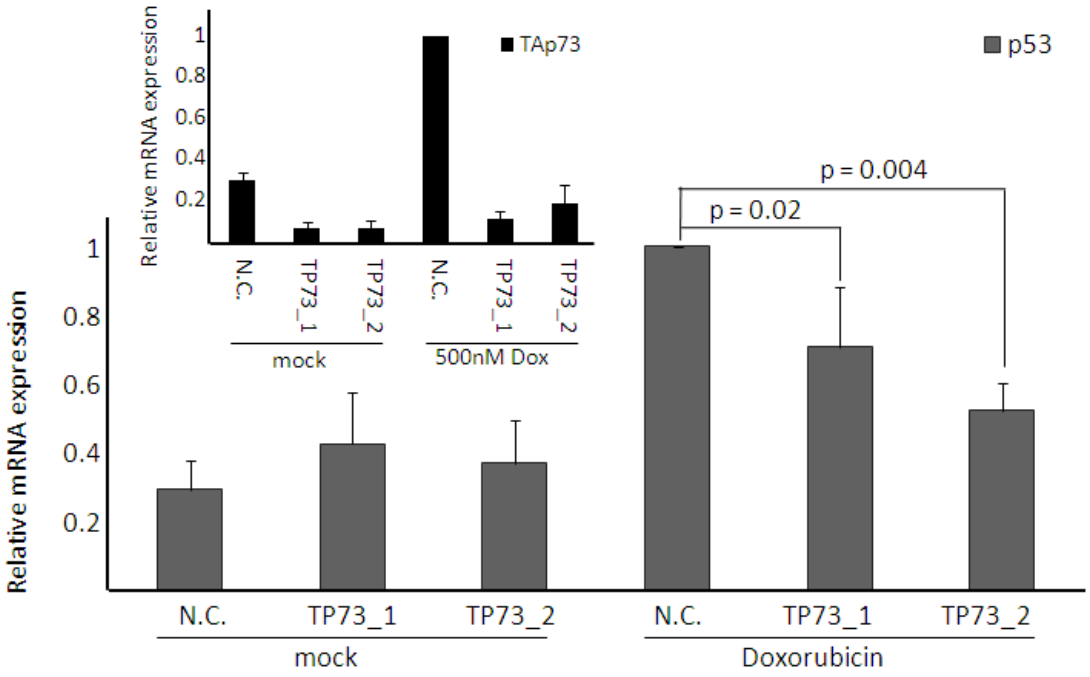


Figure 11: The induction of p53 transcription is partially dependent on p73.
U251 cells were treated with 500nM doxorubicin for 24 hours. The isolated RNA was reverse transcribed and the expression of p53 and TAp73 was quantified relative to 36B4 using real-time PCR. The experiment was performed in four biological replicates. The means and standard deviations were plotted. For statistical analysis a Student's t-test was performed (α=0.05).

4.3. E2F1 REGULATES P53 DIRECTLY AS A TRANSCRIPTION FACTOR

The finding that E2F1 knock-down has a stronger effect on the doxorubicin induced augmentation of p53 transcription, compared to p73, pinpoints to the additional involvement of E2F1 in the regulation of p53. To identify potential E2F1 binding sites and their biological relevance we first used bioinformatical tools to *in silico* predict potential binding sites that were then confirmed by chromatin immunoprecipitation analysis (ChIP).

4.3.1. *IN SILICO* IDENTIFICATION OF POTENTIAL E2F1 BINDING SITES WITHIN THE P53 PROMOTER REGION

E2F1 is an important transcription factor regulating the expression of various genes in response to its activation through DNA damage or other stimuli. The DNA sequence TTTSSCGC is described to be the canonical binding site motif for E2F1; nevertheless, some ChIP-on-chip studies revealed that a large proportion of E2F binding occurs at sites where this recognition sequence cannot be found (Bieda *et al.*, 2006). In collaboration with Martin Haubrock (Department of Bioinformatics, University of Göttingen) all these ChIP-on-chip data sets were used to generate a scoring matrix, helping to identify potential E2F1 binding sites (Table 1).

Table 1: Scoring matrix for the identification of potential E2F1 binding sites
Sequence information from known E2F1 binding site motifs were integrated to generate a scoring matrix for the identification of potential new E2F1 binding sites. The resulting consensus motif is displayed in the left column. N: any (A, C, G, or T); K: ketone (G or T); S: strong bonds (C or G)

Nucleotide / Consensus	A	C	G	T
N	1	4	3	5
K	0	1	5	7
T	2	0	0	11
S	0	7	6	0
S	0	5	8	0
C	0	10	3	0
G	0	3	10	0
C	0	8	4	1

This matrix was then applied to the genomic sequence around the transcriptional start site (TSS) of p53 in order to identify potential binding sites. In Figure 12A the determined scores are plotted against the genomic region. Two sites within the analyzed sequence revealed a score greater than 0.9 and

were therefore considered as potential E2F1 binding sites. First, E2F1 BS-I, at position 7590195-7592195 (hg19 chromosome 17) with the sequence ACTGGCGC revealed a score of 0.911, and second, E2F1 BS-II, at position 7590195-7592195 (hg19 chromosome 17) with the sequence TTTCGCGG resulted in a score of 0.954. Analysis of the evolutionary conservation of the predicted binding sites showed that E2F1 BS-I close to the TSS is highly conserved, whereas E2F1 BS-II even though displaying a higher score lacks this conservation (Figure 12B).

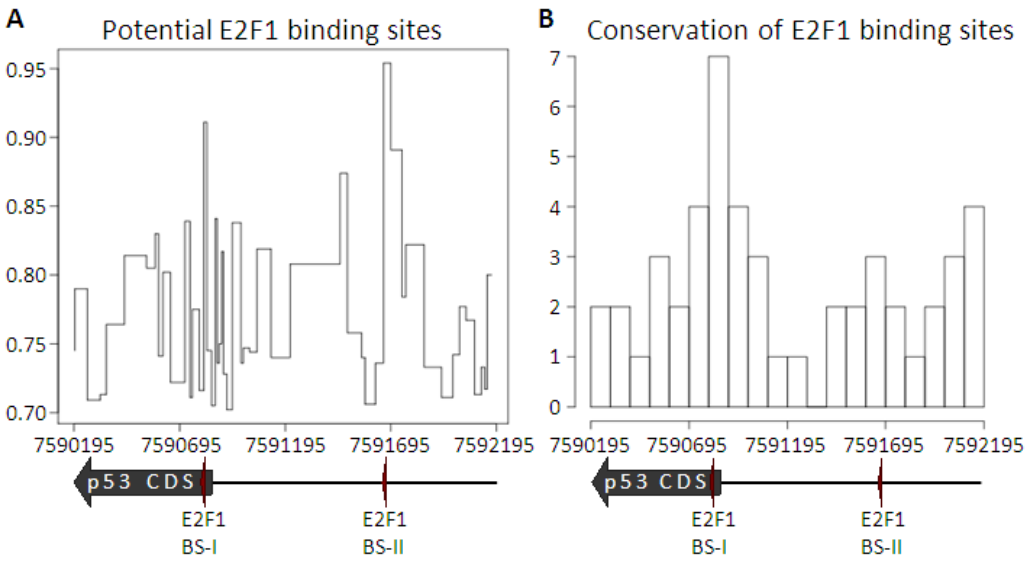


Figure 12: Bioinformatical analysis of the p53 promoter uncovers two E2F1 binding sites.
A scoring matrix (Table 1) to discover potential E2F1 binding sites was applied to the DNA sequence of the p53 promoter region (-1500 to +500 around the TSS). **A:** The calculated scores are plotted against the genomic region. **B:** The level of conservation within the analyzed genomic region is depicted. **A, B:** A schematic representation of the p53 gene locus, with the two binding motifs displaying the highest score and sequence conservation, is shown underneath both plots.

4.3.2. THE TRANSCRIPTION FACTOR E2F1 BINDS TO ONE OF THE POTENTIAL E2F1 BINDING SITES WITHIN THE P53 PROMOTER REGION

Within 2000 base pairs around the TSS of p53 two potential E2F1 binding sites were identified using bioinformatical tools. In order to confirm the biological relevance of these binding sites ChIP analysis was performed. E2F1 protein,

cross linked to its DNA binding elements, was precipitated using antibodies, generated against the transcription factor. ChIP-grade IgG antibodies were used for negative control precipitation. The recovered DNA was amplified by quantitative real-time PCR using primers spanning the two potential E2F1 binding sites on the p53 promoter. Additionally, primers spanning the well known E2F1 binding sites on the p107 and TAp73 gene loci were used as positive controls. Primers amplifying a region 19.5kb downstream of the p53 TSS, a region where no binding of E2F1 is expected, served as negative control. The data show that E2F1 is bound to the predicted E2F1 BS-I roughly 40bp downstream of the TSS, but not to the less conserved second potential binding site (Figure 13, upper panel).

As expected, E2F1 also associated with the promoters of its target genes p107 and TAp73. In contrast, the recovery of a distant fragment of the p53 genomic locus (p53 +19.5kb) was at the background level (similar to precipitation with non-specific IgG) (Figure 13, lower panel).

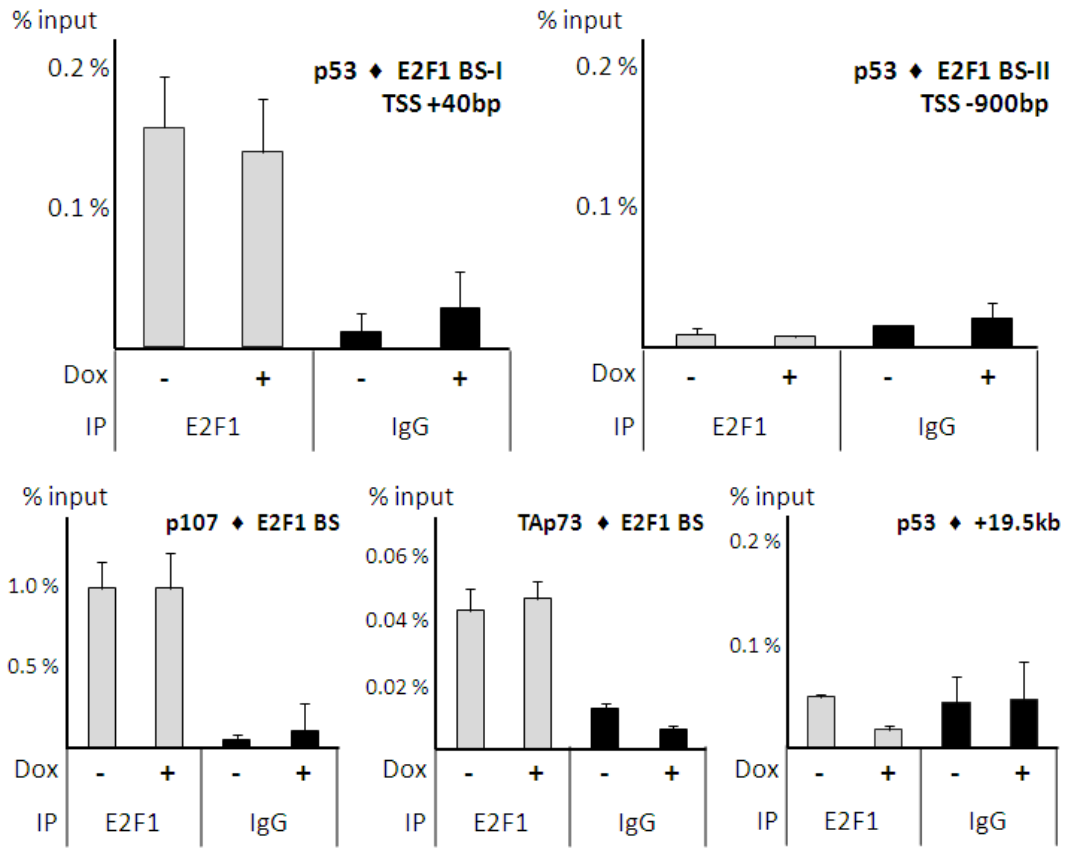


Figure 13: E2F1 is bound to one of the predicted binding sites under physiological conditions.
U251 cells were treated with 500nM doxorubicin for 24 hours. Cross linked DNA – protein complexes were precipitated using antibodies against E2F1 or IgG, as negative control. The recovered DNA was quantified by real-time PCR using primers flanking the two potential E2F1 binding sites, as well as two positive control sequences (p107 and TAp73) and a negative control region (p53 +19.5kb). The means and standard deviations of three replicates are depicted as percentile of the input DNA.

4.4. IS THE INCREASED TRANSCRIPTION OF P53 A GENERAL RESULT OF CHEMOTHERAPEUTIC TREATMENT?

4.4.1. THE INFLUENCE OF DIFFERENT CHEMOTHERAPEUTIC DRUGS ON THE TRANSCRIPTIONAL REGULATION OF P53 EXPRESSION

Treatment of U251 cells with the topoisomerase I inhibitor camptothecin (CPT), the topoisomerase II inhibitor etoposide, as well as the nucleoside analog 5-fluorouracil revealed that the induction of p53 transcription is specific to doxorubicin, rather than a general result of the evoked DNA damage response.

The treatment of the cells with CPT resulted in a very strong induction of NOXA, another target gene of E2F1, whereas the induction of TAp73 and p53 was not detected under these circumstances (Figure 14A). Also 5-fluorouracil (5'FU), as nucleoside analog, caused similar to CPT an induction of NOXA, but neither TAp73 nor p53 were elevated in their expression (Figure 14A).

Additionally, we tested etoposide, a chemotherapeutic drug more closely related to doxorubicin, since it also acts through the inhibition of topoisomerase II. In contrast to CPT and 5'FU this drug led to the induction of TAp73, as it was previously observed for doxorubicin. But, to our surprise, the levels of p53 messenger RNA remained unaffected (Figure 14A). Other than the mentioned induction of TAp73 transcription, we also observed that etoposide treatment mediates an increase in Serine 15 phosphorylation of p53 in U251 cells (Figure 14B). This could be explained by the evoked DNA damage response and the accompanied activation of ATM. Nevertheless, we observed in the same experiment that the total levels of p53 protein remain unchanged in response to etoposide treatment (Figure 14C).

In conclusion, these data show that the induction of TAp73 in the DNA damage response seems not to be sufficient to augment the transcription of p53, although we demonstrated before (4.2.1) that it is necessary.

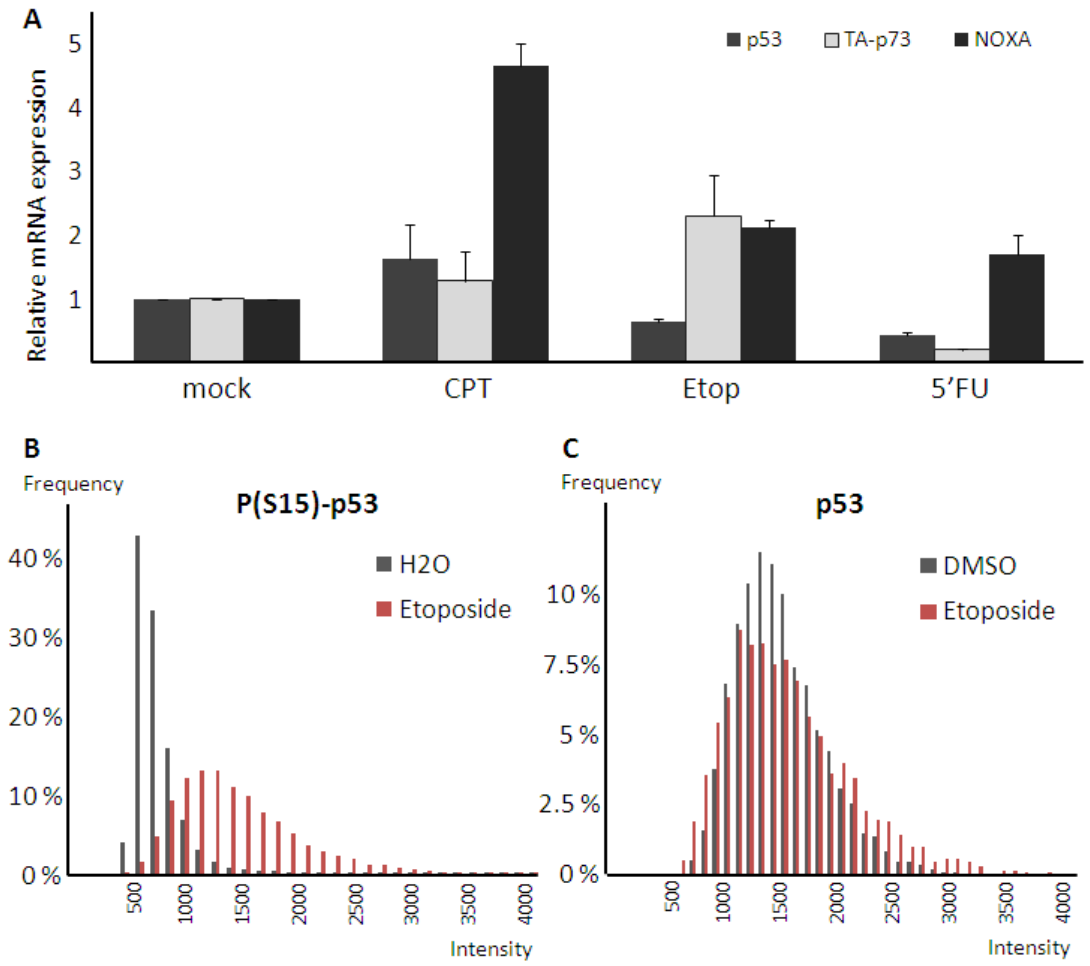


Figure 14: The induction of p53 is not simply caused by the inhibition of topoisomerase. **A:** U251 cells were treated with CPT (2,87µM), etoposide (100µM), or 5'FU (500µM) for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. The means and standard deviations of three biological replicates are depicted. **B, C:** U251 cells were treated with 100µM etoposide for 24 hours each. Upon fixation, the cells were stained for immunofluorescence analysis using antibodies against phospho(S15)-p53 (**B**) or total p53 (**C**). Single nuclei were identified using 10µM doxorubicin. The average fluorescence intensity per nucleus was determined. The results are represented in histograms.

4.4.2. THE IMPACT OF DIFFERENT ANTHRACYCLINES ON THE TRANSCRIPTIONAL REGULATION OF P53

The mechanism by which doxorubicin acts in the cell is controversially discussed in the literature, some reports claim that its ability to intercalate into DNA leads to decreased transcription through inhibition of helicase activity (Bachur *et al.*, 1992) or through DNA cross linking (Swift *et al.*, 2006), others claim that doxorubicin mainly functions by stalling of topoisomerase II on the

DNA and the stabilization of a reaction intermediate in which the DNA strands are cut and covalently linked to the enzyme (Tewey *et al.*, 1984a; Tewey *et al.*, 1984b). Additionally, there are mechanisms of free radical formation, DNA alkylation, direct membrane effects, and direct induction of apoptosis discussed (Gewirtz, 1999). The observation that etoposide, in contrast to doxorubicin, lacks the ability to induce p53 transcription, argues against the theory that the general inhibition of topoisomerase II activity is sufficient to induce the transcription of p53. In addition to doxorubicin, there are other anthracyclines currently used in the clinics, namely daunorubicin, epirubicin, and idarubicin. These drugs are described to have slightly different sequence specificities, but are all believed to interfere with DNA transcription through intercalation as well as topoisomerase II inhibition (Minotti *et al.*, 2004).

These 4 structurally related drugs share a common tetracyclic ring system containing an anthraquinone chromophore with a daunosamine moiety attached to the A-ring (C7), but they differ in their substitutions to this basic structure (Figure 15A, differences compared to doxorubicin are highlighted with red circles). Interestingly, analysis of their ability to induce the transcription of p53 revealed that dauno-, doxo-, and epirubicin induced p53 in a comparable manner. Whereas idarubicin, differing from daunorubicin only in a methoxy-group at C4 (D-ring), has no effect on p53 transcription, even though it induces TAp73 the same way as the other three compounds (Figure 15B).

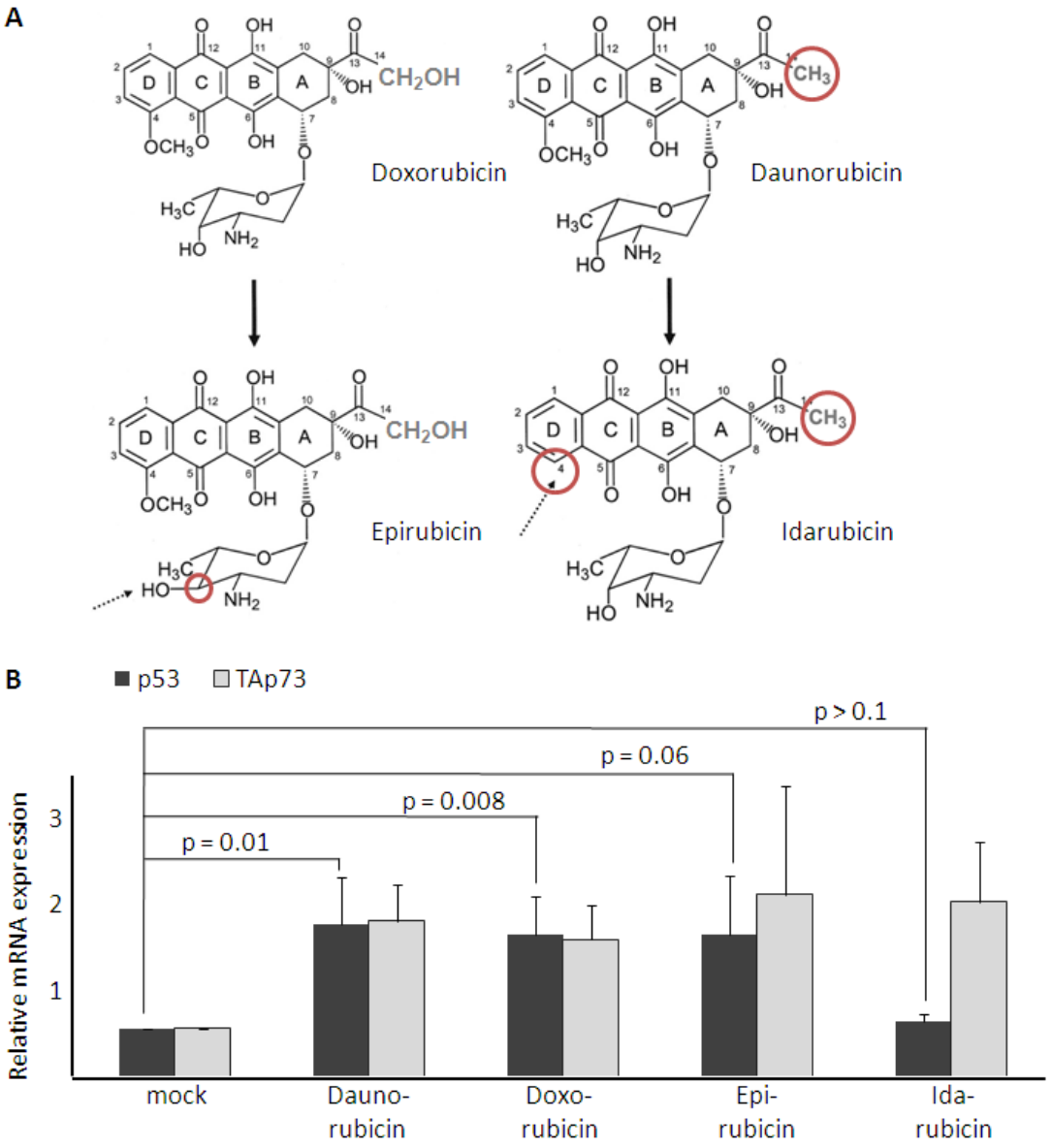


Figure 15: Three out of four anthracyclines activate the transcription of p53.
A: Chemical structure of the four anthracyclines doxorubicin, daunorubicin, epirubicin and idarubicin [Adopted from Minotti *et al.* (2004)]. Differences with respect to doxorubicin are highlighted with red circles. **B:** U251 cells were treated with 500nM dauno-, doxo-, epi-, or idarubicin for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. The experiment was performed in triplicate. The means and standard deviations of the three experiments are diagramed. For statistical analysis a Student's t-test was performed (α=0.05).

4.4.3. ANTHRACYCLINE MEDIATED ACTIVATION OF P53 TRANSCRIPTION

Quantification of the p53 pre-mRNA expression revealed that upon doxorubicin treatment not only the levels of mature p53 mRNA are elevated, which could as well be explained by increased mRNA stability, but also the direct product of

transcription, the pre-mRNA. This argues in favor of the hypothesis that the two transcription factors TAp73 and E2F1 facilitate the active transcription of the p53 gene (Figure 16).

Interestingly, we found that idarubicin, the anthracycline that is structurally related to doxorubicin, but not capable of augmenting the levels of p53 mRNA, induced the expression of p53 pre-mRNA the same way as the other anthracyclines (Figure 16; data not shown).

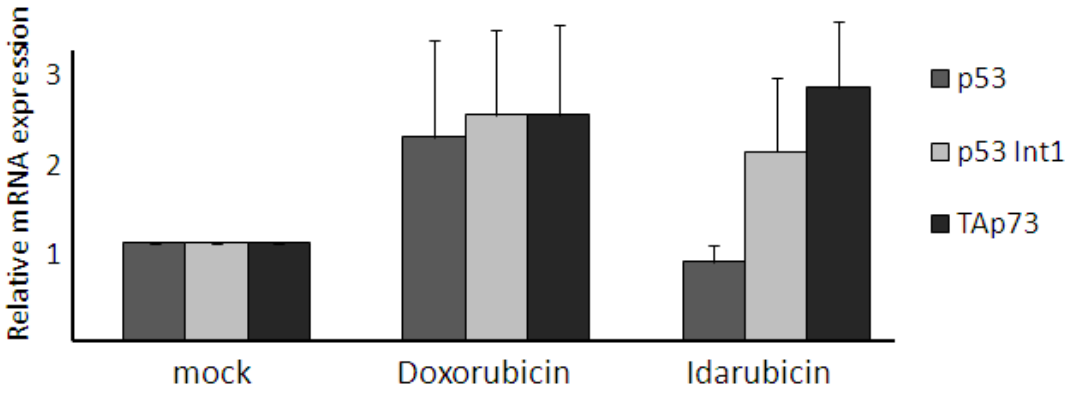


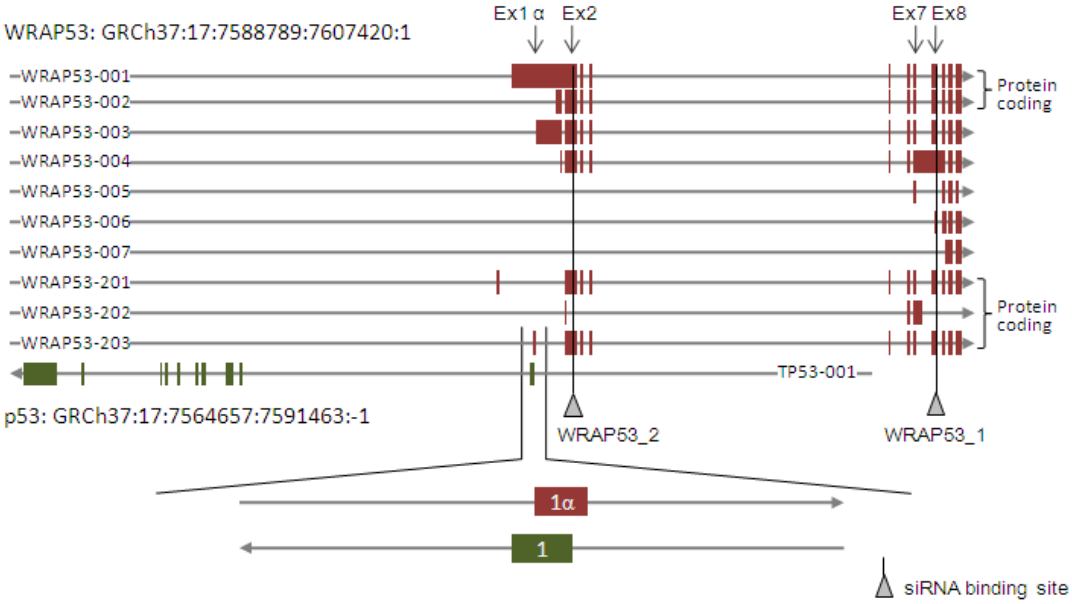
Figure 16: p53 pre-messenger RNA is elevated in response to all anthracyclines.
U251 cells were treated with 500nM doxorubicin or idarubicin for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. Means and standard deviations of three biological replicates are depicted.

4.5. WRAP53 AS REGULATOR OF P53 MRNA EXPRESSION

The recent finding of Mahmoudi *et al.* (2009) that the pre-mRNA stability of p53 is regulated through a natural antisense transcript prompted us to investigate whether the expression of the described WRAP53 (WD repeat containing, antisense to p53) gene contributes to the regulation of p53 in response to anthracyclines.

WRAP53 was identified as a gene located immediately upstream of *TP53* on the opposite strand. Mahmoudi *et al.* (2009) found at least 17 variants of this gene, generated through alternative splicing, as well as three different TSS. In Figure 17 the 10 transcripts listed in the Ensembl database were aligned with the p53 gene. Two of the transcripts (WRAP53-001 and WRAP53-203) contain

exon 1α, which is overlapping with a large portion of exon 1 of *TP53*. So far the function of WRAP53 protein is unknown; whereas it is claimed by Mahmoudi *et al.* (2009) that exon 1α of the WRAP53 mRNA contributes to the induction of p53 in response to DNA damage.



Transcript \ Primer	WRAP53 1α	WRAP53 1α-2	WRAP53 7-8
WRAP53-001	X	—	X
WRAP53-002	—	—	X
WRAP53-003	—	—	X
WRAP53-201	—	—	X
WRAP53-203	X	X	X

Figure 17: Schematic representation of the *TP53* genomic locus on chromosome 17
The 10 transcripts of WRAP53 listed in the Ensembl database are aligned with *TP53*. Both genes are encoded within the same genomic locus on opposite strands of the DNA. Exon 1α of WRAP53 and exon 1 of p53 overlap with the majority of their sequence, as depicted in the zoom-in. A table of the transcripts amplified using three different primer pairs is depicted in the lower part.

To explore the functional significance of WRAP53 in the context of anthracycline induced transcription of p53, we analyzed its mRNA expression using three different primer pairs: WRAP53 7-8 is used to amplify the majority of the transcripts independent of the TSS; WRAP53 1α is used to amplify both transcripts containing exon 1α; and WRAP53 1α-2 is used to specifically amplify WRAP53-203 (an overview of the transcripts targeted by the primer pairs is listed in Figure 17, lower part).

In response to doxorubicin we observed a slight up-regulation of WRAP53 1α. In contrast to that, our analysis yielded a massive induction of the WRAP53 transcripts containing exon 1α in response to idarubicin (Figure 18A), the anthracycline that was shown before to induce the pre-mRNA levels of p53 (4.4.3), but keeps the levels of mature mRNA low (4.4.2). An even stronger induction of WRAP53 1α was observed in response to etoposide treatment (Figure 18B), the topoisomerase II inhibitor that as well induced the DNA damage response through TAp73, but failed to augment the expression levels of p53 (4.4.1).

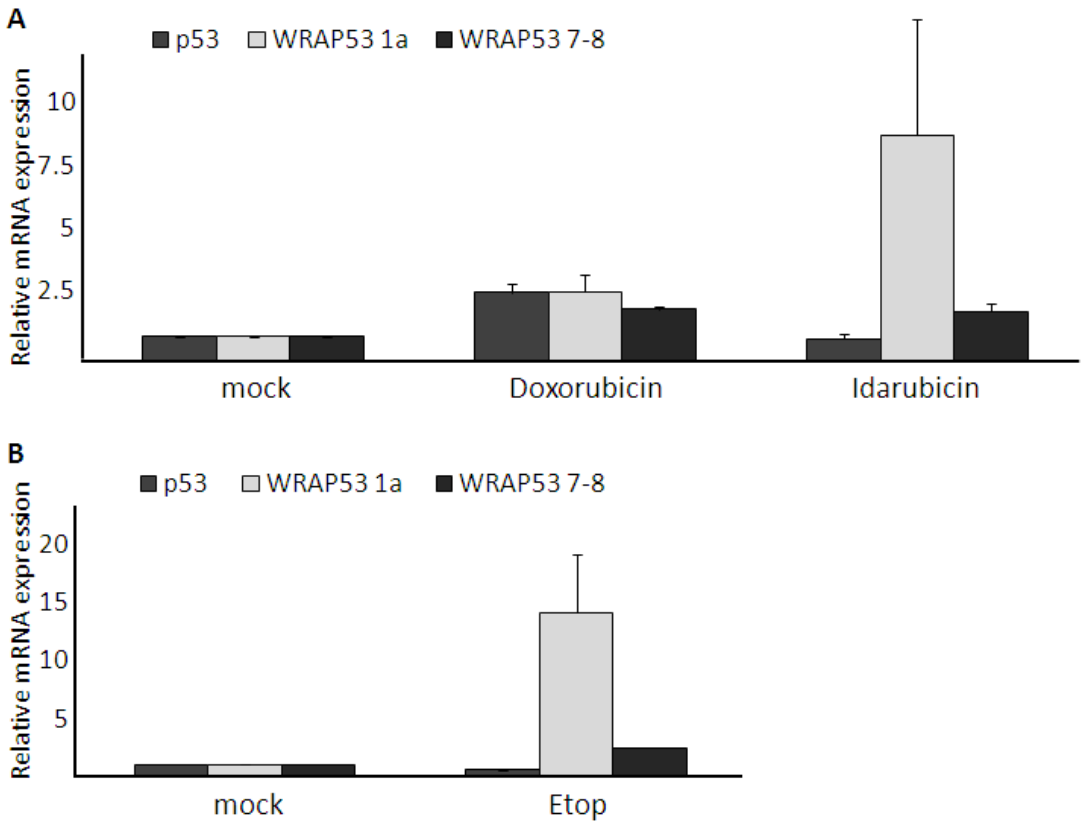


Figure 18: The natural antisense transcript of p53 is expressed antagonistic to p53 itself. U251 cells were treated with 500nM doxorubicin or idarubicin (**A**) or 100µM etoposide (**B**) for 24 hours each. The isolated RNA was reverse transcribed and quantified relative to 36B4 by real-time PCR. The depicted diagram represents the means and standard deviations of four biological replicates (**A**), experiment **B** was performed in triplicate.

The total levels of WRAP53 are slightly induced in response to any kind of DNA damage that was subjected to the cells, but remained the same for idarubicin

and etoposide, when compared to doxorubicin treatment (Figure 18A, B; black bars). These data show a clear correlation between the induction of WRAP53 transcripts containing exon 1α and the diminished response of p53 mRNA expression to E2F1 activation. Nevertheless, it should be mentioned that these data stand in contrast to the study published by Mahmoudi *et al.* (2009), where WRAP53 was shown to stabilize p53 mRNA in response to DNA damage.

Quantification of the p53 mRNA levels, upon siRNA mediated knock-down of WRAP53 (the siRNA target sites are depicted in Figure 17) and subsequent treatment with idarubicin was used to elicit the role of WRAP53 in the regulation of p53. To our surprise, the levels of p53 mRNA remained low upon knock-down of the antisense transcript WRAP53 and subsequent idarubicin treatment (Figure 19, white bars). Nevertheless, when we checked for the knock-down efficiency of the used siRNAs, we observed a discrepancy depending on the primer pair that was used for the analysis. Quantification of WRAP53 mRNA using primers to amplify either all transcripts, or specifically WRAP53-203 led to a reduction upon siRNA transfection of about 90%. Whereas, the usage of primers amplifying all transcripts containing exon 1α revealed that almost 60% of mRNA escaped the knock-down (Figure 19). A possible explanation for this discrepancy is that a WRAP53 transcript exists that contains exon 1α, but lacks exon 2 and 8, where the used siRNAs bind. Alternatively, it could be suggested that due to RNA masking or inhibited nuclear export, this WRAP53 transcript escapes the siRNA mediated knock-down.

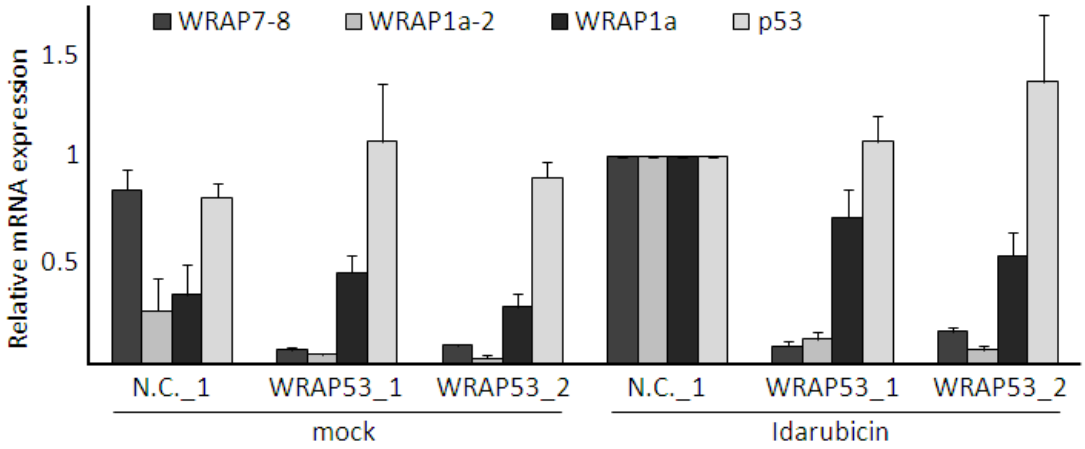


Figure 19: Not all transcripts of WRAP53 are targeted by the used siRNAs.
U251 cells were long-term transfected with two different siRNAs targeting WRAP53. Total RNA was isolated and reverse transcribed. Quantitative real-time PCR was used to determine the expression of the different WRAP transcripts as well as p53 mRNA. 36B4 was used as reference gene. Means standard deviations of three biological replicates are diagramed.

4.6. IS THE OBSERVED TRANSCRIPTIONAL REGULATION OF P53 THROUGH TOPOISOMERASE II INHIBITORS LIMITED TO U251 CELLS?

An important feature of tumor derived cell lines is their individual collection of mutations, rendering their physiological balance between different pathways. Thus, the behavior of cell lines might be different when they get exposed to certain stimuli. To substantiate that the presented mechanisms of p53 transcriptional regulation have general validity, we analyzed the effect of doxorubicin treatment using additional cell lines, also harboring different p53 mutations, as well as wild type p53.

A431 cells, derived from an epidermoid carcinoma (p53 R273H) and the bladder carcinoma cell line 5637 (p53 R280T) displayed an up-regulation of p53 mRNA levels in response to doxorubicin. In both cases the transcription of p53 is induced even stronger than that of TAp73, the gene that served throughout the study as a positive control for the triggered DNA damage response (Figure 20A). In contrast to these results we observed that the osteosarcoma cell line U2OS, that was used as a representative of wt p53

expressing cells, responded to doxorubicin treatment with an invariant expression of p53 mRNA (Figure 20A). Similar data were obtained for the colon carcinoma derived cell line HCT116 that as well expresses wt p53 (data not shown).

According to the general knowledge of the pathways within the p53 network, we hypothesized that a negative feedback loop from p53, via the cyclin dependent kinase inhibitor p21 and the Retinoblastoma protein, to E2F1 explains the decreased response of p53 mRNA expression to doxorubicin treatment. Of note, due to p53 loss of function, this feedback loop is permanently silenced in cells expressing mutant p53. To investigate the impact of the mentioned feedback loop in U2OS cells, we used siRNA mediated knock-down of p21, to intercept the pathway. The treatment of U2OS cells with doxorubicin after silencing of p21 resulted in the same slight up-regulation of p53 mRNA levels, as it was observed upon control siRNA transfection (Figure 20B). This indicates that the mentioned negative feedback loop, from transcriptionally active p53 via p21 to E2F1, is not causing the diminished response of p53 mRNA expression to doxorubicin treatment.

The result, we obtained in U2OS cells upon doxorubicin treatment, reminded us of, what we have seen in U251 cells with idarubicin before, the levels of TAp73 increase, but there is almost no change in the p53 expression. This prompted us to investigate, whether the expression of the p53 natural antisense transcript WRAP53 might again be involved in the regulation of p53. Strikingly, this theory was approved, treatment of U2OS cells with doxorubicin leads as well to the up-regulation of WRAP53 1α (Figure 20C) and thereby resembles another example of inverse correlation between the expression of opposing transcripts from the *TP53* locus.

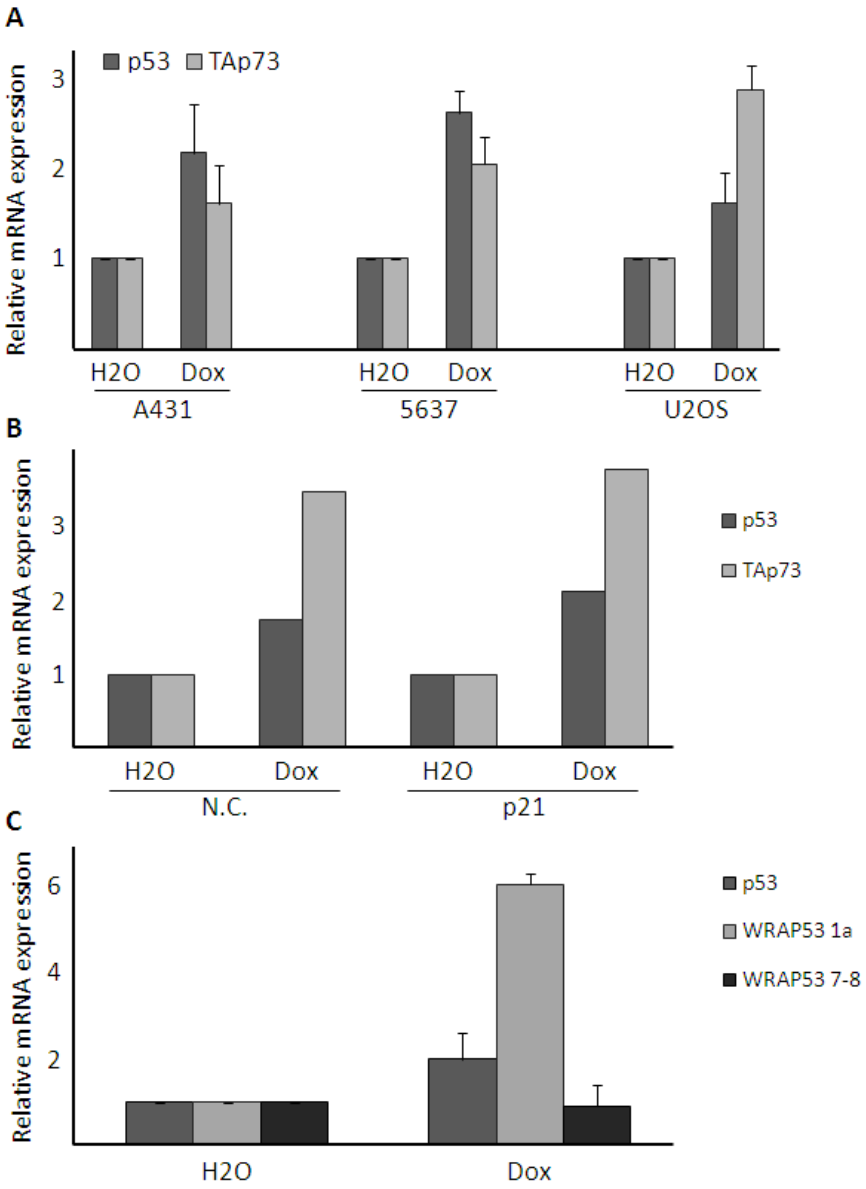


Figure 20: The discovered mechanisms of p53 regulation also apply to other cell lines
A: A431, 5637, and U2OS cells were treated with 500nM doxorubicin for 24 hours. **B:** U2OS cells were transfected with siRNAs as indicated for 48 hours. The last 24 hours the samples were additionally treated with 500nM doxorubicin. **C:** U2OS cells were treated with 500nM doxorubicin for 24 hours. **A-C:** Total RNA was isolated and subjected to reverse transcription. The levels of p53, TAp73, and WRAP53 were quantified relative to 36B4 using real-time PCR. Means and standard deviations of the experiments are diagramed.

Having elucidated important parts of mutant p53 expression regulation in response to different chemotherapeutics, the question remains whether these pathways also contribute to the general regulation of mutant p53 accumulation, as it is observed during tumor progression. The finding that E2F1, as well as TAp73 knock-down keeps the expression of p53 mRNA and protein levels more

or less constant in the absence of doxorubicin (4.2.1) pinpoints to the requirement of additional mechanisms regulating the expression of mutant p53.

4.7. A KINASE SCREEN DISCLOSES FURTHER CANDIDATES INVOLVED IN THE EXPRESSION REGULATION OF MUTANT P53 IN TUMOR DERIVED CELLS

Already 30 years ago, p53 was identified as a protein frequently accumulated in tumor cells and served as a diagnostic marker (Crawford *et al.*, 1981; DeLeo *et al.*, 1979; Rotter *et al.*, 1980). Until now, it is not clear why mutant p53 accumulates so strongly, whenever it is mutated. For many years, it was believed that the loss of p53 function goes along with low levels of Mdm2, the E3 ubiquitin ligase that is on the one hand a direct target gene of p53, but on the other hand its most important inducer of proteasomal degradation. This was a perfect explanation for the increased half-life of mutant p53 until Lang *et al.* (2004) and Olive *et al.* (2004) generated transgenic mouse models harboring various p53 hotspot mutations. Using these mouse models, they demonstrated that mutant p53 is specifically accumulated in the cells of advanced tumors, but remained at low levels in the surrounding tissue. These observations prompted us to investigate which pathways, imbalanced through tumor specific mutations, contribute to the expression regulation of p53.

The mutational spectrum in cancer cells mostly affects the pathways regulating cell cycle progression as well as DNA damage response, in order to keep a cell proliferating and alive. These pathways involve a series of constitutive phosphorylation events as to multiply the signal. Kinases, the enzymes that perform all these phosphorylations, are therefore central players and common targets of deregulation in the progression of tumor formation.

Investigating, whether these imbalanced pathways entail the accumulation of mutant p53, we performed a high content siRNA screen in 5637 cells. The so far known mutational spectrum of this bladder carcinoma derived cell line is

limited to the p53 mutation R280T and a nonsense mutation within the Rb gene. In addition to that, we found the cells to be siRNA transfected with a very high efficiency and perfectly shaped for single-cell based immunofluorescence analysis. The *Silencer* Human Kinase siRNA Library V3 targets 719 human kinases and kinase subunits with three individual siRNAs per gene, including validated siRNAs for more than half of the targets.

After 48 hours of siRNA transfection the cells were fixed and stained for single cell based immunofluorescence analysis. Hoechst 33342, as a nuclear stain, was used to define the individual nuclei of the cells. The average p53 staining in each of these nuclei was measured and used to calculate the mean expression of p53 upon each of the individual siRNA transfections. For the final hit determination Z-scores were calculated (Figure 21).

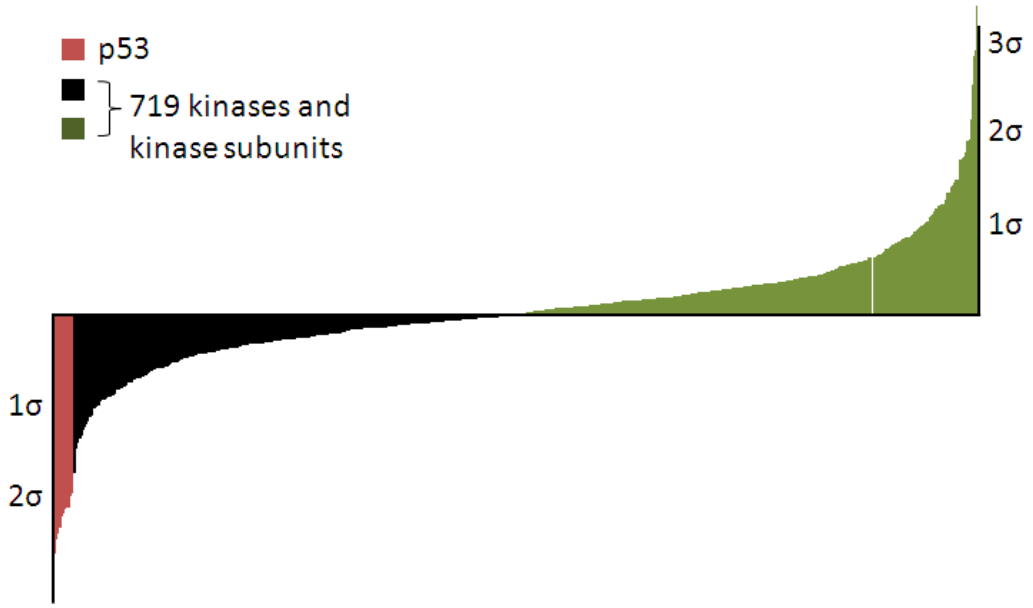


Figure 21: The influence of 719 human kinases on the expression of mutant p53 protein. 5637 cells were transfected with siRNAs targeting 719 human kinases and kinase subunits. Each target was silenced by three different siRNAs in individual wells. 48 hours after transfection the cells were fixed and stained using antibodies against p53. Hoechst 33342 was used to identify the individual nuclei within which the average expression levels of p53 were determined. Z-scores were assigned to each of the targeted kinases as a measure of p53 induction/ repression. For detailed description of the analysis please see 3.2.4. On each plate two wells were transfected with siRNAs targeting p53 itself as positive control for the down-regulation of mutant p53; the results of these controls are depicted in red.

The knock-down of 18 kinases revealed a Z-score lower than -1.1. These targets were considered as potential hits and analyzed in more detail. For 4 of these kinases it turned out that only one of the siRNAs had a striking effect, whereas the other two did not influence the expression of p53 at all. The chances that the effects of these kinases are caused by an off-target effect are very high and the targets were excluded from further analysis.

We assigned the remaining 14 hits to the pathways or cellular processes, where they were previously found to be involved in (Table 2).

Table 2: Hits from the screen.
The knock-down of the listed genes displayed a down-regulation of p53 in 5637 cells. Three siRNAs per gene were used, in column 3-5 the individual scores are listed and in column 6 the score of the average signal determined for the three siRNAs. The last column assigns the identified kinase to the pathway in which it is known to act.
PI5K: Phosphatidylinositol 5-kinase; PI3K: Phosphatidylinositol 3-kinase; mTOR: mammalian target of Rapamycin; PKC: Protein kinase C

	Kinase	Score A	Score B	Score C	Ø Score	Pathway
1	PIP5K1A	-1,32	-1,65	-1,75	-1,58	PI5K
2	PIK3C2G	-1,52	-1,58	-1,56	-1,55	PI3K → mTOR
3	RPS6KL1	-1,48	-1,56	-1,53	-1,52	PI3K → mTOR
4	PIM2	-1,28	-1,55	-1,64	-1,49	Cell proliferation, Meiosis
5	PIM3	-1,28	-1,08	-1,91	-1,43	Cell proliferation, Meiosis
6	PIP5K1C	-1,08	-1,49	-1,56	-1,38	PI5K
7	PGK2	-1,65	-0,65	-1,78	-1,36	Glycolysis, testis-specific
8	PINK1	-1,84	-1,76	-0,47	-1,35	Mitochondrial stress response
9	PIK3R3	-1,40	-1,14	-1,39	-1,31	PI3K → mTOR
10	PIK3CB	-1,20	-1,34	-1,25	-1,26	PI3K → mTOR
11	PIP5K2B	-0,73	-1,04	-2,02	-1,26	PI5K
12	PIP5K1B	-1,28	-0,99	-1,46	-1,24	PI5K
13	PKN2	-0,75	-1,34	-1,47	-1,19	PKC related, function unknown
14	RPS6KB2	-0,84	-0,84	-1,81	-1,16	PI3K → mTOR

Besides the two kinases PGK2 (Phosphoglycerate kinase 2) and PKN2 (Protein kinase N2) that are either very tissue specific or functionally unknown, three groups of kinases remained, as potential targets to be followed up on. First, the

PIM kinases, they are as well largely unknown in their function, but it became evident that two out of three paralogs appeared within the hit list. Second, the PI5K, they are represented by four members in the hit list, nevertheless, so far they are rather known to regulate cellular polarity and membrane trafficking, processes, in which the regulation of p53 expression would not be expected to happen. Third, the PI3K → mTOR pathway, it is represented by three members of the PI3K family and two members downstream of mTOR. Additionally, it should be mentioned that PINK1 (PTEN induced kinase 1) gets activated by members of the PI3K → mTOR pathway, even though its described function is so far restricted to the mitochondrial stress response in Parkinsons disease. Only very recently, it was observed by Morimoto *et al.* (2010) that the up-regulation of PINK1 expression in amyotrophic lateral sclerosis patients is positively correlated with the phosphorylation and stabilization of wt p53, suggesting that there is a link between the two pathways.

Taking all these information together, we decided to follow up on RPS6KL1 and –B2. These are two kinases that act quite far downstream in a pathway that is represented by 5 kinases within the hit list. In addition to this, there is one report claiming that some members of the large protein family of RPS6 kinases directly phosphorylate wild type p53, which was shown using an *in vitro* kinase assay (Cho *et al.*, 2005).

The S6 kinases were named according to their primary function of phosphorylating the ribosomal protein S6. The protein family consists of 10 members that can be grouped in three functionally more related subfamilies. First the RSKs (Ribosomal S6 kinases) consisting of RPS6KA1, RPS6KA2, RPS6KA3, and RPS6KA6, second the MSKs (Mitogen- and stress-activated kinases) with RPS6KA4 and -5, and third the S6Ks (S6 kinases) namely RPS6KB1 and RPS6KB2. Additionally, RPS6KC1 and RPS6KL1, as structurally related, but functionally mostly unknown kinases, belong to this family. Similar as described earlier for the E2Fs, also the RPS6 kinases fulfill

partially opposing activities and it can therefore not be expected that all 10 members of the protein family have an impact on the mutant p53 expression regulation.

4.7.1. VALIDATION OF THE IDENTIFIED HITS: RPS6KB2 AND RPS6KL1

RPS6KL1 and RPS6KB2 were identified in the performed kinase screen as potential regulators of mutant p53 expression in the tumor cell line 5637 by quantitative immunofluorescence microscopy. This is a very sensitive method that was on the one hand enabling us to detect kinases that have slight effects on the expression of mutant p53, but on the other hand also requires optimization until the effects can get validated by less sensitive methods like immunoblotting.

First, we tried to find another cell line, still harboring a p53 mutation, but expressing higher levels of the identified kinases, in order to observe whether S6 kinases have an even stronger impact on p53 levels in such a system. Nakamura *et al.* (2008) published the two glioma cell lines U251 and U373 to express detectable levels of the kinases RPS6KB1 and -2. Since RPS6KL1 is largely unknown and the available antibodies fail to specifically detect the protein, we could not optimize the cell line with respect to the expression level and activity of this kinase, which originally showed a stronger effect on the regulation of p53. In order to confirm that the knock-down of RPS6KB2, as well as RPS6KL1, contributes to the expression regulation of p53 in U251 cells we performed immunofluorescence analysis. The experiment was carried out the same way, as in the screen, but using U251 cells. This revealed that U251 cells are a good model system to analyze the impact of S6 kinases on the expression regulation of mutant p53. The impact of RPS6KL1 on mutant p53 levels was as well confirmed under these circumstances (Figure 22).

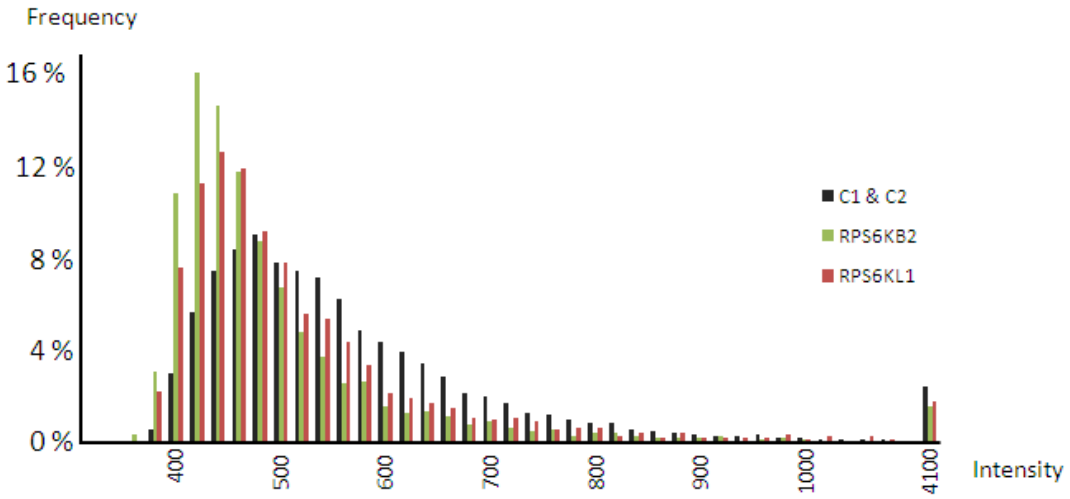


Figure 22: The knock-down of RPS6KB2 and RPS6KL1 decreases the expression levels of mutant p53 protein in U251 cells.
U251 cells were transfected with siRNAs against RPS6KB2 and RPS6KL1 for 48 hours. The fixed cells were stained for immunofluorescence analysis using antibodies recognizing total p53. Hoechst 33342 was used to identify the individual nuclei. The average expression of p53 per nucleus was determined; the data are diagramed in a histogram.

Taking into account that mutant p53 proteins in tumor cells have a much longer half-life than wild type p53, we further optimized the assay with respect to the duration of the knock-down. Using a double siRNA transfection protocol (3.2.1 LONG-TERM SIRNA TRANSFECTION), we confirmed the role of RPS6KB2 and RPS6KL1, as well as RPS6KB1, in the regulation of mutant p53 expression (Figure 23). The knock-down was performed for 5 days before the cells were harvested for immunoblot analysis. The structurally related kinase RPS6KA1 was used as an additional negative control, since it was found in the screen to keep the expression levels of p53 constant compared to non-targeting scrambled siRNAs.

The knockdown efficiency was monitored indirectly, by staining for the expression of Serine 235/236 phosphorylated S6, a well known target of the analyzed kinases RPS6KB1 and -2, due to the lack of specific antibodies recognizing the S6 kinases. This way, we observed that for RPS6KB1, as well as RPS6KB2 the first siRNAs mediated a stronger knock-down, compared to the second. This reflects the same pattern, as it is observed for the expression

of mutant p53. The knock-down of RPS6KL1 as well diminished the expression of mutant p53 to a large extent. Unfortunately, we could not monitor the knock-down efficiency of the protein, since there are neither specific antibodies available, nor a well characterized substrate that could be used for this analysis (Figure 23).

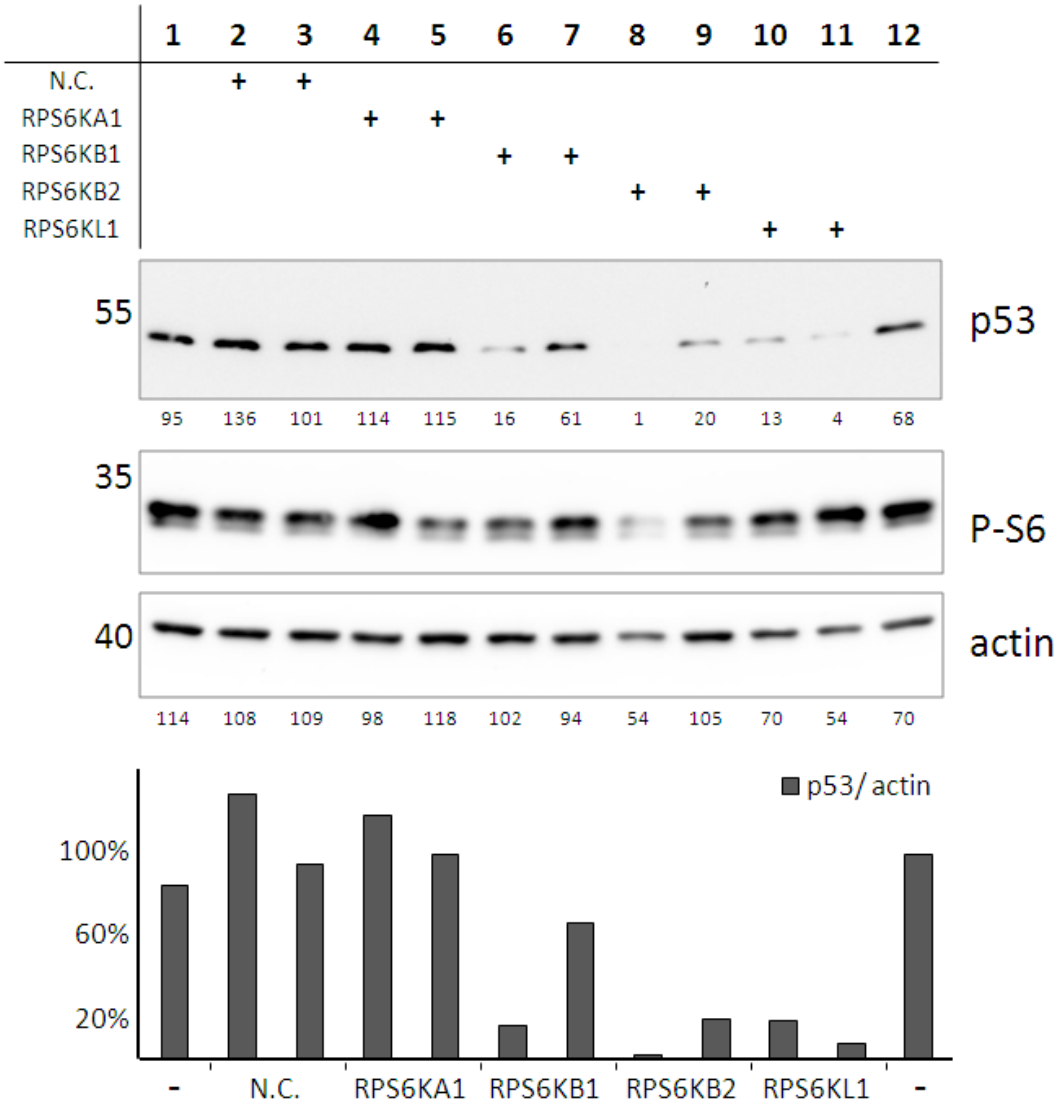


Figure 23: The S6 kinases RPS6KB2 and -B1, as well as RPS6KL1 contribute to the regulation of mutant p53 expression in tumor cells
U251 cells were double-transfected with siRNAs targeting four members of the RPS6 kinase family. Five days after the first transfection the cells were harvested and whole cell lysates were subjected to immunoblot analysis. The expression of p53, P(Ser 235/236)-S6, and actin was detected. The blots were quantified using LabImage 1D (lower part).

To test whether the knockdown of the identified kinases affects mutant p53 expression on the protein level, and not, as previously observed, on the transcriptional level, we isolated total RNA from cells upon siRNA mediated knockdown of the S6 kinases. This experiment clearly revealed that all three RPS6 kinases, that were shown to have an impact on mutant p53 expression, do not change its mRNA levels (Figure 24). This strongly argues, in line with previous observations, that the accumulation of mutant p53 during tumor progression happens on the protein level.

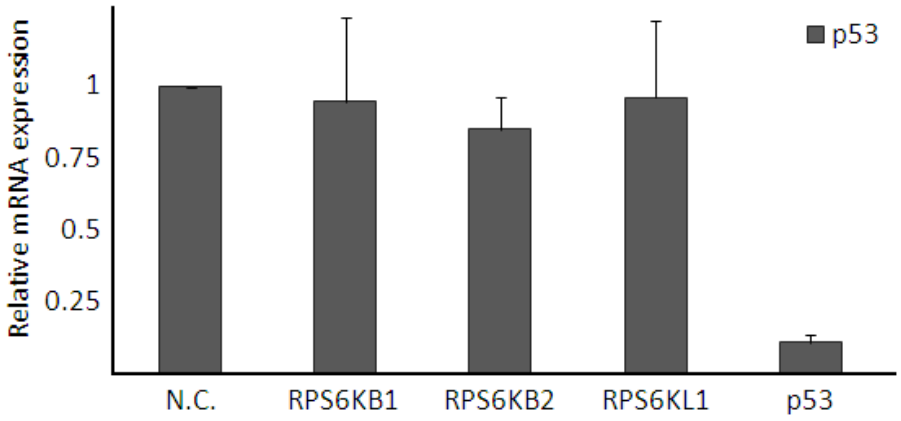


Figure 24: S6 kinases do not regulate the expression of p53 on the transcriptional level. U251 cells were siRNA transfected for 48 hours. Total RNA was isolated and quantified relative to 36B4 using real-time PCR. Means and standard deviations of four biological replicates were diagrammed.

Other than the depletion of S6 kinases, we also investigated, whether modulation of their activity would result in decreased expression of mutant p53. As described by Nobukuni *et al.* (2005) and Hidayat *et al.* (2003), inactivation of the kinases can be achieved by the withdrawal of FCS and the two amino acids Arginine and Lysine and can be reverted by 30min incubation with fresh medium containing FCS (Figure 25; P-S6 staining). The levels of mutant p53 decreased as well upon starvation and recovered after 30min of incubation in full medium supplemented with 10% FCS (Figure 25).

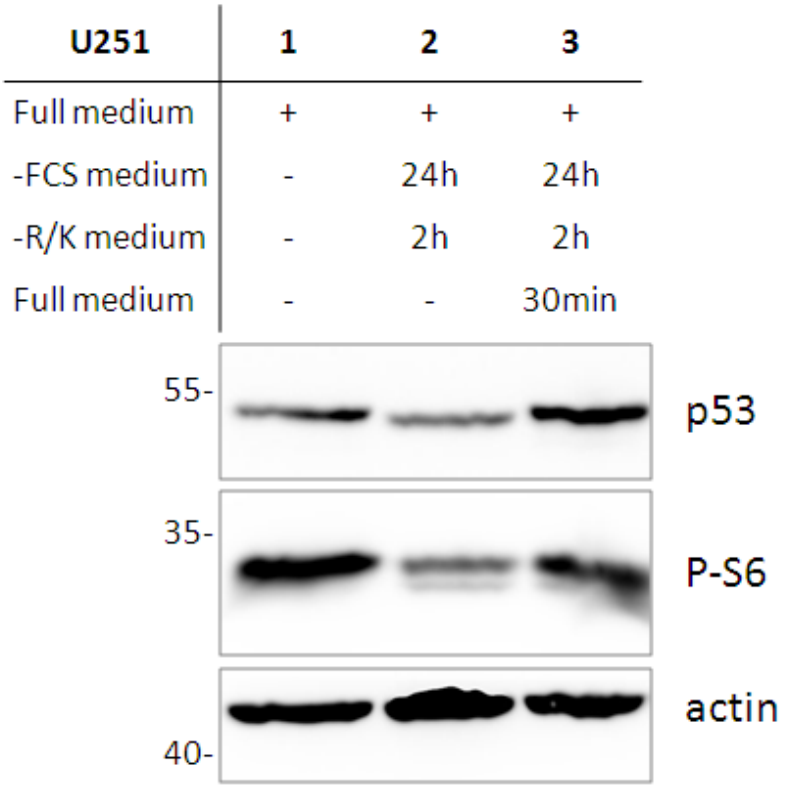


Figure 25: Serum starvation abolishes S6 kinase activity and leads to the down-regulation of mutant p53 expression.
U251 cells were seeded in 12 wells. After the cells attached the medium was changed, and the cells were kept in serum deprived medium. 24 hours later the medium was again changed to Arg/ Lys free medium without FCS, to further starve the cells. After 2 hours the cells were supplemented with fresh medium containing 10% FCS for 30 minutes. The cells were harvested and whole cell lysates were subjected to immunoblot analysis, using antibodies against p53 and P(Ser235/ 236)-S6. Actin staining was used as loading control.

This experiment argues that the protein level of p53 is regulated through the activity of S6 kinases and not simply through their abundance. Nevertheless, we don't know yet, whether this is a direct activity of the S6 kinases, as it was claimed by Cho *et al.* (2005) or, whether it still might be indirectly affected by other intermediates.

DISCUSSION

It is characteristic for most tumor cells that they proliferate rapidly in an uncontrolled fashion. The mechanistic principle behind most chemotherapeutic agents takes advantage of this feature, by preferentially inducing apoptosis in rapidly dividing cells. In most cases this is achieved through the induction of a DNA damage response, going along with the stabilization and activation of the tumor suppressor p53. This, depending on the severity and the nature of the damage, leads to cell cycle arrest or apoptosis. Sequence analysis of thousands of tumor samples revealed that p53 is mutated in more than 50% of all human solid tumors. In contrast to other tumor suppressors, p53 is mainly inactivated through single point mutations within the central DNA binding region of the protein. This mutation leads not only to the loss of tumor suppressor activity, but at the time confers oncogenic properties to the expressed gene product. According to different studies, this comprises increased chemoresistance, as well as a higher frequency of metastasis formation. It has previously been shown that knock-in mice, harboring one of the p53 hotspot mutations, differ from p53 null mice with respect to the frequency of metastasis formation (Lang *et al.*, 2004; Olive *et al.*, 2004). Histochemical analysis of the tumors revealed that mutant p53 was specifically over-expressed in high grade tumors, whereas its expression was at the background level in the surrounding tissue (Terzian *et al.*, 2008). The molecular signals that cause this accumulation are not known so far, nevertheless, studies of various groups indicate that Mdm2, as well as other E3 ubiquitin ligases like Cop1, ARF-BP1, and CHIP (C terminus of Hsc70-interacting protein) become inactivated due to the deregulation of tumor specific pathways (Lukashchuk and Vousden, 2007).

Within this study we identified three different levels of mutant p53 expression regulation. Due to different stimuli, either DNA damage responsive transcription factors were identified as central players of mutant p53 expression regulation, or the natural antisense transcript of p53, recently identified by Mahmoudi *et al.* (2009), or kinases involved in the PI3 kinase → mTOR pathway.

5.1. TRANSCRIPTIONAL REGULATION OF MUTANT P53 EXPRESSION IN RESPONSE TO CHEMOTHERAPEUTIC TREATMENT

In response to genotoxic stress, conferred by chemotherapeutic agents, like the three anthracyclines daunorubicin, doxorubicin, and epirubicin, the expression of mutant p53 in tumor cells is augmented due to increased transcription (Figure 15). Within the performed experiments, we have shown that the transcription factors HOXA5, RREB1, TP73, and E2F1 are all necessary for the observed up-regulation in response to treatment with the named anthracyclines, while they have no impact on the basal transcriptional expression regulation of mutant p53 in the absence of DNA damage (Figure 9, Figure 11, data not shown). The transcription factor E2F1 was further shown, to directly bind to a, so far not described binding site within the p53 promoter (Figure 13). Interestingly, ChIP analysis revealed no differences in E2F1 binding to the promoter of p53 upon doxorubicin treatment. Nevertheless, for the well known DNA damage responsive E2F1 target gene TAp73, we as well observed invariant E2F1 binding regardless of doxorubicin treatment. These data indicate that the E2F1 that we recover in our ChIP experiments is bound to the promoter of its target genes independently of their transcriptional activation through chemotherapeutic treatment. As Pediconi *et al.* (2003) showed, this could most probably be explained by posttranslational modifications of E2F1 that are necessary to promote active transcription. To further look into this matter, we would need to establish an antibody specific to acetylated E2F1 and thereby restrict the analysis to the active form of the transcription factor. Additionally, it could be tested whether RNA polymerase II in its active form

(Serine 2 phosphorylated) is more abundant around the transcriptional start site, in response to doxorubicin treatment, compared to untreated cells in a re-ChIP experiment, where the eluate of an E2F1 ChIP is used as starting material.

5.2. WRAP53 - A NATURAL ANTISENSE TRANSCRIPT OF P53 PREVENTS MRNA MATURATION

Natural antisense transcripts (NATs) are a class of non-coding RNAs that were shown to have an impact on the expression regulation of genes. It is estimated that in mammals about 70% of all transcripts have antisense partners that can alter the expression of the sense genes (Katayama *et al.*, 2005). For example, in about 70% of tumor samples, the antisense transcript of the tumor suppressor p15 (p15AS) is highly accumulated, whereas the tumor suppressor itself is silenced; in normal cells this is observed vice versa (Yu *et al.*, 2008). NATs have been proposed to regulate the expression of their target genes at several levels, including transcription, messenger RNA processing, splicing, stability, cellular transport, and translation (Lapidot and Pilpel, 2006). To understand the mechanisms of NAT regulation, Chen *et al.* (2005b) analyzed the expression profiles of sense and corresponding antisense transcripts on a genome-wide scale. This revealed that sense-antisense pairs tend to be co-expressed or inversely correlated more frequently, than would be statistically expected. Furthermore, they found that most of these pairs and their expression regulation is evolutionary conserved.

Within this study we observed that three topoisomerase II inhibitors, daunorubicin, doxorubicin, and epirubicin, induced the p53 mRNA expression in an E2F1 and TAp73 dependent manner. Two other topoisomerase II inhibitors, idarubicin and etoposide, were found to evoke a similar DNA damage response leading to the activation of the transcription factors E2F1 and TAp73, but, to our surprise, did not increase p53 mRNA expression levels (Figure 14, Figure 15). Over and above, we even found that etoposide treatment slightly decreased the

mRNA levels of p53 in U251 cells. Further analysis revealed that idarubicin and etoposide, but none of the other three analyzed topoisomerase II inhibitors, induced the expression of WRAP53-1α, a NAT encoded on chromosome 17 opposite of p53 (Figure 18). To elucidate the mechanism behind these controversial regulatory activities of highly related chemotherapeutic drugs, we on the one hand analyzed the pre-mRNA expression of p53 and on the other hand used siRNA mediated knock-down of WRAP53. Within the first experiment we found that the E2F1 and TAp73 dependent transcriptional activation of p53 occurred upon idarubicin treatment the same way, as upon treatment with any of the other anthracyclines (Figure 16, data not shown). This indicates that the maturation of p53 mRNA is affected upon idarubicin treatment, possibly mediated through the elevated expression of WRAP53-1α. The knock-down experiment of WRAP53 revealed that the used siRNAs targeted the mRNAs of most WRAP53 isoforms efficiently. Nevertheless, we also observed that transcripts, that either lack exon 2 and 8 or escape the knock-down by other means, are specifically induced in response to idarubicin treatment (Figure 19). Since siRNAs are believed to mainly act in the cytoplasm, absent nuclear export of the transcript should be considered as a possible way of inefficient knock-down of mRNAs, containing the siRNA target sequence. Additionally to the data we obtained in mutant p53 expressing cell lines, we observed that doxorubicin treatment strongly induces the expression of WRAP53-1α in the wt p53 expressing cell lines U2OS and HCT116 (Figure 20 C, data not shown), while the mRNA levels of p53 remained unaffected from the treatment (Figure 20 A).

When interpreting all these results from the tumor cells point of view: It appears advantageous for wt p53 expressing cells to circumvent the accumulation of p53 in response to DNA damage, in order to escape the induction of apoptosis. Elevated expression of WRAP53-1α could serve as one mechanism to achieve this. For mutant p53 expressing cells, on the contrary, this induction of

WRAP53-1α in response to doxorubicin is needless if not disadvantageous, since they induce a protein with oncogenic activities, leading to tumor progression and chemoresistance, rather than apoptosis. Nevertheless, this still owes a rational for the WRAP53-1α induction in response to idarubicin and etoposide in mutant p53 expressing cells.

The following NAT related mechanisms could serve to explain our data:

DNA METHYLATION AND HETEROCHROMATIN FORMATION:

Antisense-induced DNA methylation and silencing was described to play an important role for example in thalassaemia, where the haemoglobin 2 gene is efficiently silenced through the expression of an antisense transcript (Tufarelli *et al.*, 2003). Several studies have indicated that this is not mediated through the formation of RNA duplexes, but through the modification of chromatin structure or DNA methylation patterns (Lee and Lu, 1999; Wutz *et al.*, 1997). According to studies from Tufarelli *et al.* (2003) and Yu *et al.* (2008) antisense induced DNA methylation leading to efficient silencing of target genes should be considered a widespread mechanism of tumor suppressor silencing. Nevertheless, this concept stands in conflict with our observation that idarubicin treatment entails the same induction of p53 pre-mRNA synthesis, as it is observed in response to doxorubicin.

RNA MASKING:

Sense-antisense RNA duplex formation masks cis-regulatory elements within the p53 mRNA hindering proteins involved in polyadenylation, splicing, or nuclear export to bind to the pre-mRNA (Hastings *et al.*, 1997). This would in turn lead to less efficient maturation and thereby explain the observed phenotype of increased pre-mRNA levels that do not affect the overall mRNA levels of p53. Furthermore, this would explain the inefficient knock-down that was observed for WRAP53-1α, since the p53 mRNA is only exported into the cytoplasm after successful polyadenylation and splicing.

One central question that remains is how the inversely correlated transcription of p53 and WRAP53-1α is regulated. Collision of two RNA polymerase II enzymes is discussed as a possible mechanism of inverse regulation of antisense gene expression (Crampton *et al.*, 2006; Prescott and Proudfoot, 2002). Collision of RNA polymerase II with stalled topoisomerase II is not described so far. Nevertheless, we would like to suggest this to explain the generation of WRAP53-1α transcripts lacking exon 2. If, due to the collision, a shortened transcript of WRAP53 is released, it would resemble a natural antisense transcript to p53 and might then get stabilized in the nucleus through its interaction with the pre-mRNA or the DNA of the sense gene p53. This hypothesis is summarized in Figure 26.

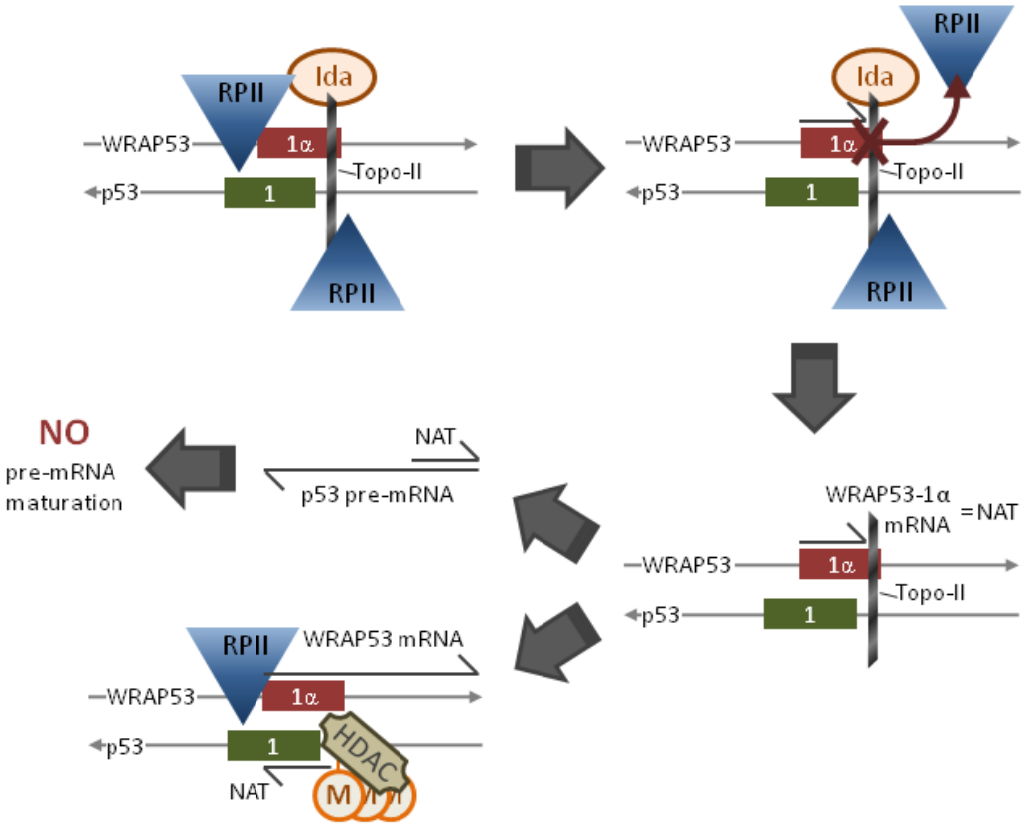


Figure 26: Model of the expression regulation within the *TP53* genomic locus.
Topoisomerase II (Topo-II) is stalled on the DNA through idarubicin (Ida). Thereby it prevents RNA polymerase II (RP-II) from binding to the p53 promoter, as well as elongation of WRAP53 transcription due to collision. The short WRAP53-1α mRNA acts as natural antisense transcript (NAT) on p53. This NAT could either bind to p53 pre-mRNA, preventing its further maturation, or NAT-DNA interactions could feature promoter methylation and heterochromatin formation, leading to the silencing of p53.

To experimentally elucidate the underlying mechanisms it would be important to first identify the WRAP53-1α transcript that is accumulating in response to treatment with these chemotherapeutic drugs and its cellular localization. Subcellular fractionation prior to RNA isolation would help to get insights into the localization of idrubicin induced WRAP53-1α transcripts. Additionally, RACE-PCR could be used to identify a potential truncated WRAP53-1α transcript. To investigate whether topoisomerases, inhibited through idarubicin or etoposide, get stalled within the *TP53* genomic locus at positions different from those where daunorubicin, doxorubicin, and epirubicin inhibited topoisomerases get stalled, ChIP technology could be used (Cashman and Kellogg, 2004). Furthermore, it could be tested whether HDAC inhibition would lead to similar impacts of all five topoisomerase II inhibitors on the p53 transcription and thereby rescue the defect in pre-mRNA maturation upon treatment with idarubicin or etoposide.

5.3. KINASES REGULATING MUTANT P53 EXPRESSION IN TUMOR CELLS

We found that the three kinases RPS6KL1, RPS6KB2, and RPSKB1 contribute to the expression regulation of mutant p53 in tumor cells (Figure 21, Figure 22, and Figure 23). We identified these kinases in a microscopy based siRNA screen and confirmed their roles in the performed follow-up experiments. The knock-down of the named kinases decreased the expression levels of mutant p53 in tumor cells that otherwise express the protein, without further stimuli, at high levels. Our data additionally suggest that the kinase activities of RPS6KB1 and -B2 are necessary for their impact on p53, rather than their simple abundance (Figure 25). For RPS6KL1 this can currently not be analyzed, since it is neither known, whether this protein exhibits intrinsic kinase activity, nor ways to alter it.

According to the data from Cho *et al.* (2005), some kinases of the RPS6K family directly phosphorylate p53 at Serine 15. Additionally, Melnikova *et al.* (2003) observed mutant p53 to be constitutively phosphorylated at Ser15 in

UV-induced skin tumor cells and correlated this with decreased susceptibility to Mdm2-mediated degradation. Taking these data together, it could be hypothesized that the knock-down or inactivation of the S6 kinases decreases the levels of mutant p53 phosphorylation, which in turn leads to the destabilization of the protein. Nevertheless, therefore the overall levels of posttranslationally modified mutant p53 would need to be high, even in the absence of DNA damage, a prerequisite that we did not observe in our studies (Figure 7A, B). Hence, we hypothesize that the mechanism, underlying this regulation of transformation induced accumulation of mutant p53, does not depend on posttranslational modifications and their impact on the susceptibility of p53 towards Mdm2, or other ubiquitin ligases. To further exclude this as a possible mechanism, we would like to investigate, whether ectopically over-expressed mutant p53 is regulated by the identified kinases. And, whether an additional mutation of known phosphorylation sites within p53 (Serine or Threonine to Alanine) would abolish this effect.

The presented results could also be explained with the help of reports published by Fu and Benchimol (1997) and Takagi *et al.* (2005), finding that p53 expression is regulated on the translational level in response to DNA damage. In the work of Fu and Benchimol (1997), the 3'UTR of p53 itself was identified to posses inhibitory activity on p53 translation. They further showed that γ-irradiation abolishes this translational inhibition. Whereas, Takagi *et al.* (2005) claims that irradiation leads to an increased binding of RPL26 to the 5'UTR of p53, which in turn promotes p53 mRNA association with heavy polysomes, augmenting the rate of its translation. Even though these data were obtained in wild type p53 expressing cells, in response to irradiation, we aimed to test whether RPL26 contributes to the regulation of mutant p53 expression in the absence of DNA damage. But, upon siRNA mediated knock-down of RPL26 in U251 cells neither the posttranslational modification of p53, nor its overall expression levels were affected (data not shown). This argues against

translational regulation of mutant p53 expression mediated by RPL26. Nevertheless, to determine the impact of translational regulation on mutant p53 accumulation mediated through the identified S6 kinases, it should be tested whether the amount of p53 mRNA associated with heavy polysomes changes upon the knock-down of the kinases.

The mTOR pathway was shown to contain sensors for nutrient and amino acid availability (Kim, 2009). Cells that lack essential amino acids often use autophagy to degrade cellular proteins, thereby increasing the pool of amino acids that can be used to translate new proteins of greater importance for their survival (Jung *et al.*, 2010). We would like to test, whether the highly accumulated mutant p53 protein might get degraded through autophagy upon RPS6K knock-down or starvation, since it was previously shown that Arginine deprivation, which was used in our experiments to inhibit mTOR signaling, induces autophagy (Savaraj *et al.*, 2010). Along that line, there are several ways to induce or block autophagy independently of mTOR that could be used to analyze the impact of this degradative pathway within the regulatory network of mutant p53 expression. According to Munafo and Colombo (2001), autophagy is efficiently blocked through treatment with 3-methyladenine or N-ethylmaleimide (NEM), while the incubation with the microtubule depolymerizing agent vinblastine could be used to accumulate autophagic vacuoles, by preventing their degradation. Furthermore, the knock-down of Belcin1, as a central regulator of autophagy could be used, to more specifically analyze the impact of this pathway on mutant p53 expression regulation (Liang *et al.*, 1999). Microscopic analysis of mutant p53 expression upon modulation of autophagy could be used to test, whether this pathway is involved in mutant p53 expression regulation in tumor cells. If this hypothesis turns out to be true, we will not have identified one of the tumor specific pathways that lead to the accumulation of mutant p53 at the first place. Nevertheless, this finding could help to decrease the expression levels of the oncogenic mutant p53 protein in

cancer cells and it should be tested, whether the induction of autophagy could be used to prevent tumor progression and metastasis formation.

5.3.1. METASTASIS FORMATION AS MUTANT P53 GAIN OF FUNCTION

It can be hypothesized that the activity of the mTOR pathway kinases is decreased in areas of the tumor that lack sufficient nutrient supply and elevated in the outer cells. According to the data we have obtained, this would result in high levels of accumulated mutant p53 in cells at the outer rim of the tumor cell mass and in close proximity to blood vessels. The fact that exactly these cells are the ones that detach from a primary tumor to form new metastases, pinpoints to the importance of finding ways to actively suppress the oncogenic gain of function of accumulated mutant p53. Rapamycin (Rapamune®), a small molecule that was originally isolated from *Streptomyces hygroscopicus* is FDA approved as immunosuppressant. This molecule, which can efficiently inhibit the mTOR pathway, is recently more and more described to have a tumor protective function (Guertin and Sabatini, 2005; Sudarsanam and Johnson, 2010). In many tumors the prognosis, especially in terms of tumor proliferation and metastasis formation, seems to be correlated with the activity of the mTOR pathway (Zhou and Huang, 2010). We believe that the expression of mutant p53 could be a mechanistic explanation for this observed correlation. To investigate the *in vivo* role of mutant p53 accumulation, sections of larger tumors, harboring a p53 point mutation, could be histochemically analyzed, in order to test whether the expression levels of mutant p53 are indeed higher at the outer rim of the tumor cell mass and whether they decrease in response to treatment with rapamycin.

5.3.2. CHEMORESISTANCE AS MUTANT P53 GAIN OF FUNCTION

One aspect that should be tested as a link between mutant p53 gain of function and the use of anthracyclines for chemotherapeutic treatment is the expression

of the multi-drug resistance gene 1 (MDR1). It was on the one hand shown by several groups that in malignancies, expressing high levels of mutant p53 protein, chemoresistance is often conferred through transcriptional activation of MDR1 (Blandino *et al.*, 1999; Brosh and Rotter, 2009; Bush and Li, 2002). It was on the other hand clinically observed that doxorubicin treatment often leads to multi-drug resistance, going along with increased MDR1 levels, as a side effect. Thereby it was further observed that idarubicin, despite its structural homology to the other anthracyclines is the only representative of its kind that lacks this side effect (Hargrave *et al.*, 1995; Lotfi *et al.*, 2002). These observations do not only reflect another example of different phenotypes conferred by the structurally almost identical anthracyclines doxorubicin and idarubicin. Rather, correlated with our data, it can be hypothesized that the clinically observed chemoresistance upon doxorubicin treatment is mediated by an accumulation of mutant p53 in the cells, conferred by increased expression of MDR1. Whereas idarubicin lacks the ability to induce this accumulation and thereby does not exhibit MDR1 over-expression and the observed side effect of chemoresistance.

5.4. WHICH CONSEQUENCES CAN BE DRAWN FROM THESE FINDINGS

It should be the aim of mutant p53 research to elucidate the mechanisms of its accumulation and to get more insights into the cellular concepts underlying the oncogenic gain of function. Within this study we obtained data indicating that the expression of mutant p53 is regulated on different levels depending on the stimuli that cause its accumulation. Bearing in mind the disadvantageous side effects of mutant p53 accumulation that were published by a number of groups (Barlev *et al.*, 2001; Di Agostino *et al.*, 2006; Lang *et al.*, 2004; Muller *et al.*, 2009; Strano *et al.*, 2007) it should be as well aimed to prevent this accumulation, or at least to decrease the expression levels.

We observed that the accumulation of mutant p53 is increased upon topoisomerase II inhibitor treatment. We further demonstrated that the

transcription of p53 is activated in response to this treatment, which is inversely regulated to the expression of the natural antisense transcript WRAP53. Therapeutically it should be considered to use idarubicin or etoposide more widely in the tumors expressing mutant p53. It could also be tested whether the transcription of WRAP53-1α can exogenously be stimulated to prevent the accumulation of mutant p53 in response to one of the other topoisomerase II inhibiting drugs, possibly through a combinational treatment with idarubicin or etoposide.

Regarding the accumulation of mutant p53 that occurs during cellular transformation, we found that inhibition of ribosomal S6 kinase activity decreases the expression of mutant p53 in the used cell lines. It was furthermore recently shown that mTOR plays a critical role in the regulation of tumor cell motility and cancer metastasis. It would now be important to analyze whether the mTOR activity in tumor cells promotes tumor progression through the oncogenic activities gained by p53 through its point mutation. Therapeutically, it should then be tested whether the metastatic gain of function of mutant p53 can be abolished by treatment with rapamycin.

SUMMARY

The tumor suppressor p53 in its mutant form was previously shown to massively accumulate in tumor cells. Furthermore, enhanced tumor progression, as well as chemoresistance were associated with its expression. Within this study, we observed that chemotherapeutic treatment with some, but not all topoisomerase II inhibitors, currently used in the clinics, leads to a further up-regulation of mutant p53 expression and thus might favor unwanted tumor progression of tumor cells that escape the apoptosis induction at the first place. The network to regulate the expression of mutant p53 includes different mechanisms in response to various stimuli. The mediators range from transcription factors, over non-coding RNAs, to kinases.

All topoisomerase II inhibitors that we tested within our study augmented mutant p53 transcription. We showed that this was mediated by several transcription factors, including E2F1 and its target gene TAp73, that itself is known to exhibit activities similar to wt p53. While it was previously shown that TAp73 binds to a responsive element with the p53 promoter we observed here for the first time that E2F1 also binds directly to the p53 promoter in close proximity to the transcriptional start site. This was first found using *in silico* methods and confirmed by chromatin immunoprecipitations.

Nowadays, non-coding RNAs are recognized as another level of gene expression regulation. Recently, it was identified that within the *TP53* genomic locus, a natural antisense transcript is encoded, partially overlapping with exon 1 of the p53 mRNA. We observed that idarubicin and etoposide, but none of the other topoisomerase II inhibitors, strongly induced the expression of this antisense transcript, WRAP53. Furthermore, it became evident that this

expression is inversely correlated with proper pre-mRNA maturation of p53. Therefore, we hypothesize that the expression of this natural antisense transcript efficiently inhibits p53 mRNA maturation, possibly through RNA masking. We further hypothesize that the inversely correlated expression of sense and antisense transcripts might be caused by the collision of RNA polymerase II with idarubicin- or etoposide-inhibited, stalled topoisomerase II.

The accumulation of mutant p53, as it is observed during tumor progression, seems to be mostly regulated on the protein level, where we identified the ribosomal S6 kinases to be involved in. We found that the kinase activity of RPS6KB2 is necessary, to regulate the amounts of mutant p53 protein, as it was determined by serum and amino acid starvation. The mechanistic details that form the basis of this regulation were not determined, but we would like to suggest several hypotheses to be investigated. While our data can be explained by translational defects that the knock-down or inhibition of RPS6KB2 might cause, we favor the model that the induction of autophagy in response to mTOR pathway deregulation causes an enhanced degradation of mutant p53. A role of direct phosphorylation of mutant p53 through RPS6KB2 can also not be excluded.

In conclusion, we found that tumor cells accumulate mutant p53 protein through the activity of kinases that transduce mTOR signaling. Surprisingly, some chemotherapeutics further enhance mutant p53 levels through an entirely different mechanism, i.e. the regulation of p53 sense and antisense transcription.

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CURRICULUM VITAE

PERSONAL INFORMATION

	Monika Bug
Adress	Am Hasengraben 5 37075 Göttingen Germany
Email	mbug@gwdg.de
Date of Birth	September, 12th 1982
Nationality	German

EDUCATION

	Georg August University Göttingen, Germany
2006 - 2010	PhD in the Department of Molecular Oncology Thesis: “The accumulation of mutant p53 in human cancer cells”
	Georg August University Göttingen, Germany
2005 - 2006	Molecular Biology (MSc/PhD program – Max-Planck International Research School)
	University of Lübeck, Germany
2002 - 2005	Molecular Life Science (BSc) Thesis in the Department of Biology: “Characterization of the cytosolic protein p97 binding to the membrane of rough endoplasmic reticulum during retrograde transport”

TEACHING

	Tutor of Lectures
2007 - 2010	“Apoptosis and Cancer” within the Max-Planck International Research School MSc/PhD Program “Molecular Biology”

Supervisor of Labrotations

2008 - 2009 Golnaz Aghazadeh: Master-student in “Molecular Medicine”, University of Göttingen
Anke Hellrung: Master-student in “Biomedizin”, University of Würzburg

Tutor of Advanced Methods Courses

2008 - 2009 “Assessing promoter activity by luciferase assays” within the Max-Planck International Research School MSc/PhD Program “Molecular Biology”

HONORS AND AWARDS

2005 - 2006 Stipend Max-Planck International Research School

2004 Student exchange scholarship at the University of New Mexico

2003 - 2006 Stiftung der deutschen Wirtschaft (Foundation of German Economy)

CONFERENCES

2009 First AACR International Conference on Frontiers in Basic Cancer Research, Boston, MA, USA (poster)

2007 PhD Symposium “Horizons in Molecular Biology” Göttingen, Germany (poster)

EXTRACURRICULAR ACTIVITIES

Student representative

2007 - 2009 Max-Planck International Research School MSc/PhD Program “Molecular Biology”, Georg August University Göttingen, Germany

PhD representative

2007 - 2009 Göttingen Center for Molecular Biosciences (GZMB), Georg August University Göttingen, Germany