

The Department of Microbiology Tumor and Cell Biology,
Karolinska Institutet, Stockholm, Sweden

**MOLECULAR MECHANISMS OF
GROWTH SUPPRESSION BY
PHARMACOLOGICALLY ACTIVATED p53**

Elisabeth Hedström



**Karolinska
Institutet**

Stockholm 2009

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by

Cover illustration by Patricia Erica Candia-Arraya. “Assembling the pieces”

© Elisabeth Hedström, 2009

ISBN 978-91-7409-482-4

To Alex and my Family

ABSTRACT

The tumor suppressor p53 is a transcription factor that is crucial for protecting cells from cancer development. The importance of p53 tumor suppression function is highlighted by the fact that the p53 pathway is inactivated in most, if not all cancers. Mutation of the p53 gene occurs in about 50% of all tumors, whereas in the tumors which retain wild-type p53, the function of p53 is abolished due to deregulation of the p53 pathway. Due to the potency of p53 in suppressing tumors, p53 is considered to be an attractive therapeutic target. This is further strengthened by the *in vivo* mouse models which demonstrated regression of already established tumors upon reinstatement of p53.

The aims of this thesis were to identify new p53 reactivating compounds and to investigate the mechanisms of action of the previously identified p53-reactivating molecule RITA. The NCI library of low molecular weight compounds was screened for molecules that suppressed cell growth in a p53-dependent manner. We identified the small molecule MITA, which induces p53-dependent cell death in a variety of human tumor cells. The p53/MDM2 interaction is blocked by MITA which results in accumulation of p53 in cells. The expression of p53 target genes *MDM2*, *BAX*, *PUMA* and *GADD45* is induced upon activation of p53 by MITA. Importantly, MITA does not induce p53 or its target genes in normal human diploid fibroblasts (NHDF), which correlates with the absence of growth suppression.

The low molecular weight compound RITA was previously identified in a cell-based screen. RITA has been shown to bind p53 and subsequently inhibit its interaction with MDM2. RITA induces p53-dependent apoptosis in cancer cells and *in vivo* in human tumor xenografts in mice. In the studies presented in this thesis we further elucidated the mechanisms of RITA action. Oligonucleotide microarray analysis of gene expression profiles revealed that RITA targets p53 with high specificity and that it mainly induces pro-apoptotic targets of p53. In line with these results, p53 wild-type expressing cell lines of different origin revealed a predominant apoptotic response. We demonstrate that MDM2 released from p53 by RITA promotes degradation of p21 and the p53 cofactor hnRNP K, required for p21 transcription. This leads to downregulation of p21 on both transcriptional and protein level. Consequently, MDM2 acts as a switch between growth arrest and apoptosis upon p53 activation.

The preferential induction of apoptotic response by RITA is further facilitated by the targeting of the p53 regulator HDMX. RITA inhibits the p53/HDMX interaction and induces p53-dependent downregulation of HDMX on protein and on mRNA level. This ensures a robust activation of p53 leading to induction of apoptosis.

Additionally, a new cellular target of RITA was identified, namely the redox protein TrxR1. We demonstrated that RITA binds purified TrxR1 protein directly and inhibits its activity *in vitro* and in cells. Notably, the inhibition of TrxR by RITA appeared to be p53-dependent and correlated with the induction of a covalently linked TrxR1 dimer and induction of ROS. We believe that inhibition of TrxR1 in a tumor-dependent and p53-dependent manner might contribute to the tumor-specific cell killing properties of RITA.

In conclusion we believe that compounds such as RITA and MITA may not only be used as lead compounds for anti-cancer therapy, but may also be useful tools to study of p53 functional activity.

LIST OF PUBLICATIONS

This thesis is based on the following papers referred to in the text by their Roman numerals:

- I. **Elisabeth Hedström**, Natalia Issaeva, Martin Enge, and Galina Selivanova
Tumor-specific induction of apoptosis by a p53-reactivating compound
Experimental Cell Research, 2009 **315**: 451-461
- II. Martin Enge, Wenjie Bao, **Elisabeth Hedström**, Stephen P. Jackson, Abdeladim Moumen, and Galina Selivanova
MDM2-dependent down-regulation of p21 and hnRNPK provides a molecular switch between apoptosis and cell cycle arrest induced by pharmacologically reactivated p53
Cancer Cell, 2009 **15**: 171-183
- III. **Elisabeth Hedström**, Clemens Spinnler, and Galina Selivanova
Pharmacologically activated p53 downregulates HDMX gene transcription and induces HDMX protein degradation
Manuscript
- IV. **Elisabeth Hedström**, Sofi Eriksson, Joanna Zawacka-Pankau, Elias Arnér, and Galina Selivanova
p53-dependent inhibition of TrxR1 contributes to the tumor-specific induction of apoptosis by RITA
Manuscript

CONTENTS

INTRODUCTION	1
Cancer	1
p53.....	2
Regulation of p53.....	7
The functions of p53	7
Pharmacological reactivation of p53	10
The Thioredoxin system and redox regulation.....	12
Thioredoxin.....	12
Thioredoxinreductase	13
p53 and the thioredoxin system.....	14
AIMS OF THE THESIS	15
RESULTS AND CONCLUSIONS	16
Paper I	16
Paper II	17
Paper III	19
Paper IV.....	25
DISCUSSION AND FUTURE PERSPECTIVES	23
ACKNOWLEDGEMENTS	25
REFERENCES	27

LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
ALDH-4	Aldehyde dehydrogenase-4
ATM	Ataxiatelangiectasia mutated
BER	Base excision repair
CDK	Cyclin dependent kinases
Chk2	Checkpoint kinase 2
DNA-PK	DNA-dependent protein kinase
DSB	Double strand break
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FCS	Fluorescence correlation spectroscopy
FDXR	Ferredoxin reductase
GADD45	Growth arrest and DNA-damage inducible gene 45
GPX	Glutathione peroxidase
GR	Glutathione reductase
HAUSP	Herpes virus-associated ubiquitin-specific protease
HIPK2	Homeodomain-interacting protein kinase 2
HPV	Human papilloma virus
HR	Homologous recombination
HSA	Human serum albumin
HU	Hydroxyurea
MDM2	Mouse double minute 2
NER	Nucleotide excision repair
NHDF	Normal human diploid fibroblast
qPCR	Quantitative real-time PCR
Rb	Retinoblastoma protein
Ref-1	Redox factor-1
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
SOD2	Superoxidase dismutase 2
TIGAR	Tp53-inducible glycolysis and apoptosis regulator
TGR	Thioredoxin glutathione reductase
Trx	Thioredoxin
TrxR	Thioredoxin reductase

INTRODUCTION

CANCER

Human cancer is a genetic disease caused by sequential accumulation of mutations each conferring growth advantage which leads to the progressive conversion of normal cells to cancer cells (Vogelstein and Kinzler, 2004). Six essential acquired capabilities have been suggested to be required to transform a normal cell to malignant, including self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Furthermore, statistic analysis of mutations detected by sequencing of breast and colon carcinomas suggest that most of the mutations found in an individual tumor are so called “passenger” mutations, which are not directly involved in cancer development, and that <15 mutations are likely to be responsible for driving the initiation, progression and maintenance of the tumor (Wood et al., 2007). These mutations appear to reflect alterations in signaling pathways, showing that deregulation of pathways rather than individual genes govern the course of tumorigenesis. These changes involve activation of oncogenes, inactivation of tumor suppressor genes and genome stability genes (genes involved in DNA damage repair). Alterations can occur in the genome through point mutations, deletions, gene amplifications, chromosomal aberrations, such as chromosomal translocations, or through epigenetic changes including DNA hypo- or hypermethylation and histone modification.

The data demonstrating that defects in a relatively small number of pathways underlie many different tumor types suggests that therapies targeting major tumor-related pathways could be efficient against a broad range of cancers (Wood et al., 2007).

p53

In 1979 two reports were published describing the discovery of a new oncogene, named p53, which was found to bind the large T-antigen of the SV40 DNA tumor virus (Lane and Crawford, 1979; Linzer and Levine, 1979). Its overexpression in tumor cells as well as its ability to cooperate with the oncogene Ras in immortalizing and transforming cells further supported p53 oncogenic potential (Parada et al., 1984). However, ten years later the true function of p53 as a tumor suppressor was discovered when it was demonstrated that the previous studies had been performed using cells expressing mutated p53 gene (Finlay et al., 1989; Hinds et al., 1989). Further evidence of tumor suppressive function by p53 was uncovered by the findings that p53 null mice are susceptible to spontaneous tumors at early age (Donehower et al., 1992). Furthermore, patients with the dominantly inherited Li-Fraumeni syndrome which leads to early onset of tumorigenesis carry germ line mutations in the p53 gene (Malkin et al., 1990).

p53 is a tetrameric, sequence-specific transcription factor with a defined consensus for optimal DNA binding consisting of two RRRCWWGYYY palindromes (el-Deiry et al., 1992; Fields and Jang, 1990; Funk et al., 1992; Kern et al., 1991; Raycroft et al., 1990; Wei et al., 2006). Upon stress signals, p53 is activated in a specific manner by post-transcriptional modification and triggers a variety of anti-proliferative processes including cell cycle arrest, cellular senescence or apoptosis (Levine et al., 2006). Thus, disruption of p53 function promotes checkpoint defects, cellular immortalization, genomic instability, and inappropriate survival, allowing the continued cell

proliferation and malignant evolution of damaged cells. Therefore, it is not surprising that p53 is the most commonly inactivated tumor suppressor gene in human cancer (Beroud and Soussi, 2003; Hussain and Harris, 1998).

Localized on the short arm of chromosome 17 (17q13), the p53 encodes a protein of 393 amino acid residues. p53 is evolutionary conserved in *Drosophila* and *C. elegans*, where it is an important component in damage surveillance (Brodsky et al., 2000; Frantz, 2001; Nordstrom and Abrams, 2000). Based on structural and functional analysis, the p53 protein is subdivided into five different domains; the N-terminal transactivation domain, the proline-rich domain, the DNA binding domain, the oligomerization domain and the regulatory domain in the C-terminus (May and May, 1999) (Fig 1).



Figure 1. Schematic presentation of the five domains of the p53 protein

Regulation of p53

Due to the important role of p53 in controlling life and death, the expression of p53 is tightly regulated in cells. In unstressed normal cells p53 is kept at low levels, mainly through p53 protein degradation. Upon stress signaling, p53 undergoes a series of post-translational modifications, leading to stabilization of the protein. Thus the major regulation of p53 occurs on the level of protein stability, whereas, fine-tuning of the p53 response is achieved by different post-translational modifications.

p53 activation

Various post-translational modifications appear to influence the p53 response. These post-translational events include phosphorylation, acetylation, glycosylation, ribosylation, ubiquitination and sumoylation. Different types of DNA damage activate diverse enzyme activities that modify the p53 protein at specific amino acid residues (Appella and Anderson, 2001). p53 molecules with distinct modifications may have different preferences or recruit distinct transcriptional co-activators thus leading to the activation of a certain pattern of p53 target genes and different cellular responses.

Phosphorylation of p53 at Ser15 produced by kinases such as ATM and DNA-PK in response to DNA damage has been shown to stimulate p53-dependent transactivation, leading to growth arrest and apoptosis in cell-based studies (Canman et al., 1998; Lees-Miller et al., 1992). Phosphorylation of Ser15 appears to initiate the phosphorylation of other residues such as Ser20 and Thr18. These two phosphorylations can in turn stabilize p53 by interfering with the binding to the negative regulator MDM2, thus preventing the ubiquitination of p53. Additionally, the phosphorylation of Ser46 is induced upon DNA damage signals and has been demonstrated to selectively induce the apoptotic function of p53 (Bulavin et al., 1999).

Different histone acetyltransferases (HAT), such as p300/CBP, PCAF and TIP60, acetylate p53 lysine residues. *In vitro* studies have demonstrated that acetylation of the lysine residues in the C-terminal domain results in stabilization and activation of p53 (Rodriguez et al., 2000). However, mice in which all the C-terminal lysines had been

exchanged for arginine are viable and have only mild abnormalities (Krummel et al., 2005). This study questions the functional significance of C-terminal lysines. Studies on Lys120 and Lys164 located in the DNA binding domain have shown that acetylation at these sites is required for suppression of cell growth in cell culture models (Tang et al., 2006; Tang et al., 2008).

Despite the accumulated *in vitro* data on modulation of p53 function by post-translational modifications, the exact biological significance of these modifications remains uncertain. Genetic mouse models used to study phosphorylation and acetylation sites have failed to offer any clarification, due to the minimal phenotypes exhibited. These results suggest that not one single modification on its own is important, but rather a combination of several modifications cooperating. Distinct combinations of post-translational modifications might be required for different protein-protein interactions, and thus different outcomes upon p53 activation.

There are a vast number of p53 binding proteins, some of which have been demonstrated to dictate the behavior of p53 upon stress signals. A few examples follow below.

The ASPP family has three members: ASPP1, ASPP2 and inhibitory ASPP (iASPP), which can all bind and regulate p53 activity through their C-terminus (Samuels-Lev et al., 2001). ASPP1 and ASPP2 stimulate the apoptotic function of p53 by selectively promoting p53 binding to promoters of pro-apoptotic genes, such as *BAX*, and *TP53I3* (*PIG3*), but not *p21* and *MDM2*. In contrast, iASPP inhibits p53-mediated apoptosis and is overexpressed in various tumor cells (Bergamaschi et al., 2003).

The prolyl isomerase Pin1 induces conformational changes by catalyzing cis/trans isomerization at phosphorylated Ser-Pro or Thr-Pro motifs in many proteins, including p53 (Mantovani et al., 2004). Pin1-mediated isomerization of p53 results in induction of p53 pro-apoptotic targets (Zacchi et al., 2002).

One of the most recently discovered p53 cofactors hnRNP K, is required for DNA-damage induced cell cycle arrest and serves as a co-activator for p53 in regulating cell-cycle arrest genes such as *CDKN1A* (p21), *GADD45* and *14-3-3 σ* (Moumen et al., 2005). Upon DNA damage, hnRNP K is induced due to protection from MDM2-mediated proteasomal degradation and is recruited to the promoters of p53-responsive genes (Moumen et al., 2005).

The homeodomain interacting protein kinase 2 HIPK2 activates p53 by direct binding and phosphorylation at Ser46, which is thought to promote changes in the affinity of p53 for pro-apoptotic genes. It has been demonstrated that HIPK2 and p53 cooperate in the activation of p53-dependent transcription and apoptotic pathways (D'Orazi et al., 2002). Furthermore, in growth-arrested cells HIPK2 is degraded by MDM2, suggesting that the cell cycle arrest functions of p53 include the suppression of its apoptotic functions (Rinaldo et al., 2007).

The acetyltransferase TIP60 acetylates p53 at Lys120 and induces pro-apoptotic function of p53, by promoting transcription of *BAX* and *BBC3* (*PUMA*) (Sykes et al., 2006; Tang et al., 2006).

The histoneacetyltransferases p300/CBP and PCAF acetylate the C-terminal lysine residues of p53 upon DNA damage, which is associated with the stabilization of p53 and induction of sequence-specific DNA binding and transactivation of p53 (Grossman, 2001). In conclusion, the complexity of p53 regulation ensures the right processing of stress signals by p53 and a diversity of p53-induced biological responses.

The MDM2-HDMX network

MDM2 (mouse double minute 2) is the main repressor of p53, which was originally demonstrated to be amplified in a tumor mouse cell line where it induced

tumorigenicity (Fakharzadeh et al., 1991). MDM2 gene amplifications have also been found in various human tumors, particularly in sarcomas expressing wild-type p53 (Oliner et al., 1992). MDM2 interacts with p53 through its N-terminal domain, inhibiting p53 transactivation function (Momand et al., 1992). Further, MDM2 functions as an E3-ligase, which ubiquitinates p53 and targets it for proteasomal degradation (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Polyubiquitination of p53 leads to its degradation, whereas monoubiquitination of p53 instead functions as an export-signal (Li et al., 2003). Monoubiquitinated p53 is sent out of the nucleus to the mitochondria, where it is thought to be involved in transcription-independent induction of apoptosis (Li et al., 2003; Marchenko et al., 2007). However, ubiquitination and degradation of p53 can occur both in the nucleus and the cytoplasm (Stommel et al., 1999; Yu et al., 2000). Furthermore, MDM2 can auto-ubiquitinate itself (Fang et al., 2000).

The promoter of MDM2 contains a p53-binding site and is transcribed in a p53-dependent manner. Consequently, MDM2 and p53 form an autoregulatory feedback loop (Wu et al., 1993). A critical role of MDM2 for p53 regulation in development is supported by the rescue of the embryonic lethal phenotype of MDM2-null mice by deletion of p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995). The importance of the MDM2-p53 regulation is demonstrated in yet another mouse model, which expresses extra copies of the MDM2 gene under the control of the endogenous promoter. Mice overexpressing MDM2 are significantly predisposed to tumor formation (Jones et al., 1998). Furthermore, a single nucleotide polymorphism (SNP) in the human MDM2 promoter region leads to elevated MDM2 expression and an earlier onset of both hereditary tumors in Li-Fraumeni patients, as well as sporadic tumors in the normal human population (Bond et al., 2004).

The HDMX protein (also known as MDM4) was found in a screen for p53-binding proteins and was demonstrated to share structural homology with MDM2 (Shvarts et al., 1996). MDM2 and HDMX share three well conserved domains: the N-terminal domain involved in p53 binding, a Zinc finger domain whose function is unclear, and the C-terminal RING domain (Fig 2). The central regions of MDM2 and HDMX include an acidic domain. In addition, MDM2 has a nuclear localization signal (NLS) and a nuclear export signal (NES), that HDMX lacks. HDMX is therefore dependent on other proteins, such as MDM2 and p53, for nuclear localization. The amino acid residues in the N-terminal domain required for interaction with the N-terminal of p53 are highly conserved in the two proteins. Therefore the hydrophobic clefts which they form are similar, although that of HDMX is smaller due to protruding side chains of two residues (Macchiarulo et al., 2008; Popowicz et al., 2008). The C-terminal RING domains of MDM2 and HDMX are essential for the proteins to form homo- and heterodimers (Tanimura et al., 1999). An important difference between the MDM2 and HDMX RING domains is a missing cysteine residue in HDMX, resulting in the lack of E3-ligase activity. However, similarly to MDM2, HDMX can inhibit p53 transcriptional activity by binding p53, and possibly interfering with the recruitment of the basal transcription machinery and co-activators to p53-dependent promoters (Tanimura et al., 1999). Additionally, HDMX hinders p300/CBP-mediated acetylation of p53, which is thought to contribute to the inhibition of p53 activity (Sabbatini and McCormick, 2002). The importance of HDMX control of p53 activity in development is illustrated in HDMX knockout mice, which are embryonic lethal due to deregulated p53 (Parant et al., 2001). This further demonstrates that HDMX is critical and non-redundant in keeping p53 activity in check during embryogenesis. Furthermore, HDMX is amplified or overexpressed in various types of tumors, but most strikingly in

retinoblastomas, where 65% of the tumors show deregulated HDMX levels (Laurie et al., 2006; Toledo and Wahl, 2006).

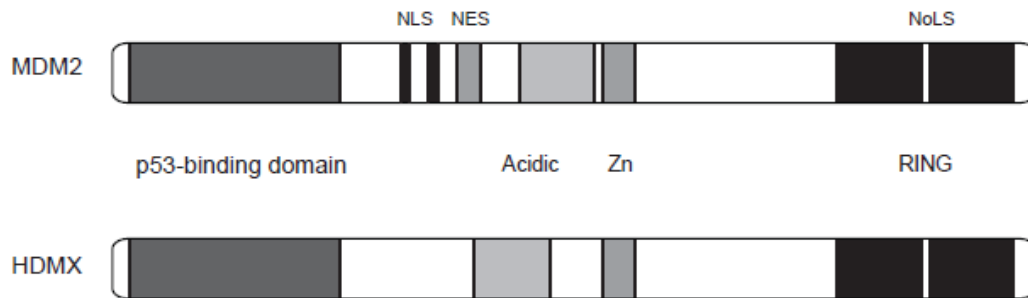


Figure 2. Schematic comparison of the MDM2 and HDMX protein structures. The p53-binding domain, Zinc finger (Zn) and RING finger domain are well conserved. MDM2 additionally contains nuclear localization signal (NLS), nuclear export signal (NES) and nucleolar localization signal (NoLS).

In contrast to MDM2, which is a known target gene of p53, no p53 response elements have been found in the *hdmx* locus, and no p53-dependent induction of *hdmx* expression is detected after DNA damage.

As mentioned above, the RING domain of MDM2 mediates the formation of homodimers or heterodimerization with HDMX. However, homodimers of HDMX have so far not been observed *in vitro* or *in vivo*, suggesting that free HDMX is monomeric. Studies investigating the binding affinities between the proteins revealed that the formation of MDM2/HDMX heterodimers is favored (Kawai et al., 2007; Tanimura et al., 1999). This correlates with the finding that HDMX enhances MDM2-dependent ubiquitination and degradation of p53 (Gu et al., 2002; Linares et al., 2003). However, these results are based on cell culture studies and should therefore be further explored with mouse models. The biological functions of MDM2 and HDMX on p53 have been much studied and discussed. However, the recent *in vivo* models indicate that MDM2 and HDMX have distinct, but complementary roles in regulation of p53; while MDM2 mainly regulates p53 stability, MDM4 has a major role in regulating the activity of p53 (Francoz et al., 2006; Toledo and Wahl, 2006; Xiong et al., 2006) (Fig 3).

Clearly, the inhibition of MDM2 and HDMX activities is essential for p53 stabilization and activation. As mentioned above, the activation of p53 is not fully controlled by any single phosphorylation or other single modification. Kinases, such as DNA-PK, ATM, ATR, Chk2 and Chk1 phosphorylate p53, MDM2 and HDMX at various sites. The phosphorylation of p53 on Ser15, Thr18 and Ser20 are all thought to disrupt the p53/MDM2 and p53/HDMX interactions (Giaccia and Kastan, 1998; Meek, 1999; Prives and Hall, 1999; Unger et al., 1999). Phosphorylations of MDM2 and HDMX also lead to inhibition of their binding to p53 (Chen et al., 2005b; Maya et al., 2001; Mayo et al., 1997; Pereg et al., 2005; Shinozaki et al., 2003). Moreover, regulated MDM2 auto-degradation appears to be an important mechanism by which p53 is activated in cells upon certain types of DNA damage (Stommel and Wahl, 2004). Induction of MDM2 expression combined with triggering of MDM2 auto-ubiquitination and degradation results in an oscillation of MDM2 protein levels upon DNA damage (Lev Bar-Or et al., 2000). HDMX has also been demonstrated to be ubiquitinated and degraded by MDM2 upon DNA damage (de Graaf et al., 2003;

Kawai et al., 2003; Pan and Chen, 2003). The switch by which MDM2 initiates ubiquitination of itself and HDMX instead of p53 is not fully elucidated, but appears to involve the deubiquitination enzyme Herpes virus-associated ubiquitin-specific protease (HAUSP). Upon DNA damage MDM2 and HDMX are released from HAUSP, resulting in impaired deubiquitination and subsequent degradation (Li et al., 2004; Meulmeester et al., 2005). HAUSP can instead bind p53, rescuing it from MDM2-mediated degradation (Li et al., 2003).

In addition to checkpoint kinases, the protein stability of HDMX and MDM2 is regulated by the serine/threonine protein kinase Akt, which stabilizes both MDM2 and HDMX by phosphorylation (Ashcroft et al., 2002; Feng et al., 2004; Lopez-Pajares et al., 2008).

Upon oncogene activation p53 is up-regulated through p14ARF (de Stanchina et al., 1998; Palmero et al., 1998; Zindy et al., 1998). p14ARF interacts with MDM2 and inhibits its p53-ubiquitin ligase activity, thus stabilizing p53. The p14ARF gene is positively regulated by oncogenes such as E2F1 and β -catenin, whereas negative regulation is exerted by p53 (Harris and Levine, 2005). Inactivation of p14ARF through epigenetic alterations occurs frequently in human tumors and appears to inversely correlate with p53 status, i.e. inactivation of p14ARF correlates with wild-type p53 (Sharpless, 2005; Stott et al., 1998). Recent studies in mice demonstrate that the key tumor-suppressive function of p53 is to respond to oncogene activation. p14ARF-dependent response of p53 to oncogenic signaling is therefore critical for the tumor-suppressor activity (Christophorou et al., 2006; Efeyan et al., 2006). However, other studies have shown that the genotoxic stress is the key signal for activating p53 and tumor suppression in pre-cancerous lesions and that DNA damage can be induced by the activation of several oncogenes in a p14ARF-independent manner (Bartkova et al., 2005; Gorgoulis et al., 2005).

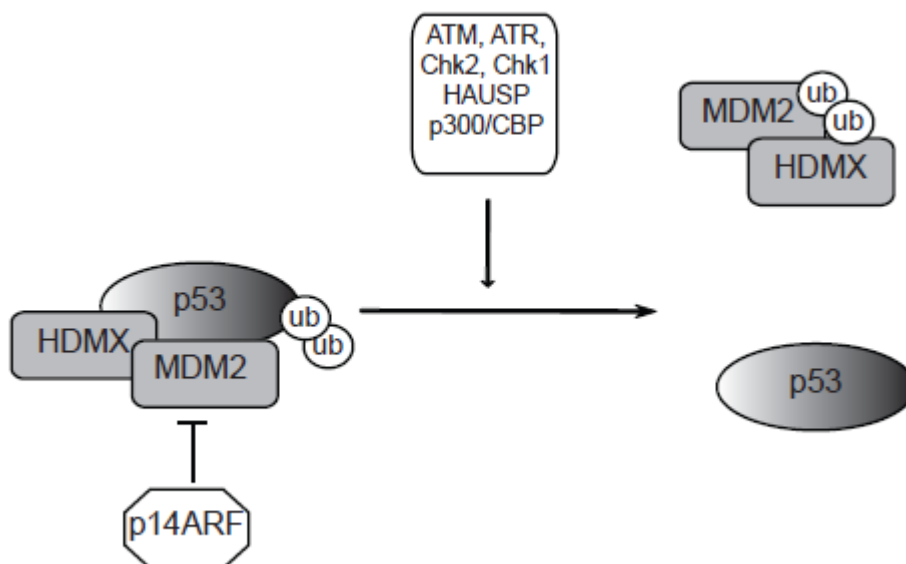


Figure 3. Regulation of p53 stability by the MDM2-HDMX network

In addition to controlling p53, MDM2 has a number of p53-independent functions, some of which are listed below (Zhang et al., 2005a). MDM2 can bind and inhibit Rb, leading to its ubiquitination and degradation. As a result E2F1 is relieved from inhibition by Rb and induces cell cycle progression by entry to S-phase (Hsieh et al., 1999; Uchida et al., 2005). Furthermore, MDM2 can bind E2F1 and increase its transcriptional activity subsequently promoting cells into S phase (Zhang et al., 2005b). Another binding partner of MDM2 is Nbs1, a member of the Mre11/Rad50/Nbs1 DNA DSB repair complex. The binding between MDM2 and Nbs1 results in the inhibition of DNA repair and an increase of chromosome breaks. This might explain how elevated MDM2 levels can promote genomic instability and transformation independent of p53 (Bouska and Eischen, 2009)

Additionally, MDM2 can promote the degradation of a number of proteins in a p53-independent manner. Via its E3-ligase activity MDM2 ubiquitinates several targets, such as hnRNP K, IGF-1R and TIP60 (Girnita et al., 2003; Legube et al., 2002; Moumen et al., 2005). Furthermore, MDM2 binds the p53 CDK inhibitor p21 and facilitates its binding with the proteasome, thus promoting its degradation (Jin et al., 2003; Zhang et al., 2004).

Additional ubiquitin ligases

Several other ubiquitin ligases have been identified to target p53 for degradation, including COP1, Pirh-2 and ARF-BP1 (Chen et al., 2005a; Dornan et al., 2004; Leng et al., 2003). ARF-BP1 is induced upon oncogene activation through p14ARF. COP1 and Pirh-2 function similarly to MDM2, both forming an autoregulatory loop by promoting p53 degradation. However, the relative impact of these E3 ligases to the regulation of p53 levels in cancer is unclear at the moment.

Redox modulation

Several studies have demonstrated that the activity of p53 and its ability to bind DNA is influenced by its redox status. This is due to the presence of cysteine residues in the DNA binding domain which contain redox sensitive thiol groups. Cys176, 238 and 242 together with His179 make up a binding site for a zinc atom (Hainaut and Mann, 2001). The presence of zinc is required for proper folding of p53 *in vitro* and upon exposure to metal chelators such as EDTA recombinant p53 loses its wild-type conformation (Hainaut and Milner, 1993). Furthermore, there is a cluster of cysteines in the proximal part of the DNA binding domain, which appears to be regulated by redox modulation and subsequently influences the binding of p53 to the DNA consensus sequence (Parks et al., 1997).

Redox factors such as redox factor-1 (Ref-1) and thioredoxin can affect the DNA binding activity of p53. Ref-1 binds to p53 and enhances its binding activity and promotes p53 tetramerization (Hanson et al., 2005). Furthermore, Ref-1 can cooperate with thioredoxin in activating p53 DNA-binding and transcriptional activity, which is further discussed below (Seemann and Hainaut, 2005; Ueno et al., 1999).

The functions of p53

In the absence of cellular stress, p53 levels are kept low due to constant degradation. However, upon stress stimuli, including DNA damage, hypoxia, telomerase shortening, nutrient deprivation or oncogene activation, p53 becomes stabilized and activated which in turn leads to cellular responses such as DNA repair, cell cycle arrest, cellular senescence and apoptosis. This occurs through transcriptional activation and repression

of target genes and by transcription-independent mechanisms (May and May, 1999; Vousden and Lu, 2002). (Fig 4) It is not clear how p53 chooses which pathway to activate, but it appears to depend on the nature of the stress signal, the types and location of p53 post-translational protein modifications, the proteins associated with p53 and the type of cell.

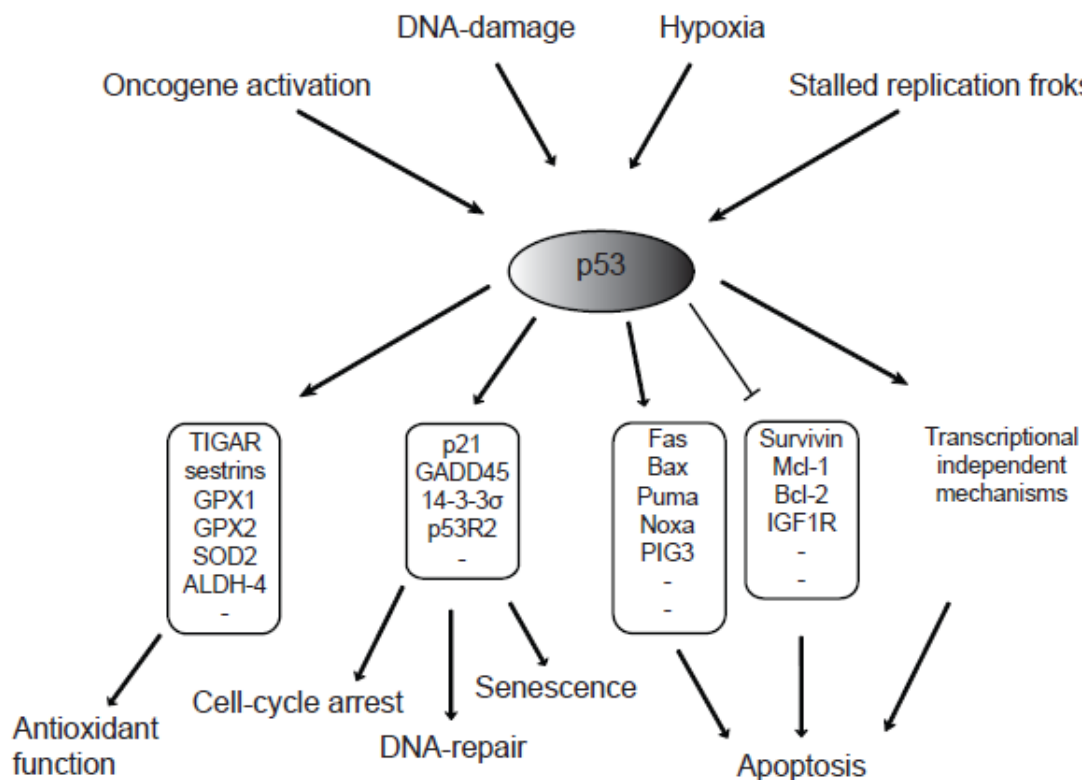


Figure 4. In response to different stress signals, p53 is stabilized and induces cellular responses through transcriptional activation and repression of target genes.

Apoptosis

p53-dependent apoptosis involves transactivation and transrepression of target genes, but also transcription-independent mechanisms. Apoptosis induction by p53 is mainly achieved through the two major apoptotic pathways, the extrinsic pathway, involving death receptors, and the intrinsic pathway, which is also called the mitochondrial pathway. p53 activates the extrinsic pathway by induction of the genes encoding the transmembrane receptors Fas and DR5. Fas is activated upon binding of Fas ligand (FasL), whereas DR5 is activated by binding of TNF-related apoptosis-inducing ligand (TRAIL). Activation of the receptors leads to a chain of events, which in the end activate the effector caspases.

The intrinsic pathway is regulated by the Bcl-2 family, which consists of the pro-survival proteins, such as Bcl-2, Bcl-XL and Mcl-1, the pro-apoptotic proteins Bax, Bak, and the BH3-only proteins PUMA, Noxa and Bid. Several members of the family are transcriptional targets of p53 including Bax, PUMA, Noxa and Bid (Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda et al., 2000; Sax et al., 2002). In

addition, Bcl-2 and Mcl-1 are transrepressed by p53 (Miyashita et al., 1994; Pietrzak and Puzianowska-Kuznicka, 2008). The pro-apoptotic proteins are located at the mitochondria and can trigger the release of cytochrome c into the cytoplasm, resulting in activation of caspases. Bax expression is activated by p53 and upon induction, Bax undergoes a conformational change, forms homodimers and translocates to the mitochondrial membrane where it promotes cytochrome c release (Adams and Cory, 1998; Miyashita and Reed, 1995). Another target of p53, PUMA, triggers translocation and oligomerization of Bax, resulting in cell death (Yu et al., 2003). Interestingly, Bid appears to function as a link between the extrinsic and intrinsic pathways since caspase-8, which is involved in death receptor signaling, can activate Bid by cleavage. Truncated Bid can in turn induce Bax activation.

Additionally, induction of p53 leads to the production of pro-oxidant genes such as *TP53I3* (PIG3) and ferredoxin reductase (*FXDR*) (Hwang et al., 2001; Polyak et al., 1997). These increase the levels of reactive oxygen species (ROS) which in turn might interfere with mitochondrial function and/or integrity, contributing to cell death. In addition, the higher levels of ROS appear to be part of a feed-forward loop that stabilizes p53 resulting in further p53 activation.

p53 can also, as mentioned, promote transrepression of survival genes, including *Bcl-2*, *BIRC5* (survivin) and *Mcl-1*, which may contribute to apoptosis; (Hoffman et al., 2002; Miyashita et al., 1994; Pietrzak and Puzianowska-Kuznicka, 2008).

Several studies have demonstrated that p53 also possesses a transcription-independent apoptotic activity. Activated p53 translocates to the mitochondria, where it can directly induce Bak or Bax oligomerization, and/or interact with the anti-apoptotic members Bcl-2 and Bcl-XL, thereby displacing Bak and Bax which instead can homooligomerize and cause cytochrome c release (Erster et al., 2004; Mihara et al., 2003). This pathway probably works in synergy with the transcription-dependent action of p53.

By simultaneously targeting several levels of the apoptotic program, p53 increases the probability that the process progresses and ensures a well-coordinated program once it is initiated.

Cell-cycle arrest

The cell cycle is tightly regulated by a variety of actors, where cyclins, their corresponding cyclin dependent kinases (CDK), and the CDK inhibitors are the key regulators. The cell cycle is monitored and controlled by different cell cycle checkpoints, which block progression upon certain signals, such as DNA damage. In response to stress signals p53 can activate the expression of components of both the G1/S and G2/M checkpoints.

The most famous p53-induced cell cycle inhibitory gene is *CDKN1A* (p21), which induces both G1 and G2 arrest. p21 initiates G1 arrest by binding to cyclinD/CDK4 and cyclinE/CDK2, thus inhibiting downstream phosphorylation of the tumor suppressor Rb (Harper et al., 1993). Unphosphorylated Rb represses E2F1 thus blocking the transcription of genes required for S-phase entry. Similarly, p21 can also inhibit cyclinB/CDK2 and cyclinB/Cdc2 (CDK1), which leads to G2 arrest (Bunz et al., 1998). Additionally, p21 has been demonstrated to be a negative regulator of apoptosis, a property which appears to be distinct from its growth arrest function (Baptiste-Okoh et al., 2008; Steinman and Johnson, 2000; Suzuki et al., 1998).

G2 arrest can also be induced by the p53 targets GADD45 and 14-3-3 σ . GADD45 dissociates the cyclinB/Cdc2 complex (Wang et al., 1999b), whereas 14-3-3 σ sequesters Cdc2 in the cytoplasm (Chan et al., 1999).

Senescence

Cellular senescence can be triggered by DNA damage, oncogene activation or by shortening of telomeres. Upon entering senescence, cells cease to divide and undergo a number of morphological, and metabolic changes. It has been experimentally demonstrated that p53 is involved in senescence. However, it is not clear which target genes are involved in triggering senescence. In a study by Sugrue et al wild-type p53-triggered senescence was shown to be associated with induction of p21 and repression of mitotic cyclins (Sugrue et al., 1997).

Genetic stability

Various insults such as chemotherapeutical drugs, ROS and irradiation lead to DNA damage, which can be repaired by a number of different pathways. Interestingly, p53 is involved in several of these pathways including nucleotide excision repair (NER), base excision repair (BER) and in homologous recombination (HR) (Helton and Chen, 2007). The ribonucleotide reductase p53R2, is a p53 target gene, and can supply nucleotides to repair damaged DNA (Yamaguchi et al., 2001). Facilitation of DNA repair by p53 serves to maintain genomic stability and thus prevent tumor development. On the other hand, induction of DNA repair by p53 in tumor cells upon chemo- and radiotherapy can lead to increased survival of cancer cells (Moreno et al., 2007).

Antioxidant activity

A number of reports have made it evident that the everyday strains of normal mammalian life induce low levels of p53 activity which promote survival (Lassus et al., 1996; Sablina et al., 2005). Various studies have shown that one such survival function of p53 is keeping intracellular ROS levels low via the activity of p53-inducible genes such as the Tp53-inducible glycolysis and apoptosis regulator (*TIGAR*), sestrins, glutathione peroxidases (*GPX1*, *GPX2*), superoxidase dismutase 2 (*SOD2*), aldehyde dehydrogenase-4 (*ALDH-4*) and others (Bensaad and Vousden, 2005; Budanov et al., 2004; Hussain et al., 2004; Yan and Chen, 2006; Yoon et al., 2004). This antioxidant function of p53 appears to be important to prevent the accumulation of DNA damage in response to day-to-day incidents (Sablina et al., 2005). One can therefore speculate that the low levels of DNA damage that are encountered daily are dealt with through p53 by keeping ROS levels low and thus promoting survival. However, in response to more severe and sustained stress, p53 function switches and instead induces ROS, leading to apoptosis.

Pharmacological reactivation of p53

Inactivation of p53 occurs in the majority, if not all tumors, either by mutation or by deregulation of the p53 pathway, for example by overexpression of its negative regulators.

Mutations of the p53 gene occur in about 50% of all sporadic tumors, making it one of the most mutated genes in cancer (Beroud and Soussi, 2003; Olivier et al., 2002). In contrast to many other tumor suppressors, deletions in the p53 gene are rare whereas missense point mutations occur with extremely high frequency (Soussi and Lozano, 2005). A majority of all the mutations target the DNA binding domain of the protein, affecting p53 binding to DNA and the transactivation of downstream targets, thus abolishing the p53 tumor suppressor function. Mutations occur at a higher frequency in the six hotspot codons; R175, R245, R248, R249, R273 and R282 (see IARC TP53 mutation database <http://www-p53.iarc.fr>).

In the other half of the tumors, which do not carry p53 gene mutations, the p53 function is compromised by defects in the upstream pathways or by deregulation of p53 protein stability. As mentioned earlier, both MDM2 and HDMX are overexpressed in a number of tumors. The tumor suppressor p14ARF, which inhibits MDM2 binding to p53, is frequently inactivated in tumors due to epigenetic alterations. The upstream modulators of p53, such as for example the protein kinases ATM and Chk2 have been found to be mutated in certain tumors (Stankovic et al., 2002; Sullivan et al., 2002).

Additionally, there are a number of viruses which encoding viral oncogenes that can inactivate p53. The SV40 large T-antigen binds the p53 core domain thus blocking its DNA binding activity (O'Reilly, 1986). The E6 protein of the human papilloma virus (HPV) binds p53 and targets it for proteasomal degradation (Scheffner et al., 1990). Numerous studies suggest that inactivation of p53 is important for the survival of tumor cells. Thus, re-instatement of p53 function could be an attractive strategy for cancer therapy. The findings that already established tumors in mice are vulnerable to the restoration of p53 (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007) further support the idea of reactivation of p53 tumor suppressor function to combat cancer. Several different approaches have been developed in order to find specific and efficient activators of wild-type p53. A majority of these have been focused on antagonizing MDM2 function to prevent p53 degradation.

Antisense inhibition of MDM2 expression results in p53 stabilization and activation in tumor cells *in vitro* and in tumor xenografts in mice, supporting the validity of this approach (Wang et al., 1999a; Zhang et al., 2005a).

Another approach is targeting the ubiquitin ligase activity of MDM2 to block p53 ubiquitination and degradation. Small molecule inhibitors have been identified that specifically target the E3 ligase activity of MDM2 and induce apoptosis in a p53-dependent manner (Lai et al., 2002; Yang et al., 2005). However, these compounds have relatively low potency and selectivity, therefore further optimization is required in order to develop a candidate drug based on these compounds.

Several labs have described the identification of small molecules inhibiting p53/MDM2 interaction. The nutlins were found in a biochemical screen of a library of synthetic chemicals (Vassilev et al., 2004). Nutlins bind the p53-binding pocket of MDM2 and can therefore displace p53. Nutlins inhibit the p53/MDM2 interaction in cells, leading to stabilization of p53 and activation of downstream targets. Tumor xenograft growth in animal models was suppressed upon treatment with nutlins, without apparent toxicity to the normal tissue (Vassilev et al., 2004).

The benzodiazepines is another class of MDM2 antagonists which can disrupt the p53/MDM2 binding *in vitro* and have been demonstrated to suppress the growth of wild-type p53 expressing cells (Grasberger et al., 2005; Koblisch et al., 2006). However, due to low cellular potency and selectivity it is difficult to evaluate their anti-tumor potential.

The small molecule RITA (reactivation of p53 and induction of tumor cell apoptosis) was found by screening the NCI library of low molecular weight compounds for compounds that suppressed cell growth in a p53-dependent manner. RITA can bind the N-terminal domain of p53, disrupting its interaction with MDM2 and other negative regulators (Issaeva et al., 2004; Zawacka-Pankau et al., manuscript in preparation). RITA restores the p53 transcriptional function and induces p53-dependent apoptosis in tumor cells, but not in non-transformed cells. Further, *in vivo* experiments using human tumor xenograft models in mice demonstrated a p53-dependent anti-tumor effect. In our recent studies we addressed the mechanisms of the tumor-specific apoptotic response after RITA treatment. We have demonstrated that RITA mainly induces pro-apoptotic target genes and that apoptosis induction s

facilitated by downregulation of p21 on two different levels: transcription and protein stability (Enge et al., 2009). Furthermore, RITA-activated p53 inhibits crucial oncogenes by transcriptional repression of anti-apoptotic proteins Mcl-1, Bcl-2, MAP4, and survivin, by blocking Akt pathway on several levels, and by downregulating c-Myc, cyclin E and β -catenin (Grinkevich et al., 2009).

The p53-reactivating compound MITA was discovered using the same screening assay as for RITA, and is discussed further below.

Other p53 activating compounds include roscovitine, a CDK inhibitor, which suppresses MDM2 expression (Lu et al., 2001), and tenovins, which activate p53 through the inhibition of deacetylase activities of SirT1 and SirT2 (Lain et al., 2008).

Although much effort has been made on targeting MDM2, recent reports showing that downregulation of HDMX by means of siRNA in cell lines induces p53, and suppresses colony outgrowth (Danovi et al., 2004), and that HDMX knockdown leads to reduced tumor xenograft growth (Gilkes et al., 2006), highlight the importance of developing inhibitors towards HDMX. So far, only short peptides which inhibit the interaction between HDMX and p53 have been reported (Hu et al., 2007; Pazgier et al., 2009), but small molecules targeting HDMX have not yet been discovered. The clinical application of peptides is not optimal due to their proteolytic degradation and the insufficient cellular uptake.

Another strategy to reinstate p53 tumor suppression is to restore the wild-type function of mutant p53. One of the most promising compounds which can rescue the p53 tumor suppressor function of mutant p53 is the small molecule PRIMA-1, which was found in a cell-based screen of low molecular weight compounds (Bykov et al., 2002). PRIMA-1 inhibits cell growth in a mutant p53-dependent manner and restored the transcription of p53 target genes. Furthermore, PRIMA-1 has a mutant p53-dependent *in vivo* anti-tumor activity in human xenografts in mice (Bykov et al.). The mechanisms behind PRIMA-1 function are not fully elucidated yet, but seem to involve induction of apoptosis by transcription-dependent activity of p53 and transcription-independent functions of p53 involving caspase activation (Wang et al., 2007a).

THE THIOREDOXIN SYSTEM AND REDOX CONTROL

Low levels of reactive oxygen species (ROS) are constantly generated in all aerobic organisms as by-products of cellular metabolism. However, excessive levels of ROS can damage several cellular components including DNA, protein and lipid membranes (Hensley et al., 2000; Pelicano et al., 2004). DNA damage caused by ROS leads to the generation of oxidized bases, DNA strand breaks, DNA intra-strand adducts, and DNA-protein cross-links (Lloyd et al., 1997; Pelicano et al., 2004; Randerath et al., 1996). Oxidative modifications of DNA bases may result in mutations during replication due to base pair mismatching (Lloyd et al., 1997; Pelicano et al., 2004; Randerath et al., 1996). It is therefore of importance to have a balance between oxidant and antioxidant intracellular systems. The major redox control in cells is conducted by the thioredoxin and glutathione systems. The thioredoxin system consists of thioredoxin reductase (TrxR) and thioredoxin (Trx), where Trx is reduced by TrxR at the expense of NADPH. The thioredoxin system is involved in several important biological activities, such as redox regulation of cellular function, generation of deoxyribonucleotides for DNA synthesis, regulation of apoptosis induction and in antioxidant defense (Arner and Holmgren, 2000; Nordberg and Arner, 2001).

Thioredoxin

Thioredoxin (Trx) is a small 12 kDa protein that is ubiquitous from Archea to humans. It has a well conserved active site with two cysteine residues which form a disulfide bond that is reduced to a dithiol via TrxR (Holmgren et al., 1968). TrxR is in fact the only known enzyme that can reduce Trx. The major cytosolic form of Trx, Trx1 is the most studied form so far. However there is also a Trx2, which is found in the mitochondria, as well as some tissue-specific isoforms (Miranda-Vizuete et al., 1999; Miranda-Vizuete et al., 1998).

Trx1 has many biological activities in the cell, such as functioning as an antioxidant, directly or through thioredoxin peroxidases, also called peroxiredoxins (Chae et al., 1994; Spector et al., 1988). One of the earliest functions described for Trx1 is the reduction of ribonucleotide reductase (Laurent et al., 1964), which catalyzes the conversion of ribonucleotides to deoxyribonucleotides. This is the first step in DNA synthesis and is essential for cellular proliferation. In each catalytic cycle of ribonucleotide reductase a disulfide is formed in the enzyme, which is reduced back to a dithiol by Trx1 (Avval and Holmgren, 2009). Furthermore, Trx1 can regulate the DNA binding of a number of transcription factors such as NF- κ B, AP-1 and p53 (Gaiddon et al., 1999; Hirota et al., 1997; Jayaraman et al., 1997). Trx1 has also been shown to bind directly to proteins without catalyzing redox reactions. One such example is the binding to the apoptosis signal-regulating kinase 1 (ASK1). Reduced Trx1 can bind ASK1 and thereby inhibit its MAPKKK activity (Fujisawa et al., 2007).

Thioredoxin reductase

TrxR is a homodimeric selenocysteine-containing flavoprotein that supports Trx reduction using NADPH (Cheng et al., 2009; Gladyshev et al., 1996; Powis and Kirkpatrick, 2007). TrxR has many similarities to glutathione reductase (GR), in its overall structure and function. However, TrxR has a prolonged C-terminal end, containing an additional redox active motif including the selenocysteine residue, which is essential for the catalytic activity of TrxR (Arscott et al., 1997; Gasdaska et al., 1995; Tanimura et al., 1999; Zhong et al., 1998). TrxR has broad substrate specificity and can reduce many other targets in addition to its support of Trx function (Arner and Holmgren, 2000). Three different human TrxR genes have been identified, encoding the cytosolic TrxR1 (Holmgren and Luthman, 1978), the mitochondrial TrxR2 (Chae et al., 1999; Miranda-Vizuete et al., 1998), and the testis-specific thioredoxin glutathione reductase TGR (Sun et al., 2001). In addition, several different splice variants have been described (Rundlof et al., 2000).

Thioredoxin reductase and cancer

Overexpression or constitutive activation of TrxR has been reported in human cancers as well as in a number of different cancer cell lines and may be associated with aggressive tumor growth and poor survival (Arner and Holmgren, 2000; Rundlof and Arner, 2004). The upregulation of TrxR confers growth advantage to tumor cells probably due to the growth promoting affect of the thioredoxin system via facilitation of deoxyribonucleotide generation, but also via protection of cells from the increased basal oxidative stress. The elevated ROS levels in tumor cells are thought to be a result of oncogenic signaling and malfunctioning of the respiratory chain (Szatrowski and Nathan, 1991; Toyokuni et al., 1995). Therefore, a good strategy to kill cancer cells might be by tipping this balance through enhancing the production of ROS in tumor cells, thus exhausting the antioxidant systems of the cells, or alternatively, inhibiting

antioxidants (Nordberg and Arner, 2001; Ozben, 2007). TrxR is therefore thought to be a promising target for cancer therapy (Arner and Holmgren, 2006; Becker et al., 2000; Biaglow and Miller, 2005; Nguyen et al., 2006; Urig and Becker, 2006). Several studies have demonstrated an inhibition of tumor growth upon downregulation of TrxR using siRNA (Yoo et al., 2006). Furthermore, inhibitors of TrxR, such as the gold-containing compound auranofin, have been shown to induce cell death in cancer cells (Kim et al., 2004; Krishnamurthy et al., 2008; Marzano et al., 2007; Simon et al., 1981).

Inhibitors of thioredoxin reductase

The accessible and reactive selenocysteine residue makes TrxR a very susceptible target for inhibition by different compounds. The gold compound auranofin is one of the most effective and selective inhibitors of TrxR (Hill, McCollum et al 1997; Gromer, Ascott et al, 1998), and as mentioned above proven to be effective as an anti-neoplastic agent. Furthermore, a number of clinically used chemotherapeutic anticancer drugs inhibit TrxR, including arsenicals, which are used in leukemia treatment (Lu et al., 2007), cisplatin, which irreversibly inhibits TrxR (Sasada, Nakamura et al 1999) and other alkylating agents, such as nitoureas used in treatment of brain tumors and lymphomas, and cyclophosphamide (Witte et al., 2005, Wang, Zang et al 2007).

Notably, inhibiting TrxR with electrophilic compounds may induce more severe cell death than using siRNA depletion (Cassidy et al.). TrxR1 which is derivatized at the selenocysteine residue, or which lacks the selenocysteine, yields a protein which can induce rapid and potent cell death in tumor cells, which is a gain-of-function property (Anestål et al 2006). These forms of TrxR1 are called SecTRAPS, for *selenium compromised thioredoxin reductase-derived apoptotic proteins*, and demonstrate that the effects of inhibiting TrxR are complex (Arner, 2009).

p53 and the thioredoxin system

There have been a number of reports linking p53 to the thioredoxin system in several ways. The first studies performed in yeast reported that the p53 induced growth arrest which normally occurs upon expressing human p53 in yeast was significantly decreased upon mutation or deletion of the *trr1* gene, a gene which bears functional overlap with the human TrxR genes (Pearson and Merrill, 1998). These data suggest that TrxR function is required for p53 function.

In contrast, a more intricate network between the two proteins appears to occur in human cells. Studies have shown that p53 is directly regulated by the thioredoxin system and becomes impaired by depletion of Trx or TrxR, or upon targeting TrxR with electrophilic compounds (Cassidy et al., 2006; Moos et al., 2003; Ravi et al., 2005). In line with these studies, it has been demonstrated that Trx together with Ref-1, an activator of p53 (Jayaraman et al., 1997), can induce p53 DNA binding activity *in vitro* and also enhance the p53-dependent expression of its target gene p21 (Ueno et al., 1999). However, in a study where the effect of endogenous TrxR on p53 in cells was explored without using overexpression vectors, it was shown that both TrxR and Ref-1 are important for the control of basal p53 stability and activity, but not for p53 activity upon stress conditions (Seemann and Hainaut, 2005). This correlates well with the antioxidant functions of p53 in unstressed cells, which is important to prevent the accumulation of DNA damage in response to everyday strains (Sablina et al., 2005). Additionally, it is logical to suppose that increased ROS formation due to inhibition of TrxR activates p53 and induces its pro-oxidant functions, which involve induction of target genes increasing the levels of ROS further, facilitating p53-mediated apoptosis.

AIMS OF THE THESIS

The general aims of this thesis were to identify new p53 reactivating compounds and to investigate the mechanisms of action of the p53-reactivating small molecule RITA.

The specific aims were:

- To identify small molecules that can suppress the growth of human tumor cells in a wild type p53-dependent manner (Paper I).
- To elucidate the molecular mechanism of p53 response upon RITA treatment using oligonucleotide microarray analysis and to investigate the mechanisms leading to preferential induction of apoptosis by RITA (Paper II).
- To study the effect of RITA on the p53 regulator HDMX (Paper III).
- To investigate the impact of TrxR1 inhibition by RITA (Paper IV).

RESULTS

PAPER I

Tumor-specific induction of apoptosis by a p53-reactivating compound.

Inactivation of p53 in tumors, by mutation or by overexpression of its negative regulators occurs in the majority, if not all tumors. Reconstitution of p53 results in regression of already established tumors, as shown in mouse models (Issaeva et al., 2004; Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). Therefore, p53 is considered as a promising target for cancer therapy. We have earlier identified a low molecular weight compound RITA in a cell-based screen for wild-type p53-reactivating molecules (Issaeva et al., 2004). In the present study we identified and characterized another p53-reactivating compound NSC 162908. Our in-depth analysis revealed that NSC 162908, which we named MITA, restored the function of p53 by targeting the p53/MDM2 interaction.

The HCT116 cell line harboring wild type p53 and its isogenic derivative, HCT116 *TP53*^{-/-}, in which p53 has been knocked out by means of homologous recombination (Bunz et al., 1998) were used in a cell-based screen of a chemical library from NCI. We identified a compound which suppressed tumor cell growth in a wild-type p53-dependent manner. MITA induced apoptosis in a p53-dependent manner in tumor cells, but did not affect the growth of normal cells such as human diploid fibroblasts (NHDF) or mammary epithelial cells, MCF10A and 184A1.

Treatment of MITA resulted in p53 accumulation in tumor cells, due to its increased half-life. Notably, we did not observe an induction of p53 in NHDF, which correlated with the absence of growth suppression and apoptosis. The stabilization of p53 in tumor cells was not associated with p53 phosphorylation of Ser15 or phosphorylation of γ H2AX, two typical events triggered by treatment with DNA damaging agents. This indicates that induction of p53 by MITA did not involve DNA damage signaling. Instead we showed that MITA treatment prevented the binding between p53 and MDM2 *in vitro* and in cells, thus blocking p53 ubiquitination and proteasomal degradation. This resulted in accumulation of p53 in cells.

Furthermore, the transcriptional activation function of p53 was restored in cells upon treatment with MITA as evidenced by the induction of p53 responsive *lacZ* reporter gene and by transcriptional activation of endogenous p53 target genes, *MDM2*, *PUMA*, *BAX*, and *GADD45*. Surprisingly, p53 target gene p21 was induced not only in p53-positive tumor cells, but also in p53-negative cells as well as in NHDF. Small molecules such as MITA are bound to have more than one target in cells which could account for the p53-independent p21 induction. It is indeed known that p21 is regulated by several other transcription factors such as E2F1 (Hiyama et al., 1998), p73 (Goldschneider et al., 2004), AP2 (Zeng et al., 1997), BRCA1 (Somasundaram et al., 1997), and STAT1 (Chin et al., 1996). The increase of p21 could explain the weak G2-arrest observed in HCT116 *TP53*^{-/-} cells. However, the long-term growth suppression assay suggests that the arrest was transient and did not affect the survival of p53-null cells.

In summary, we described a tumor-specific p53-reactivating molecule which might serve as a lead compound for novel non-genotoxic anti-cancer drugs.

Conclusions from paper I

- MITA induces apoptosis in tumor cells in a wild-type p53-dependent manner.
- Treatment with MITA prevented the interaction between p53 and MDM2 *in vitro* and in cells
- The p53 protein was stabilized and activated upon treatment with MITA, leading to the transcription of p53 target genes such as *MDM2*, *PUMA*, *BAX*, and *GADD45*.

PAPER II

MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53.

We have previously described the wild-type p53-reactivating compound RITA (Issaeva et al., 2004). RITA targets p53 directly, inhibiting its binding to MDM2 and other negative regulators, resulting in p53 activation and induction of apoptosis in tumor cells and in human tumor xenografts (Issaeva et al., 2004; Zawacka-Pankau et al., manuscript in preparation).

To explore the molecular mechanism of action of RITA in more detail we conducted oligonucleotide microarray analysis using the HCT116 cell line and its p53-null derivative, HCT116 *TP53*^{-/-}. We hybridized cDNA prepared from parental and p53-null HCT116 cells untreated or treated with 1 μ M RITA for 12 hours to Affymetrix hgu133a chips containing ~22,000 probe sets corresponding to ~15,000 known human genes. Using a false discovery rate of 5% and the Significance Analysis of Microarrays (SAM) procedure (Tusher et al., 2001) to assess the statistical significance of differential expression of individual genes, on a two-sample *t*-statistic, we identified 800 differentially regulated genes in the HCT116 cells treated with RITA compared to untreated samples (the 0.8k list). The same procedure on the HCT116 *TP53*^{-/-} samples yielded zero differentially regulated genes, revealing that the activity of RITA was completely specific to the wild type p53-expressing cell line with negligible effect on the p53-null cell line. The results from the microarray analysis of differentially regulated genes were confirmed by quantitative real-time PCR (qPCR), which showed induction of *FAS*, *PMAIP1* (Noxa), *BBC3* (PUMA), and *GADD45* in HCT116 cells, but not in HCT116 *TP53*^{-/-}.

To further investigate the nature of the responses to RITA, we analyzed differential expression at the level of pathways, applying a procedure based on (Tian et al., 2005). The highest up-regulated pathway was that of p53 pro-apoptotic targets, followed by pathways related to apoptosis and DNA damage signaling. By inhibiting Fas or PUMA, we examined the functional significance of individual target genes representing the extrinsic and intrinsic apoptotic pathways, and concluded that both pathways were involved in apoptosis induced by RITA.

Notably, other p53-activating stimuli, 5-fluorouracil (5-FU), hydrogen peroxide (H₂O₂), nitric oxide (NO), hypoxia, and hydroxyurea (HU) did not produce the same pattern of gene expression as RITA treatment did. Instead, induction of cell-cycle arrest genes was more profound for most treatments, except for 5-FU. In line with above results, assessment of RITA induction of apoptosis and/or growth arrest in p53 wild-type expressing cell lines of different origin, A549, A498, HCT116 and U2OS revealed a predominant apoptotic response. In contrast, the p53 activator nutlin3a predominantly

induced growth arrest in these cell lines. Comparison of p53 target genes induced by nutlin3a (Tovar et al., 2006) and RITA further demonstrated that nutlin3a induced a greater fraction of p53 target genes involved in cell-cycle control, compared to RITA. Considering that these two molecules both prevent p53/MDM2 interaction, we further investigated the mechanisms for preferential induction of pro-apoptotic genes by RITA. We reasoned that MDM2 released from p53 by RITA could target other proteins. Upon further investigation we found that the p53 transcriptional cofactor hnRNP K (Moumen et al., 2005) was degraded in a MDM2-dependent manner upon RITA treatment, but not by nutlin3a treatment. The major known target for hnRNP K as a p53 transcriptional cofactor is the *CDKN1A* gene encoding p21 (Moumen et al., 2005). Furthermore, p21 has been demonstrated to be a negative regulator of apoptosis (Baptiste-Okoh et al., 2008; Steinman and Johnson, 2000; Suzuki et al., 1998) RITA induced p21 mRNA in a p53-dependent manner as determined by qPCR, microarray analysis and Northern blot (Issaeva et al., 2004). However, in comparison to nutlin3a and 5-FU treatment, the induction of p21 was deficient after RITA. We showed that the inefficient transcriptional induction of p21 was due to the absence of hnRNP K on the *CDKN1A* (p21) promoter.

In line with these results we found that p21 protein levels were induced after nutlin3a treatment, whereas after RITA treatment, the level of p21 was not increased, but rather reduced. The absence of efficient transactivation of p21 cannot solely explain the strong reduction of the p21 protein which we observed, suggesting the involvement of additional level(s) of regulation. Further studies revealed that p21 protein stability was compromised due to proteasomal degradation in MDM2-dependent manner. These results uncovered downregulation of p21 by RITA on two different levels: transcription and protein stability and that MDM2 is a crucial player in both these processes. MDM2 induced proteasomal degradation of hnRNP K and p21, thus affecting p21 transcription and protein turnover.

In summary we propose a model (Fig 4) in which functional MDM2, released from p53 after treatment with RITA, contributes to the apoptotic response by promoting proteasomal degradation of the p53 transcriptional cofactor hnRNP K, which impairs p53-mediated induction of transcription of pro-arrest genes, such as p21. In addition, the proteasomal degradation of p21 protein is enhanced by MDM2, eliminating p21 anti-apoptotic function. In turn, transcriptional upregulation of MDM2 induced by p53 upon RITA treatment amplifies the negative effect of MDM2 on hnRNP K and p21.

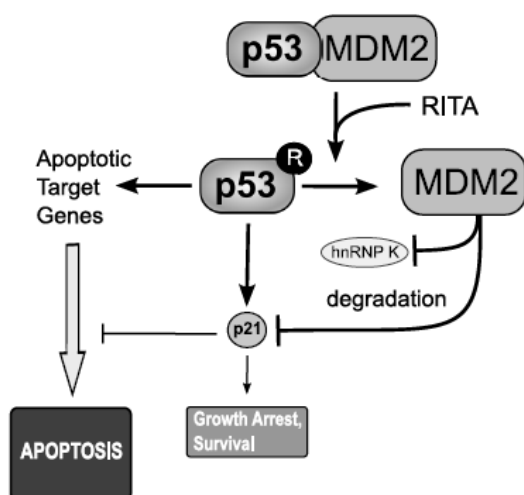


Figure 4. RITA induces dissociation of active MDM2 from p53. Released MDM2 is free to promote degradation of its other targets such as p21 and hnRNP K. p53 transactivates expression of proapoptotic target genes, but transactivation of p21 is deficient because of hnRNP K deficiency. p21 protein levels are thus kept low by both direct degradation by MDM2 and inefficient transcriptional induction. Therefore, p21 can exercise neither its growth-suppressing activity nor its antiapoptotic activity, and the cell undergoes programmed cell death.

Conclusions from Paper II

-RITA targets p53 with high specificity as evidenced by microarray analysis of gene expression profiles in p53-positive and p53-negative cells.

-p53 activated by RITA induces mainly pro-apoptotic target genes, which results in robust apoptosis of tumor cells.

-MDM2 acts as a switch between growth arrest and apoptosis upon p53 activation by downregulating p21 on both transcriptional and protein level.

PAPER III

Pharmacologically activated p53 downregulates HDMX gene transcription and induces HDMX protein degradation

It has been suggested that in order to get robust p53 activation, not only MDM2 has to be displaced from p53, but also HDMX (Toledo and Wahl, 2006; Wang et al., 2007b). The p53 activator nutlin3a, which binds MDM2, was shown to have a 30-fold lower efficiency in disrupting the p53/HDMX interaction than the p53/MDM2 complex, which leads to incomplete activation of p53 in cells with high HDMX levels (Hu et al., 2007; Patton et al., 2006; Wade et al., 2006; Xue et al., 2007) In contrast to small molecules targeting MDM2, the small molecule RITA binds p53 and induces a conformational change leading to the prevention of p53 interaction with several inhibitors, including MDM2, iASPP and Parc (Issaeva et al., 2004; Zawacka-Pankau et al., manuscript in preparation). Furthermore, we have demonstrated that RITA triggers apoptosis in a number of human tumor cell lines in which nutlin3a induces growth arrest (Enge et al., 2009). Therefore, we addressed the question whether RITA affects the p53/HDMX interaction.

We found that RITA indeed disrupted the p53/HDMX interaction in cells as well as *in vitro*. Furthermore, using kinetic studies we demonstrated that HDMX protein levels were decreased upon RITA treatment. The level of the closely related MDM2 protein rather oscillated, in line with previously described effect upon p53 activation (Lev Bar-Or et al., 2000). The downregulation of HDMX protein appeared to be p53-dependent, in view of the observation that it did not occur in any of the p53-null cell lines used, HCT116 *TP53*^{-/-}, Saos-2 and H1299. This conclusion was further validated using the previously described p53 inhibitor pifithrin- α (PFT- α) (Komarov et al., 1999). Inhibition of p53 by pre-treatment with PFT- α prior to RITA treatment abolished the decline of HDMX. Importantly, the p53-dependent HDMX downregulation was not confined to cell culture experiments, but was also detected *in vivo* upon RITA treatment of HCT116 tumor xenografts in mice, but was not observed upon treatment of HCT116 *TP53*^{-/-} tumor xenografts.

We have previously demonstrated that induction of p53 level and activation of p53 function by RITA requires deregulated oncogenes (Grinkevich et al., 2009; Issaeva et al., 2004). Therefore we tested whether the decline of HDMX was dependent on p53 activation and examined HDMX level upon RITA treatment in non-transformed cells. In line with the absence of p53 induction, there were no changes in HDMX levels in the non-transformed cell lines tested, including normal human diploid fibroblasts (NHDF) and mammary epithelial cells, MCF10A and 184A1.

Unlike MDM2 and p53, HDMX is a stable protein with a long half-life which, however, was destabilized upon treatment with RITA. We demonstrated that proteasomal degradation of HDMX was triggered by RITA. It is well known that DNA damage signaling induces ubiquitination and degradation of HDMX (Chen et al., 2005b; Pereg et al., 2005). We show that inhibition of ATM partially prevented the degradation of HDMX, indicating that signaling via ATM contributed to the decrease in HDMX levels. This is in line with recently discovered induction of p53-dependent DNA damage signaling by RITA (Nikulenkov et al., manuscript in preparation; Yang et al., 2009). The mRNA levels of HDMX also declined in p53-positive cells upon treatment with RITA, strongly suggesting that transcriptional repression of HDMX induced by RITA is p53-dependent.

Upon examining a panel of 10 human tumor cell lines of different origin, we observed a decline of HDMX in all, except two lines, the osteosarcoma cell line SJSA and the colon carcinoma cell line RKO. Furthermore, these two cell lines were the only ones which did not undergo apoptosis upon RITA treatment, suggesting a correlation between downregulation of HDMX levels and the induction of apoptosis by RITA.

In line with the notion that HDMX is an important p53 inhibitor and its downregulation is essential for the induction of apoptosis by p53, siRNA-mediated depletion of HDMX synergized with RITA in apoptosis induction in tumor cells. However, overexpression of HDMX did not confer protection from RITA induced apoptosis, probably due to enhanced degradation of ectopically expressed HDMX induced by RITA.

In summary, we demonstrate that inhibition of p53 by HDMX is relieved by the small molecule RITA on three different levels, resulting in robust p53 activation.

Conclusions from paper III

- RITA can inhibit the interaction between p53 and HDMX.
- RITA induces downregulation of HDMX protein level in a p53-dependent manner.
- The mRNA level of HDMX decline upon treatment with RITA in a p53-dependent manner.
- Downregulation of HDMX correlates with RITA-induced apoptosis.

PAPER IV

p53-dependent inhibition of TrxR1 contributes to the tumor-specific induction of apoptosis by RITA

The selenoprotein thioredoxin reductase 1 (TrxR1) is a key regulator of major redox-dependent cellular pathways which is often overexpressed in cancer. Several studies have identified TrxR1 as a potentially important target for anticancer therapy (Arner and Holmgren, 2006; Becker et al., 2000; Biaglow and Miller, 2005; Nguyen et al., 2006; Urig and Becker, 2006).

Based upon the known functional links between p53 and TrxR1, we explored whether RITA could target TrxR1 directly. We found that the activity of pure recombinant TrxR1 was indeed inhibited by RITA, and that RITA could bind specifically to TrxR1 in a non-covalent manner. Cellular TrxR activity was thereafter measured in HCT116 and HCT116 *TP53*^{-/-} cells. RITA treatment inhibited TrxR activity in both cell lines, but to a greater extent in p53-positive cells, implying a role of p53 in RITA-mediated TrxR inhibition. Further, the activity of TrxR was not affected in NHDF, which

correlated with the absence of p53 activation and growth suppression by RITA in normal cells (Grinkevich et al., 2009; Issaeva et al., 2004). These results suggest that RITA directly inhibits TrxR1 activity *in vitro* whereas in cells the inhibition of TrxR correlates with activation of p53 by RITA. In contrast, the gold-based TrxR inhibitor auranofin strongly inhibited TrxR activity in all cell types.

We speculated that the ability of p53 to induce pro-oxidant genes might potentially enhance the inhibitory effect of TrxR by induction of ROS. Analysis of changes in gene expression induced by RITA in p53-positive and p53-negative HCT116 cells revealed a p53-dependent induction of p53 pro-oxidant target genes *BAX*, *BBC3* (PUMA) (Enge et al., 2009), *TP53I3* (PIG3) and *FDXR*. In addition, two genes with pro-oxidant activity, which were not previously shown to be p53 targets, *NOX1* and *SHC1*, were found to be induced. It is thus possible that one or all of these genes might synergize with the inhibition of TrxR thus leading to a potent apoptosis induction.

Unexpectedly, we observed an accumulation of a ~130kDa band detected by the anti-TrxR1 antibody in HCT116 cells treated with RITA but not in the p53-null cells. Nor was this new form induced by auranofin, demonstrating a correlation between TrxR inhibition by RITA and accumulation of the ~130kDa band of TrxR1. Depletion of TrxR1 by means of siRNA prevented the formation of the band by RITA in HCT116 cells, strongly suggesting that this band represented a new RITA-induced form of TrxR1. Prevention of protein expression by cycloheximide did not block the formation of the ~130kDa band, indicating that this form did not represent the expression of a new splice variant, but instead indicated a p53-dependent formation of covalently linked TrxR1 dimers. Interestingly, such TrxR1 dimer formation was not observed in NHDF treated with RITA, which correlated with the absence of p53 activation and inhibition of TrxR1 activity. Furthermore, ectopic expression of p53 in HCT116 *TP53*^{-/-} cells also triggered the induction of the TrxR1 dimer, supporting the notion of p53-dependent modification of TrxR1 leading to the formation of the ~130 kDa form. Taken together, our data suggest a p53-dependent formation of covalently linked TrxR1 dimer upon RITA treatment.

Since TrxR1 is one of the major enzymes controlling the levels of ROS in cells we investigated the intracellular ROS levels upon treatment with RITA. ROS levels were induced in a p53-dependent manner in HCT116 cells upon RITA treatment. In contrast, auranofin treatment led to ROS induction in both p53-positive and p53-negative cell lines, in line with the auranofin-mediated TrxR inhibition. Notably, RITA did not increase ROS levels in NHDF. To further examine whether the induction of ROS by auranofin or RITA correlates with growth suppression, cells were placed at low oxygen levels (5% O₂) to reduce the formation of ROS. We observed a partial rescue of HCT116 cells from growth suppression by both RITA and auranofin at low oxygen conditions, suggesting that the increased ROS levels do contribute to growth suppression.

Thus, in this study we identified a new cellular target of the small molecule RITA, namely TrxR1. We demonstrated that RITA binds purified TrxR1 protein directly and inhibits its activity *in vitro* and in cells. Notably, the inhibition of TrxR by RITA in cells appeared to be p53-dependent and correlated with the induction of a covalently linked TrxR1 dimer, a phenomenon that has not been described previously. These effects of RITA also correlated with increased levels of ROS. Our results suggest that inhibition of TrxR1 in a tumor-dependent and p53-dependent manner might contribute to the tumor-specific cell killing properties of RITA.

Conclusions from paper IV

- RITA can inhibit the activity of TrxR *in vitro* and in cells.
- RITA can bind TrxR1 directly in a non-covalent manner.
- RITA induces the formation of a covalently linked TrxR1 dimer in a p53-dependent manner.
- RITA increases the intracellular ROS levels in tumor cells in a p53-dependent manner.

DISCUSSION AND FUTURE PERSPECTIVE

This thesis includes studies concerning identification of a new molecule targeting p53, MITA, as well as the mechanisms of action of the previously described p53 activating compound RITA (Grinkevich et al., 2009; Issaeva et al., 2004).

Both RITA and MITA were discovered using cell-based screening assays. The cellular system used was the HC116 cell line and its p53-null isogenic derivative HCT116 *TP53*^{-/-}. This strategy allows the identification of compounds that can rescue p53 function in cells irrespective of the molecular mechanism. The advantage of this approach is that compounds which are toxic in an unspecific manner will not be scored. Additionally, our screening procedure allows the identification of p53 activators irrespective of their mechanism of action (Bykov et al., 2002; Issaeva et al., 2004).

Biochemical screening is another generally used approach to find potential drugs. The discovery of the p53 activating molecules nutlins is one example of where this method was applied (Vassilev et al., 2004). Nutlins were discovered by their ability to bind the p53-binding pocket of MDM2, and were later found to inhibit the p53/MDM2 interaction and consequently activating p53 in cells. The weakest point of rational designed drugs is that due to our limited knowledge of the cellular networks it is difficult to predict how a certain drug will affect other proteins in the cells. In addition, drugs identified *in vitro* might not be able to act on target proteins in a cellular context, which might lead to unexpected and perhaps undesirable effects or, alternatively, insufficient effects. This is illustrated by nutlin3a, which although proven to be efficient in inducing cell cycle arrest and to a lesser extent apoptosis in tumor cell lines, and *in vivo* models, is ineffective in binding to HDMX, which is closely related to MDM2, resulting in a compromised p53 activation in many cells (Hu et al., 2007; Patton et al., 2006; Wade et al., 2006; Xue et al., 2007).

Although the pros and cons of these two approaches could be discussed further, we believe, that the most advantageous part of cell-based or phenotypic screening is that the exact molecular mechanism is not pre-defined by the screening assay. The molecules are not limited to interfering with a known member of the p53 pathway and may thus involve yet unknown mechanisms of protein regulation, which later can be revealed. This is exemplified by recent work from our lab in elucidating the mechanisms involved in RITA-induced apoptosis. A study by Grinkevich et al. demonstrates that there is a potent inhibition of crucial oncogenes by p53 upon RITA treatment. Activated p53 relieves transcriptional repression of the anti-apoptotic proteins Mcl-1, Bcl-2, MAP4 and survivin, blocks Akt pathway on several levels and downregulates the oncogenes c-Myc, cyclin E and β -catenin (Grinkevich et al., 2009).

Furthermore, in paper II we performed a transcriptome-wide oligonucleotide microarray experiment. The analysis revealed that the activity of RITA was completely specific to the wild type p53-expressing cell line with negligible effect on the gene expression in the p53-null cell line. Further investigation demonstrated that the biological response to RITA, in a number of tumor cell lines was mainly apoptotic, in contrast to nutlin3a, which predominantly induced growth arrest. Upon elucidating the differences between the mechanisms of action between these two compounds we found that RITA induces MDM2-dependent downregulation of p21 on both transcriptional and protein level. Thus, upon pharmacological activation of p53, MDM2 can play an important role in the switch between growth arrest and apoptosis by regulating p21. We speculate that the binding of nutlin3a to MDM2 might prevent its interaction with other substrates, such as hnRNP K and p21. However, additional studies are necessary to investigate this hypothesis.

Moreover, in paper III we show that RITA targets the p53 regulator HDMX on three different levels: by disrupting the p53/HDMX interaction, by reducing HDMX protein levels and by downregulating its mRNA levels. The targeting of both MDM2 and HDMX is thought to be necessary for full p53 activation, therefore this demonstrates another possible reason for the preferred induction of apoptosis by RITA compared to nutlin3a. In contrast to nutlins, RITA binds p53 (Issaeva et al., 2004; Zawacka-Pankau et al., manuscript in preparation), which might explain its effect on both p53/MDM2 and p53/HDMX complexes. Although the p53-binding pockets of MDM2 and HDMX are similar, that of HDMX is smaller due to the protruding side chains of two residues which are absent in MDM2 (Popowicz et al., 2008). Most likely, this is the major reason why MDM2 inhibitors such as nutlin3a (Vassilev et al., 2004) have different affinities for MDM2 and HDMX (Hu et al., 2007; Patton et al., 2006; Wade et al., 2006; Xue et al., 2007).

In conclusion, we show that RITA appears to affect many different targets in cells, but in a p53-dependent manner. This is of interest as a number of the modern targeted therapies have led to the development of drug resistance due to secondary mutations which greatly limits the ability of these drugs to significantly prolong patient survival (Fojo, 2008; Traxler, 2003). Therefore, a new concept has emerged favoring the idea that multi-targeting therapy might be more rational to efficiently eliminate tumor cells, and also to limit the emergence of drug resistance. In paper IV we identified an additional target of RITA, namely TrxR1. TrxR1 is often overexpressed in cancer and has been identified as a potential target for anticancer therapy. The upregulation of TrxR1 in tumor cells is presumably associated with elevated ROS levels due to oncogene activation and malfunctioning of the respiratory chain (Szatrowski and Nathan, 1991; Toyokuni et al., 1995). In order to retain the redox balance, cancer cells are dependent on several antioxidant systems, such as the thioredoxin system and, hence, they should be more sensitive to its inhibition (Hintze et al., 2003; Sakurai et al., 2005). Therefore, a good strategy to kill cancer cells might be by tipping this balance through enhancing the production of ROS in tumor cells thus exhausting the antioxidant systems of the cells, or alternatively, inhibiting antioxidants (Nordberg and Arner, 2001; Ozben, 2007). RITA was demonstrated to target TrxR1 and induce both pro-oxidant genes and ROS in a p53- and tumor-dependent manner. Furthermore, a presumed covalently linked dimer of TrxR1 was induced in a p53-dependent manner upon RITA treatment. We speculate that this dimerized form is inactive, however further studies are necessary to elucidate how this dimer arises upon RITA treatment and the consequences of its formation. The unexpected and unique p53-dependent effects of RITA on TrxR1 in cancer cells might be an important part of the p53-dependent induction of oxidative stress and apoptosis triggered by RITA, thus leading to a robust induction of cancer cell death. Future studies will help to elucidate these mechanisms further.

Concerning the identified p53 reactivating compound MITA in paper I, future studies will be focused on optimization of MITA. Since it was toxic upon application in mice, generation of non-toxic analogs using medicinal chemistry approach is required. In addition, it will be important to elucidate the mechanisms of MITA action. Our results so far demonstrate that MITA appears to have a distinct mechanism of action compared to RITA, possibly targeting MDM2 rather than p53.

Finally, we believe that compounds such as RITA and MITA may not only be used as lead compounds for anti-cancer therapy, but may also be useful tools for the study of p53 functional activity. Further work aimed at detailed characterization of molecular events upon p53 activation might help to guide rational development of more efficient and less toxic drugs.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to all the people who have helped me during these years. In particular I would like to thank:

My supervisor, *Galina Selivanova*, thank you for giving me the opportunity to work in your lab and introducing me to the p53 field. For being enthusiastic and always coming up with new ideas.

My co-supervisor, *Joanna Zawacka-Pankau*, without your support I would not have come so far! You have been a great help to me practically in the lab, but also in discussing scientific issues. However, you have also become a dear friend of mine, ever since you came to Stockholm that summer many years ago!

All the past and present members of the Selivanova group who have helped me in one way or another and for all the good times: *Martin*, my companion in the lab from the very start. Thanks for your help and for all the fun times. Good luck with your future work! *Clemens* for always being happy and helping me with the HDMX project. *Vera*, for being so helpful and supportive. *Ying*, for always listening and giving me a helping hand whenever. *Fedor*, the great photographer, for your good sense of humor! *Wenjie*, for your help and support in the lab. And also to *Yao, Hai, Natalya, Sabine, Diana, Andreas, Betty* and *Natalia*.

My collaborators; *Elias Arnér* for all the help and support you have given me with the TrxR-project. *Karin*, who was there in the beginning to teach me the enzymatic assays. *Sofi*, who took over from Karin, and has struggled with me last few years, it has been great working with you! I will miss my “second lab”! Also thanks to *Marcus* and *Pascal* for their experiments.

Some of the MTC friends: *Anna*, for the short and long talks in the corridor about life and work. *Emilie* for the smiles and chit-chats about sneakers. *Jacob*, for exchanging day-to-day experiences and struggles at work. *Marina* for always being happy! *Dani*, for being a great guy and for going out with Alex when I never had time, we miss you!

Thanks to the members of the Klas Wiman group at CCK, for good company at conferences and the KI retreats, special thanks to *Salah, Anna* and *Jeremy*. My mentor *Marianne*, although I haven't taken advantage of you as much as I could, I always knew that you had time for a coffee and chat if I needed it!

My biomedicine friends: *Mira, Linda, Pauline* and *Emma* for being good friends and for all the great times together – the parties and especially the dinners which always come with some good advice! Thanks for your support! *Kalle*, for being great fun!

My friends from outside Karolinska; *Elina, Karin* and *Anki*, my beloved childhood friends! The Kungsholmen girls; *Sara, Karolin* and *Sofie*, for fun nights out, dinners, holidays, flat-mating and just for being great friends! The Eugenia gang, *Alexandra*,

Emilie, Franco, Gustaf, Marcus and Thomas for all the crazy times at Marielund, Sågmyra and Korsnäs. For the “Första måndan” celebrations and much more! *Alexandra* for being a good friend although far away. *Emilie*, for the nice lunches with discussions about life in general and of course work – we just should have done it more often! *Eva* for being a true friend! *Johan* and *Emir* for the great dinners and football evenings in the good old times, and not to forget, Heads up! *Maria* and *Elisabeth* for all the fun summers in Sala, now I’ll be able to spend more time there with you and your lovely families.

Mi familia de Barcelona, *Rosi, Luis y Patty* gracias por los buenos momentos que hemos pasado juntos! Y *Patty* – muchas gracias por tu ayuda con la portada de mi tesis.

My brother, *Erik* and his family, *Cissie, Alton* and little *Vilgot*. Thank you for supporting me and also for all the fun and lovely times! I promise to come and visit you more often now!

My *Mum* and *Dad*, thanks for always believing in me and for being there, no matter what! For helping me with small and big things! For all your love, support and encouragement!

Finally, I would like to thank *Alex*, the best thing that happened to me during my PhD! Meeting you has made it all worth while! Thank you for all your love and support, and for always trying to put a smile on my face after a hard day at work.

REFERENCES

- Adams, J.M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. *Science (New York, NY)* 281, 1322-1326.
- Appella, E., and Anderson, C.W. (2001). Post-translational modifications and activation of p53 by genotoxic stresses. *European journal of biochemistry / FEBS* 268, 2764-2772.
- Arner, E.S. (2009). Focus on mammalian thioredoxin reductases -- important selenoproteins with versatile functions. *Biochimica et biophysica acta*.
- Arner, E.S., and Holmgren, A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. *European journal of biochemistry / FEBS* 267, 6102-6109.
- Arner, E.S., and Holmgren, A. (2006). The thioredoxin system in cancer. *Seminars in cancer biology* 16, 420-426.
- Arscott, L.D., Gromer, S., Schirmer, R.H., Becker, K., and Williams, C.H., Jr. (1997). The mechanism of thioredoxin reductase from human placenta is similar to the mechanisms of lipoamide dehydrogenase and glutathione reductase and is distinct from the mechanism of thioredoxin reductase from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 94, 3621-3626.
- Ashcroft, M., Ludwig, R.L., Woods, D.B., Copeland, T.D., Weber, H.O., MacRae, E.J., and Vousden, K.H. (2002). Phosphorylation of HDM2 by Akt. *Oncogene* 21, 1955-1962.
- Avval, F.Z., and Holmgren, A. (2009). Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for Mammalian S phase ribonucleotide reductase. *The Journal of biological chemistry* 284, 8233-8240.
- Baptiste-Okoh, N., Barsotti, A.M., and Prives, C. (2008). Caspase 2 is both required for p53-mediated apoptosis and downregulated by p53 in a p21-dependent manner. *Cell cycle (Georgetown, Tex)* 7, 1133-1138.
- Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldborg, P., Sehested, M., Nesland, J.M., Lukas, C., *et al.* (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 434, 864-870.
- Becker, K., Gromer, S., Schirmer, R.H., and Muller, S. (2000). Thioredoxin reductase as a pathophysiological factor and drug target. *European journal of biochemistry / FEBS* 267, 6118-6125.
- Bensaad, K., and Vousden, K.H. (2005). Savior and slayer: the two faces of p53. *Nature medicine* 11, 1278-1279.
- Bergamaschi, D., Samuels, Y., O'Neil, N.J., Trigiant, G., Crook, T., Hsieh, J.K., O'Connor, D.J., Zhong, S., Campargue, I., Tomlinson, M.L., *et al.* (2003). iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human. *Nature genetics* 33, 162-167.
- Beroud, C., and Soussi, T. (2003). The UMD-p53 database: new mutations and analysis tools. *Human mutation* 21, 176-181.
- Biaglow, J.E., and Miller, R.A. (2005). The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy. *Cancer biology & therapy* 4, 6-13.
- Bond, G.L., Hu, W., Bond, E.E., Robins, H., Lutzker, S.G., Arva, N.C., Bargonetti, J., Bartel, F., Taubert, H., Wuerl, P., *et al.* (2004). A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell* 119, 591-602.
- Bouska, A., and Eischen, C.M. (2009). Mdm2 affects genome stability independent of p53. *Cancer research* 69, 1697-1701.
- Brodsky, M.H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G.M., and Abrams, J.M. (2000). *Drosophila* p53 binds a damage response element at the reaper locus. *Cell* 101, 103-113.
- Budanov, A.V., Sablina, A.A., Feinstein, E., Koonin, E.V., and Chumakov, P.M. (2004). Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science (New York, NY)* 304, 596-600.
- Bulavin, D.V., Saito, S., Hollander, M.C., Sakaguchi, K., Anderson, C.W., Appella, E., and Fornace, A.J., Jr. (1999). Phosphorylation of human p53 by p38 kinase coordinates

N-terminal phosphorylation and apoptosis in response to UV radiation. *The EMBO journal* 18, 6845-6854.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W., and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science (New York, NY)* 282, 1497-1501.

Bykov, V.J., Issaeva, N., Selivanova, G., and Wiman, K.G. (2002). Mutant p53-dependent growth suppression distinguishes PRIMA-1 from known anticancer drugs: a statistical analysis of information in the National Cancer Institute database. *Carcinogenesis* 23, 2011-2018.

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., and Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science (New York, NY)* 281, 1677-1679.

Cassidy, P.B., Edes, K., Nelson, C.C., Parsawar, K., Fitzpatrick, F.A., and Moos, P.J. (2006). Thioredoxin reductase is required for the inactivation of tumor suppressor p53 and for apoptosis induced by endogenous electrophiles. *Carcinogenesis* 27, 2538-2549.

Chae, H.Z., Chung, S.J., and Rhee, S.G. (1994). Thioredoxin-dependent peroxide reductase from yeast. *The Journal of biological chemistry* 269, 27670-27678.

Chae, H.Z., Kang, S.W., and Rhee, S.G. (1999). Isoforms of mammalian peroxiredoxin that reduce peroxides in presence of thioredoxin. *Methods in enzymology* 300, 219-226.

Chan, T.A., Hermeking, H., Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1999). 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 401, 616-620.

Chen, D., Kon, N., Li, M., Zhang, W., Qin, J., and Gu, W. (2005a). ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. *Cell* 121, 1071-1083.

Chen, L., Gilkes, D.M., Pan, Y., Lane, W.S., and Chen, J. (2005b). ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. *The EMBO journal* 24, 3411-3422.

Cheng, Q., Sandalova, T., Lindqvist, Y., and Arner, E.S. (2009). Crystal structure and catalysis of the selenoprotein thioredoxin reductase 1. *The Journal of biological chemistry* 284, 3998-4008.

Chin, Y.E., Kitagawa, M., Su, W.C., You, Z.H., Iwamoto, Y., and Fu, X.Y. (1996). Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. *Science (New York, NY)* 272, 719-722.

Christophorou, M.A., Ringshausen, I., Finch, A.J., Swigart, L.B., and Evan, G.I. (2006). The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature* 443, 214-217.

D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., *et al.* (2002). Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nature cell biology* 4, 11-19.

Danovi, D., Meulmeester, E., Pasini, D., Migliorini, D., Capra, M., Frenk, R., de Graaf, P., Francoz, S., Gasparini, P., Gobbi, A., *et al.* (2004). Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. *Molecular and cellular biology* 24, 5835-5843.

de Graaf, P., Little, N.A., Ramos, Y.F., Meulmeester, E., Letteboer, S.J., and Jochemsen, A.G. (2003). Hdmx protein stability is regulated by the ubiquitin ligase activity of Mdm2. *The Journal of biological chemistry* 278, 38315-38324.

de Stanchina, E., McCurrach, M.E., Zindy, F., Shieh, S.Y., Ferbeyre, G., Samuelson, A.V., Prives, C., Roussel, M.F., Sherr, C.J., and Lowe, S.W. (1998). E1A signaling to p53 involves the p19(ARF) tumor suppressor. *Genes & development* 12, 2434-2442.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356, 215-221.

Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G.D., Dowd, P., O'Rourke, K., Koeppen, H., and Dixit, V.M. (2004). The ubiquitin ligase COP1 is a critical negative regulator of p53. *Nature* 429, 86-92.

Efeyan, A., Garcia-Cao, I., Herranz, D., Velasco-Miguel, S., and Serrano, M. (2006). Tumour biology: Policing of oncogene activity by p53. *Nature* 443, 159.

el-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. *Nature genetics* *1*, 45-49.

Enge, M., Bao, W., Hedstrom, E., Jackson, S.P., Moumen, A., and Selivanova, G. (2009). MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. *Cancer cell* *15*, 171-183.

Erster, S., Mihara, M., Kim, R.H., Petrenko, O., and Moll, U.M. (2004). In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Molecular and cellular biology* *24*, 6728-6741.

Fakharzadeh, S.S., Trusko, S.P., and George, D.L. (1991). Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *The EMBO journal* *10*, 1565-1569.

Fang, S., Jensen, J.P., Ludwig, R.L., Vousden, K.H., and Weissman, A.M. (2000). Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *The Journal of biological chemistry* *275*, 8945-8951.

Feng, J., Tamaskovic, R., Yang, Z., Brazil, D.P., Merlo, A., Hess, D., and Hemmings, B.A. (2004). Stabilization of Mdm2 via decreased ubiquitination is mediated by protein kinase B/Akt-dependent phosphorylation. *The Journal of biological chemistry* *279*, 35510-35517.

Fields, S., and Jang, S.K. (1990). Presence of a potent transcription activating sequence in the p53 protein. *Science (New York, NY)* *249*, 1046-1049.

Finlay, C.A., Hinds, P.W., and Levine, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. *Cell* *57*, 1083-1093.

Fojo, T. (2008). Commentary: Novel therapies for cancer: why dirty might be better. *The oncologist* *13*, 277-283.

Francoz, S., Froment, P., Bogaerts, S., De Clercq, S., Maetens, M., Doumont, G., Bellefroid, E., and Marine, J.C. (2006). Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. *Proceedings of the National Academy of Sciences of the United States of America* *103*, 3232-3237.

Frantz, S. (2001). Role model. *Nature reviews* *2*, 872-873.

Fujisawa, T., Takeda, K., and Ichijo, H. (2007). ASK family proteins in stress response and disease. *Molecular biotechnology* *37*, 13-18.

Funk, W.D., Pak, D.T., Karas, R.H., Wright, W.E., and Shay, J.W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. *Molecular and cellular biology* *12*, 2866-2871.

Gaididon, C., Moorthy, N.C., and Prives, C. (1999). Ref-1 regulates the transactivation and pro-apoptotic functions of p53 in vivo. *The EMBO journal* *18*, 5609-5621.

Gasdaska, P.Y., Gasdaska, J.R., Cochran, S., and Powis, G. (1995). Cloning and sequencing of a human thioredoxin reductase. *FEBS letters* *373*, 5-9.

Giaccia, A.J., and Kastan, M.B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. *Genes & development* *12*, 2973-2983.

Gilkes, D.M., Chen, L., and Chen, J. (2006). MDMX regulation of p53 response to ribosomal stress. *The EMBO journal* *25*, 5614-5625.

Girnita, L., Girnita, A., and Larsson, O. (2003). Mdm2-dependent ubiquitination and degradation of the insulin-like growth factor 1 receptor. *Proceedings of the National Academy of Sciences of the United States of America* *100*, 8247-8252.

Gladyshev, V.N., Jeang, K.T., and Stadtman, T.C. (1996). Selenocysteine, identified as the penultimate C-terminal residue in human T-cell thioredoxin reductase, corresponds to TGA in the human placental gene. *Proceedings of the National Academy of Sciences of the United States of America* *93*, 6146-6151.

Goldschneider, D., Blanc, E., Raguenez, G., Barrois, M., Legrand, A., Le Roux, G., Haddada, H., Benard, J., and Douc-Rasy, S. (2004). Differential response of p53 target genes to p73 overexpression in SH-SY5Y neuroblastoma cell line. *Journal of cell science* *117*, 293-301.

Gorgoulis, V.G., Vassiliou, L.V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., Venere, M., Ditullio, R.A., Jr., Kastriakis, N.G., Levy, B., *et al.* (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* *434*, 907-913.

Grasberger, B.L., Lu, T., Schubert, C., Parks, D.J., Carver, T.E., Koblisch, H.K., Cummings, M.D., LaFrance, L.V., Milkiewicz, K.L., Calvo, R.R., *et al.* (2005). Discovery and cocrystal structure of benzodiazepinedione HDM2 antagonists that activate p53 in cells. *Journal of medicinal chemistry* 48, 909-912.

Grinkevich, V., Nikulenkov, F., Shi, Y., Enge, M., Bao, W., Maljukova, A., Sangfelt, O., Gluch, A., Kel, A., and Selivanova, G. (2009). Ablation of key oncogenic pathways by RITA-reactivated p53 is required for efficient apoptosis. In *Cancer cell*.

Grossman, S.R. (2001). p300/CBP/p53 interaction and regulation of the p53 response. *European journal of biochemistry / FEBS* 268, 2773-2778.

Gu, J., Kawai, H., Nie, L., Kitao, H., Wiederschain, D., Jochemsen, A.G., Parant, J., Lozano, G., and Yuan, Z.M. (2002). Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. *The Journal of biological chemistry* 277, 19251-19254.

Hainaut, P., and Mann, K. (2001). Zinc binding and redox control of p53 structure and function. *Antioxidants & redox signaling* 3, 611-623.

Hainaut, P., and Milner, J. (1993). Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. *Cancer research* 53, 4469-4473.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* 100, 57-70.

Hanson, S., Kim, E., and Deppert, W. (2005). Redox factor 1 (Ref-1) enhances specific DNA binding of p53 by promoting p53 tetramerization. *Oncogene* 24, 1641-1647.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75, 805-816.

Harris, S.L., and Levine, A.J. (2005). The p53 pathway: positive and negative feedback loops. *Oncogene* 24, 2899-2908.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature* 387, 296-299.

Helton, E.S., and Chen, X. (2007). p53 modulation of the DNA damage response. *Journal of cellular biochemistry* 100, 883-896.

Hensley, K., Robinson, K.A., Gabbita, S.P., Salsman, S., and Floyd, R.A. (2000). Reactive oxygen species, cell signaling, and cell injury. *Free radical biology & medicine* 28, 1456-1462.

Hinds, P., Finlay, C., and Levine, A.J. (1989). Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. *Journal of virology* 63, 739-746.

Hintze, K.J., Wald, K.A., Zeng, H., Jeffery, E.H., and Finley, J.W. (2003). Thioredoxin reductase in human hepatoma cells is transcriptionally regulated by sulforaphane and other electrophiles via an antioxidant response element. *The Journal of nutrition* 133, 2721-2727.

Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997). AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proceedings of the National Academy of Sciences of the United States of America* 94, 3633-3638.

Hiyama, H., Iavarone, A., and Reeves, S.A. (1998). Regulation of the cdk inhibitor p21 gene during cell cycle progression is under the control of the transcription factor E2F. *Oncogene* 16, 1513-1523.

Hoffman, W.H., Biade, S., Zilfou, J.T., Chen, J., and Murphy, M. (2002). Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *The Journal of biological chemistry* 277, 3247-3257.

Holmgren, A., and Luthman, M. (1978). Tissue distribution and subcellular localization of bovine thioredoxin determined by radioimmunoassay. *Biochemistry* 17, 4071-4077.

Holmgren, A., Perham, R.N., and Baldesten, A. (1968). Thioredoxin. 3. Amino acid sequences of the peptic peptides from S-aminoethylated peptide B. *European journal of biochemistry / FEBS* 5, 352-358.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS letters* 420, 25-27.

Hsieh, J.K., Chan, F.S., O'Connor, D.J., Mittnacht, S., Zhong, S., and Lu, X. (1999). RB regulates the stability and the apoptotic function of p53 via MDM2. *Molecular cell* 3, 181-193.

Hu, B., Gilkes, D.M., and Chen, J. (2007). Efficient p53 activation and apoptosis by simultaneous disruption of binding to MDM2 and MDMX. *Cancer research* 67, 8810-8817.

Hussain, S.P., Amstad, P., He, P., Robles, A., Lupold, S., Kaneko, I., Ichimiya, M., Sengupta, S., Mechanic, L., Okamura, S., *et al.* (2004). p53-induced up-regulation of MnSOD and GPx but not catalase increases oxidative stress and apoptosis. *Cancer research* 64, 2350-2356.

Hussain, S.P., and Harris, C.C. (1998). Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. *Cancer research* 58, 4023-4037.

Hwang, P.M., Bunz, F., Yu, J., Rago, C., Chan, T.A., Murphy, M.P., Kelso, G.F., Smith, R.A., Kinzler, K.W., and Vogelstein, B. (2001). Ferredoxin reductase affects p53-dependent, 5-fluorouracil-induced apoptosis in colorectal cancer cells. *Nature medicine* 7, 1111-1117.

Issaeva, N., Bozko, P., Enge, M., Protopopova, M., Verhoef, L.G., Masucci, M., Pramanik, A., and Selivanova, G. (2004). Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nature medicine* 10, 1321-1328.

Jayaraman, L., Murthy, K.G., Zhu, C., Curran, T., Xanthoudakis, S., and Prives, C. (1997). Identification of redox/repair protein Ref-1 as a potent activator of p53. *Genes & development* 11, 558-570.

Jin, Y., Lee, H., Zeng, S.X., Dai, M.S., and Lu, H. (2003). MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation. *The EMBO journal* 22, 6365-6377.

Jones, S.N., Hancock, A.R., Vogel, H., Donehower, L.A., and Bradley, A. (1998). Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* 95, 15608-15612.

Jones, S.N., Roe, A.E., Donehower, L.A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 378, 206-208.

Kawai, H., Lopez-Pajares, V., Kim, M.M., Wiederschain, D., and Yuan, Z.M. (2007). RING domain-mediated interaction is a requirement for MDM2's E3 ligase activity. *Cancer research* 67, 6026-6030.

Kawai, H., Wiederschain, D., and Yuan, Z.M. (2003). Critical contribution of the MDM2 acidic domain to p53 ubiquitination. *Molecular and cellular biology* 23, 4939-4947.

Kern, S.E., Kinzler, K.W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991). Identification of p53 as a sequence-specific DNA-binding protein. *Science (New York, NY)* 252, 1708-1711.

Kim, I.S., Jin, J.Y., Lee, I.H., and Park, S.J. (2004). Auranofin induces apoptosis and when combined with retinoic acid enhances differentiation of acute promyelocytic leukaemia cells in vitro. *British journal of pharmacology* 142, 749-755.

Koblish, H.K., Zhao, S., Franks, C.F., Donatelli, R.R., Tominovich, R.M., LaFrance, L.V., Leonard, K.A., Gushue, J.M., Parks, D.J., Calvo, R.R., *et al.* (2006). Benzodiazepinedione inhibitors of the Hdm2:p53 complex suppress human tumor cell proliferation in vitro and sensitize tumors to doxorubicin in vivo. *Molecular cancer therapeutics* 5, 160-169.

Komarov, P.G., Komarova, E.A., Kondratov, R.V., Christov-Tselkov, K., Coon, J.S., Chernov, M.V., and Gudkov, A.V. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science (New York, NY)* 285, 1733-1737.

Krishnamurthy, D., Karver, M.R., Fiorillo, E., Orru, V., Stanford, S.M., Bottini, N., and Barrios, A.M. (2008). Gold(I)-mediated inhibition of protein tyrosine phosphatases: a detailed in vitro and cellular study. *Journal of medicinal chemistry* 51, 4790-4795.

Krummel, K.A., Lee, C.J., Toledo, F., and Wahl, G.M. (2005). The C-terminal lysines fine-tune P53 stress responses in a mouse model but are not required for stability control or transactivation. *Proceedings of the National Academy of Sciences of the United States of America* 102, 10188-10193.

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

Lai, Z., Yang, T., Kim, Y.B., Sielecki, T.M., Diamond, M.A., Strack, P., Rolfe, M., Caligiuri, M., Benfield, P.A., Auger, K.R., *et al.* (2002). Differentiation of Hdm2-mediated p53 ubiquitination and Hdm2 autoubiquitination activity by small molecular weight inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* *99*, 14734-14739.

Lain, S., Hollick, J.J., Campbell, J., Staples, O.D., Higgins, M., Aoubala, M., McCarthy, A., Appleyard, V., Murray, K.E., Baker, L., *et al.* (2008). Discovery, in vivo activity, and mechanism of action of a small-molecule p53 activator. *Cancer cell* *13*, 454-463.

Lane, D.P., and Crawford, L.V. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature* *278*, 261-263.

Lassus, P., Ferlin, M., Piette, J., and Hibner, U. (1996). Anti-apoptotic activity of low levels of wild-type p53. *The EMBO journal* *15*, 4566-4573.

Laurent, T.C., Moore, E.C., and Reichard, P. (1964). Enzymatic Synthesis of Deoxyribonucleotides. Iv. Isolation and Characterization of Thioredoxin, the Hydrogen Donor from Escherichia Coli B. *The Journal of biological chemistry* *239*, 3436-3444.

Laurie, N.A., Donovan, S.L., Shih, C.S., Zhang, J., Mills, N., Fuller, C., Teunisse, A., Lam, S., Ramos, Y., Mohan, A., *et al.* (2006). Inactivation of the p53 pathway in retinoblastoma. *Nature* *444*, 61-66.

Lees-Miller, S.P., Sakaguchi, K., Ullrich, S.J., Appella, E., and Anderson, C.W. (1992). Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Molecular and cellular biology* *12*, 5041-5049.

Legube, G., Linares, L.K., Lemerrier, C., Scheffner, M., Khochbin, S., and Trouche, D. (2002). Tip60 is targeted to proteasome-mediated degradation by Mdm2 and accumulates after UV irradiation. *The EMBO journal* *21*, 1704-1712.

Leng, R.P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J.M., Lozano, G., Hakem, R., and Benchimol, S. (2003). Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. *Cell* *112*, 779-791.

Lev Bar-Or, R., Maya, R., Segel, L.A., Alon, U., Levine, A.J., and Oren, M. (2000). Generation of oscillations by the p53-Mdm2 feedback loop: a theoretical and experimental study. *Proceedings of the National Academy of Sciences of the United States of America* *97*, 11250-11255.

Levine, A.J., Hu, W., and Feng, Z. (2006). The P53 pathway: what questions remain to be explored? *Cell death and differentiation* *13*, 1027-1036.

Li, M., Brooks, C.L., Kon, N., and Gu, W. (2004). A dynamic role of HAUSP in the p53-Mdm2 pathway. *Molecular cell* *13*, 879-886.

Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003). Mono-versus polyubiquitination: differential control of p53 fate by Mdm2. *Science (New York, NY)* *302*, 1972-1975.

Linares, L.K., Hengstermann, A., Ciechanover, A., Muller, S., and Scheffner, M. (2003). HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. *Proceedings of the National Academy of Sciences of the United States of America* *100*, 12009-12014.

Linzer, D.I., and Levine, A.J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* *17*, 43-52.

Lloyd, D.R., Phillips, D.H., and Carmichael, P.L. (1997). Generation of putative intrastrand cross-links and strand breaks in DNA by transition metal ion-mediated oxygen radical attack. *Chemical research in toxicology* *10*, 393-400.

Lopez-Pajares, V., Kim, M.M., and Yuan, Z.M. (2008). Phosphorylation of MDMX mediated by Akt leads to stabilization and induces 14-3-3 binding. *The Journal of biological chemistry* *283*, 13707-13713.

Lu, W., Chen, L., Peng, Y., and Chen, J. (2001). Activation of p53 by roscovitine-mediated suppression of MDM2 expression. *Oncogene* *20*, 3206-3216.

Macchiarulo, A., Giacche, N., Carotti, A., Baroni, M., Cruciani, G., and Pellicciari, R. (2008). Targeting the conformational transitions of MDM2 and MDMX: insights into dissimilarities and similarities of p53 recognition. *Journal of chemical information and modeling* *48*, 1999-2009.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Jr., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A., *et al.* (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science (New York, NY)* *250*, 1233-1238.

Mantovani, F., Gostissa, M., Collavin, L., and Del Sal, G. (2004). KeePin' the p53 family in good shape. *Cell cycle (Georgetown, Tex)* *3*, 905-911.

Marchenko, N.D., Wolff, S., Erster, S., Becker, K., and Moll, U.M. (2007). Monoubiquitylation promotes mitochondrial p53 translocation. *The EMBO journal* *26*, 923-934.

Martins, C.P., Brown-Swigart, L., and Evan, G.I. (2006). Modeling the therapeutic efficacy of p53 restoration in tumors. *Cell* *127*, 1323-1334.

Marzano, C., Gandin, V., Folda, A., Scutari, G., Bindoli, A., and Rigobello, M.P. (2007). Inhibition of thioredoxin reductase by auranofin induces apoptosis in cisplatin-resistant human ovarian cancer cells. *Free radical biology & medicine* *42*, 872-881.

May, P., and May, E. (1999). Twenty years of p53 research: structural and functional aspects of the p53 protein. *Oncogene* *18*, 7621-7636.

Maya, R., Balass, M., Kim, S.T., Shkedy, D., Leal, J.F., Shifman, O., Moas, M., Buschmann, T., Ronai, Z., Shiloh, Y., *et al.* (2001). ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes & development* *15*, 1067-1077.

Mayo, L.D., Turchi, J.J., and Berberich, S.J. (1997). Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer research* *57*, 5013-5016.

Meek, D.W. (1999). Mechanisms of switching on p53: a role for covalent modification? *Oncogene* *18*, 7666-7675.

Meulmeester, E., Maurice, M.M., Boutell, C., Teunisse, A.F., Ovaa, H., Abraham, T.E., Dirks, R.W., and Jochemsen, A.G. (2005). Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. *Molecular cell* *18*, 565-576.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U.M. (2003). p53 has a direct apoptogenic role at the mitochondria. *Molecular cell* *11*, 577-590.

Miranda-Vizuete, A., Damdimopoulos, A.E., Pedrajas, J.R., Gustafsson, J.A., and Spyrou, G. (1999). Human mitochondrial thioredoxin reductase cDNA cloning, expression and genomic organization. *European journal of biochemistry / FEBS* *261*, 405-412.

Miranda-Vizuete, A., Gustafsson, J.A., and Spyrou, G. (1998). Molecular cloning and expression of a cDNA encoding a human thioredoxin-like protein. *Biochemical and biophysical research communications* *243*, 284-288.

Miyashita, T., Harigai, M., Hanada, M., and Reed, J.C. (1994). Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer research* *54*, 3131-3135.

Miyashita, T., and Reed, J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* *80*, 293-299.

Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* *69*, 1237-1245.

Montes de Oca Luna, R., Wagner, D.S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* *378*, 203-206.

Moos, P.J., Edes, K., Cassidy, P., Massuda, E., and Fitzpatrick, F.A. (2003). Electrophilic prostaglandins and lipid aldehydes repress redox-sensitive transcription factors p53 and hypoxia-inducible factor by impairing the selenoprotein thioredoxin reductase. *The Journal of biological chemistry* *278*, 745-750.

Moreno, C.S., Matyunina, L., Dickerson, E.B., Schubert, N., Bowen, N.J., Logani, S., Benigno, B.B., and McDonald, J.F. (2007). Evidence that p53-mediated cell-cycle-arrest inhibits chemotherapeutic treatment of ovarian carcinomas. *PLoS ONE* *2*, e441.

Moumen, A., Masterson, P., O'Connor, M.J., and Jackson, S.P. (2005). hnRNP K: an HDM2 target and transcriptional coactivator of p53 in response to DNA damage. *Cell* *123*, 1065-1078.

Nakano, K., and Vousden, K.H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. *Molecular cell* *7*, 683-694.

Nguyen, P., Awwad, R.T., Smart, D.D., Spitz, D.R., and Gius, D. (2006). Thioredoxin reductase as a novel molecular target for cancer therapy. *Cancer letters* 236, 164-174.

Nikulenkov, F., Shi, Y., and Selivanova, G. (manuscript in preparation).

Nordberg, J., and Arner, E.S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free radical biology & medicine* 31, 1287-1312.

Nordstrom, W., and Abrams, J.M. (2000). Guardian ancestry: fly p53 and damage-inducible apoptosis. *Cell death and differentiation* 7, 1035-1038.

O'Reilly, D.R. (1986). p53 and transformation by SV40. *Biology of the cell / under the auspices of the European Cell Biology Organization* 57, 187-196.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science (New York, NY)* 288, 1053-1058.

Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L., and Vogelstein, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* 358, 80-83.

Olivier, M., Eeles, R., Hollstein, M., Khan, M.A., Harris, C.C., and Hainaut, P. (2002). The IARC TP53 database: new online mutation analysis and recommendations to users. *Human mutation* 19, 607-614.

Ozben, T. (2007). Oxidative stress and apoptosis: impact on cancer therapy. *Journal of pharmaceutical sciences* 96, 2181-2196.

Palmero, I., Pantoja, C., and Serrano, M. (1998). p19ARF links the tumour suppressor p53 to Ras. *Nature* 395, 125-126.

Pan, Y., and Chen, J. (2003). MDM2 promotes ubiquitination and degradation of MDMX. *Molecular and cellular biology* 23, 5113-5121.

Parada, L.F., Land, H., Weinberg, R.A., Wolf, D., and Rotter, V. (1984). Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. *Nature* 312, 649-651.

Parant, J., Chavez-Reyes, A., Little, N.A., Yan, W., Reinke, V., Jochemsen, A.G., and Lozano, G. (2001). Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. *Nature genetics* 29, 92-95.

Parks, D., Bolinger, R., and Mann, K. (1997). Redox state regulates binding of p53 to sequence-specific DNA, but not to non-specific or mismatched DNA. *Nucleic acids research* 25, 1289-1295.

Patton, J.T., Mayo, L.D., Singhi, A.D., Gudkov, A.V., Stark, G.R., and Jackson, M.W. (2006). Levels of HdmX expression dictate the sensitivity of normal and transformed cells to Nutlin-3. *Cancer research* 66, 3169-3176.

Pazgier, M., Liu, M., Zou, G., Yuan, W., Li, C., Li, C., Li, J., Monbo, J., Zella, D., Tarasov, S.G., *et al.* (2009). Structural basis for high-affinity peptide inhibition of p53 interactions with MDM2 and MDMX. *Proceedings of the National Academy of Sciences of the United States of America* 106, 4665-4670.

Pearson, G.D., and Merrill, G.F. (1998). Deletion of the *Saccharomyces cerevisiae* TRR1 gene encoding thioredoxin reductase inhibits p53-dependent reporter gene expression. *The Journal of biological chemistry* 273, 5431-5434.

Pelicano, H., Carney, D., and Huang, P. (2004). ROS stress in cancer cells and therapeutic implications. *Drug Resist Updat* 7, 97-110.

Pereg, Y., Shkedy, D., de Graaf, P., Meulmeester, E., Edelson-Averbukh, M., Salek, M., Biton, S., Teunisse, A.F., Lehmann, W.D., Jochemsen, A.G., *et al.* (2005). Phosphorylation of Hdmx mediates its Hdm2- and ATM-dependent degradation in response to DNA damage. *Proceedings of the National Academy of Sciences of the United States of America* 102, 5056-5061.

Pietrzak, M., and Puzianowska-Kuznicka, M. (2008). p53-dependent repression of the human MCL-1 gene encoding an anti-apoptotic member of the BCL-2 family: the role of Sp1 and of basic transcription factor binding sites in the MCL-1 promoter. *Biological chemistry* 389, 383-393.

Polyak, K., Xia, Y., Zweier, J.L., Kinzler, K.W., and Vogelstein, B. (1997). A model for p53-induced apoptosis. *Nature* 389, 300-305.

Popowicz, G.M., Czarna, A., and Holak, T.A. (2008). Structure of the human Mdmx protein bound to the p53 tumor suppressor transactivation domain. *Cell cycle* (Georgetown, Tex 7, 2441-2443.

Powis, G., and Kirkpatrick, D.L. (2007). Thioredoxin signaling as a target for cancer therapy. *Current opinion in pharmacology* 7, 392-397.

Prives, C., and Hall, P.A. (1999). The p53 pathway. *The Journal of pathology* 187, 112-126.

Randerath, K., Randerath, E., Smith, C.V., and Chang, J. (1996). Structural origins of bulky oxidative DNA adducts (type II I-compounds) as deduced by oxidation of oligonucleotides of known sequence. *Chemical research in toxicology* 9, 247-254.

Ravi, D., Muniyappa, H., and Das, K.C. (2005). Endogenous thioredoxin is required for redox cycling of anthracyclines and p53-dependent apoptosis in cancer cells. *The Journal of biological chemistry* 280, 40084-40096.

Raycroft, L., Wu, H.Y., and Lozano, G. (1990). Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. *Science (New York, NY)* 249, 1049-1051.

Rinaldo, C., Prodosmo, A., Mancini, F., Iacovelli, S., Sacchi, A., Moretti, F., and Soddu, S. (2007). MDM2-regulated degradation of HIPK2 prevents p53Ser46 phosphorylation and DNA damage-induced apoptosis. *Molecular cell* 25, 739-750.

Rodriguez, M.S., Desterro, J.M., Lain, S., Lane, D.P., and Hay, R.T. (2000). Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. *Molecular and cellular biology* 20, 8458-8467.

Rundlof, A.K., and Arner, E.S. (2004). Regulation of the mammalian selenoprotein thioredoxin reductase 1 in relation to cellular phenotype, growth, and signaling events. *Antioxidants & redox signaling* 6, 41-52.

Rundlof, A.K., Carlsten, M., Giacobini, M.M., and Arner, E.S. (2000). Prominent expression of the selenoprotein thioredoxin reductase in the medullary rays of the rat kidney and thioredoxin reductase mRNA variants differing at the 5' untranslated region. *The Biochemical journal* 347 Pt 3, 661-668.

Sabbatini, P., and McCormick, F. (2002). MDMX inhibits the p300/CBP-mediated acetylation of p53. *DNA and cell biology* 21, 519-525.

Sablina, A.A., Budanov, A.V., Ilyinskaya, G.V., Agapova, L.S., Kravchenko, J.E., and Chumakov, P.M. (2005). The antioxidant function of the p53 tumor suppressor. *Nature medicine* 11, 1306-1313.

Sakurai, A., Nishimoto, M., Himeno, S., Imura, N., Tsujimoto, M., Kunimoto, M., and Hara, S. (2005). Transcriptional regulation of thioredoxin reductase 1 expression by cadmium in vascular endothelial cells: role of NF-E2-related factor-2. *Journal of cellular physiology* 203, 529-537.

Samuels-Lev, Y., O'Connor, D.J., Bergamaschi, D., Trigiante, G., Hsieh, J.K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. (2001). ASPP proteins specifically stimulate the apoptotic function of p53. *Molecular cell* 8, 781-794.

Sax, J.K., Fei, P., Murphy, M.E., Bernhard, E., Korsmeyer, S.J., and El-Deiry, W.S. (2002). BID regulation by p53 contributes to chemosensitivity. *Nature cell biology* 4, 842-849.

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., and Howley, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 63, 1129-1136.

Seemann, S., and Hainaut, P. (2005). Roles of thioredoxin reductase 1 and APE/Ref-1 in the control of basal p53 stability and activity. *Oncogene* 24, 3853-3863.

Sharpless, N.E. (2005). INK4a/ARF: a multifunctional tumor suppressor locus. *Mutation research* 576, 22-38.

Shinozaki, T., Nota, A., Taya, Y., and Okamoto, K. (2003). Functional role of Mdm2 phosphorylation by ATR in attenuation of p53 nuclear export. *Oncogene* 22, 8870-8880.

Shvarts, A., Steegenga, W.T., Riteco, N., van Laar, T., Dekker, P., Bazuine, M., van Ham, R.C., van der Houven van Oordt, W., Hateboer, G., van der Eb, A.J., *et al.* (1996). MDMX: a novel p53-binding protein with some functional properties of MDM2. *The EMBO journal* 15, 5349-5357.

Simon, T.M., Kunishima, D.H., Vibert, G.J., and Lorber, A. (1981). Screening trial with the coordinated gold compound auranofin using mouse lymphocyte leukemia P388. *Cancer research* 41, 94-97.

Somasundaram, K., Zhang, H., Zeng, Y.X., Houvras, Y., Peng, Y., Zhang, H., Wu, G.S., Licht, J.D., Weber, B.L., and El-Deiry, W.S. (1997). Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. *Nature* 389, 187-190.

Soussi, T., and Lozano, G. (2005). p53 mutation heterogeneity in cancer. *Biochemical and biophysical research communications* 331, 834-842.

Spector, A., Yan, G.Z., Huang, R.R., McDermott, M.J., Gascoyne, P.R., and Pigiet, V. (1988). The effect of H₂O₂ upon thioredoxin-enriched lens epithelial cells. *The Journal of biological chemistry* 263, 4984-4990.

Stankovic, T., Stewart, G.S., Fegan, C., Biggs, P., Last, J., Byrd, P.J., Keenan, R.D., Moss, P.A., and Taylor, A.M. (2002). Ataxia telangiectasia mutated-deficient B-cell chronic lymphocytic leukemia occurs in pregerminal center cells and results in defective damage response and unrepaired chromosome damage. *Blood* 99, 300-309.

Steinman, R.A., and Johnson, D.E. (2000). p21WAF1 prevents down-modulation of the apoptotic inhibitor protein c-IAP1 and inhibits leukemic apoptosis. *Molecular medicine (Cambridge, Mass)* 6, 736-749.

Stommel, J.M., Marchenko, N.D., Jimenez, G.S., Moll, U.M., Hope, T.J., and Wahl, G.M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *The EMBO journal* 18, 1660-1672.

Stommel, J.M., and Wahl, G.M. (2004). Accelerated MDM2 auto-degradation induced by DNA-damage kinases is required for p53 activation. *The EMBO journal* 23, 1547-1556.

Stott, F.J., Bates, S., James, M.C., McConnell, B.B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K.H., *et al.* (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *The EMBO journal* 17, 5001-5014.

Sugrue, M.M., Shin, D.Y., Lee, S.W., and Aaronson, S.A. (1997). Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. *Proceedings of the National Academy of Sciences of the United States of America* 94, 9648-9653.

Sullivan, A., Yuille, M., Repellin, C., Reddy, A., Reelfs, O., Bell, A., Dunne, B., Gusterson, B.A., Osin, P., Farrell, P.J., *et al.* (2002). Concomitant inactivation of p53 and Chk2 in breast cancer. *Oncogene* 21, 1316-1324.

Sun, Q.A., Kirnarsky, L., Sherman, S., and Gladyshev, V.N. (2001). Selenoprotein oxidoreductase with specificity for thioredoxin and glutathione systems. *Proceedings of the National Academy of Sciences of the United States of America* 98, 3673-3678.

Suzuki, A., Tsutomi, Y., Akahane, K., Araki, T., and Miura, M. (1998). Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. *Oncogene* 17, 931-939.

Sykes, S.M., Mellert, H.S., Holbert, M.A., Li, K., Marmorstein, R., Lane, W.S., and McMahon, S.B. (2006). Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Molecular cell* 24, 841-851.

Szatrowski, T.P., and Nathan, C.F. (1991). Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer research* 51, 794-798.

Tang, Y., Luo, J., Zhang, W., and Gu, W. (2006). Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Molecular cell* 24, 827-839.

Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. (2008). Acetylation is indispensable for p53 activation. *Cell* 133, 612-626.

Tanimura, S., Ohtsuka, S., Mitsui, K., Shirouzu, K., Yoshimura, A., and Ohtsubo, M. (1999). MDM2 interacts with MDMX through their RING finger domains. *FEBS letters* 447, 5-9.

Tian, L., Greenberg, S.A., Kong, S.W., Altschuler, J., Kohane, I.S., and Park, P.J. (2005). Discovering statistically significant pathways in expression profiling studies. *Proceedings of the National Academy of Sciences of the United States of America* 102, 13544-13549.

Toledo, F., and Wahl, G.M. (2006). Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer* 6, 909-923.

Tovar, C., Rosinski, J., Filipovic, Z., Higgins, B., Kolinsky, K., Hilton, H., Zhao, X., Vu, B.T., Qing, W., Packman, K., *et al.* (2006). Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. *Proceedings of the National Academy of Sciences of the United States of America* 103, 1888-1893.

Toyokuni, S., Okamoto, K., Yodoi, J., and Hiai, H. (1995). Persistent oxidative stress in cancer. *FEBS letters* 358, 1-3.

Traxler, P. (2003). Tyrosine kinases as targets in cancer therapy - successes and failures. *Expert opinion on therapeutic targets* 7, 215-234.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America* 98, 5116-5121.

Uchida, C., Miwa, S., Kitagawa, K., Hattori, T., Isobe, T., Otani, S., Oda, T., Sugimura, H., Kamijo, T., Ookawa, K., *et al.* (2005). Enhanced Mdm2 activity inhibits pRB function via ubiquitin-dependent degradation. *The EMBO journal* 24, 160-169.

Ueno, M., Masutani, H., Arai, R.J., Yamauchi, A., Hirota, K., Sakai, T., Inamoto, T., Yamaoka, Y., Yodoi, J., and Nikaido, T. (1999). Thioredoxin-dependent redox regulation of p53-mediated p21 activation. *The Journal of biological chemistry* 274, 35809-35815.

Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Vogt Sionov, R., Lozano, G., Oren, M., and Haupt, Y. (1999). Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *The EMBO journal* 18, 1805-1814.

Urig, S., and Becker, K. (2006). On the potential of thioredoxin reductase inhibitors for cancer therapy. *Seminars in cancer biology* 16, 452-465.

Wade, M., Wong, E.T., Tang, M., Stommel, J.M., and Wahl, G.M. (2006). Hdmx modulates the outcome of p53 activation in human tumor cells. *The Journal of biological chemistry* 281, 33036-33044.

Wang, H., Zeng, X., Oliver, P., Le, L.P., Chen, J., Chen, L., Zhou, W., Agrawal, S., and Zhang, R. (1999a). MDM2 oncogene as a target for cancer therapy: An antisense approach. *International journal of oncology* 15, 653-660.

Wang, T., Lee, K., Rehman, A., and Daoud, S.S. (2007a). PRIMA-1 induces apoptosis by inhibiting JNK signaling but promoting the activation of Bax. *Biochemical and biophysical research communications* 352, 203-212.

Wang, X.W., Zhan, Q., Coursen, J.D., Khan, M.A., Kontny, H.U., Yu, L., Hollander, M.C., O'Connor, P.M., Fornace, A.J., Jr., and Harris, C.C. (1999b). GADD45 induction of a G2/M cell cycle checkpoint. *Proceedings of the National Academy of Sciences of the United States of America* 96, 3706-3711.

Wang, Y.V., Wade, M., Wong, E., Li, Y.C., Rodewald, L.W., and Wahl, G.M. (2007b). Quantitative analyses reveal the importance of regulated Hdmx degradation for p53 activation. *Proceedings of the National Academy of Sciences of the United States of America* 104, 12365-12370.

Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., *et al.* (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science (New York, NY)* 303, 844-848.

Wei, C.L., Wu, Q., Vega, V.B., Chiu, K.P., Ng, P., Zhang, T., Shahab, A., Yong, H.C., Fu, Y., Weng, Z., *et al.* (2006). A global map of p53 transcription-factor binding sites in the human genome. *Cell* 124, 207-219.

Ventura, A., Kirsch, D.G., McLaughlin, M.E., Tuveson, D.A., Grimm, J., Lintault, L., Newman, J., Reczek, E.E., Weissleder, R., and Jacks, T. (2007). Restoration of p53 function leads to tumour regression in vivo. *Nature* 445, 661-665.

Vogelstein, B., and Kinzler, K.W. (2004). Cancer genes and the pathways they control. *Nature medicine* 10, 789-799.

Wood, L.D., Parsons, D.W., Jones, S., Lin, J., Sjoblom, T., Leary, R.J., Shen, D., Boca, S.M., Barber, T., Ptak, J., *et al.* (2007). The genomic landscapes of human breast and colorectal cancers. *Science (New York, NY)* 318, 1108-1113.

Vousden, K.H., and Lu, X. (2002). Live or let die: the cell's response to p53. *Nat Rev Cancer* 2, 594-604.

Wu, X., Bayle, J.H., Olson, D., and Levine, A.J. (1993). The p53-mdm-2 autoregulatory feedback loop. *Genes & development* 7, 1126-1132.

Xiong, S., Van Pelt, C.S., Elizondo-Fraire, A.C., Liu, G., and Lozano, G. (2006). Synergistic roles of Mdm2 and Mdm4 for p53 inhibition in central nervous system development. *Proceedings of the National Academy of Sciences of the United States of America* 103, 3226-3231.

Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovskiy, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 445, 656-660.

Yamaguchi, T., Matsuda, K., Sagiya, Y., Iwadate, M., Fujino, M.A., Nakamura, Y., and Arakawa, H. (2001). p53R2-dependent pathway for DNA synthesis in a p53-regulated cell cycle checkpoint. *Cancer research* 61, 8256-8262.

Yan, W., and Chen, X. (2006). GPX2, a direct target of p63, inhibits oxidative stress-induced apoptosis in a p53-dependent manner. *The Journal of biological chemistry* 281, 7856-7862.

Yang, J., Ahmed, A., Poon, E., Perusinghe, N., de Haven Brandon, A., Box, G., Valenti, M., Eccles, S., Rouschop, K., Wouters, B., *et al.* (2009). Small-molecule activation of p53 blocks hypoxia-inducible factor 1alpha and vascular endothelial growth factor expression in vivo and leads to tumor cell apoptosis in normoxia and hypoxia. *Molecular and cellular biology* 29, 2243-2253.

Yang, Y., Ludwig, R.L., Jensen, J.P., Pierre, S.A., Medaglia, M.V., Davydov, I.V., Safiran, Y.J., Oberoi, P., Kenten, J.H., Phillips, A.C., *et al.* (2005). Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. *Cancer cell* 7, 547-559.

Yoo, M.H., Xu, X.M., Carlson, B.A., Gladyshev, V.N., and Hatfield, D.L. (2006). Thioredoxin reductase 1 deficiency reverses tumor phenotype and tumorigenicity of lung carcinoma cells. *The Journal of biological chemistry* 281, 13005-13008.

Yoon, K.A., Nakamura, Y., and Arakawa, H. (2004). Identification of ALDH4 as a p53-inducible gene and its protective role in cellular stresses. *Journal of human genetics* 49, 134-140.

Yu, J., Wang, Z., Kinzler, K.W., Vogelstein, B., and Zhang, L. (2003). PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* 100, 1931-1936.

Yu, Z.K., Geyer, R.K., and Maki, C.G. (2000). MDM2-dependent ubiquitination of nuclear and cytoplasmic P53. *Oncogene* 19, 5892-5897.

Zacchi, P., Gostissa, M., Uchida, T., Salvagno, C., Avolio, F., Volinia, S., Ronai, Z., Blandino, G., Schneider, C., and Del Sal, G. (2002). The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. *Nature* 419, 853-857.

Zawacka-Pankau, J., Grinkevich, V., Riddarstråle, K., Okorokov, A.L., Hossain, S., Larsson, L.-G., Pramanik, A., and Selivanova, G. (manuscript in preparation).

Zeng, Y.X., Somasundaram, K., and el-Deiry, W.S. (1997). AP2 inhibits cancer cell growth and activates p21WAF1/CIP1 expression. *Nature genetics* 15, 78-82.

Zhang, R., Wang, H., and Agrawal, S. (2005a). Novel antisense anti-MDM2 mixed-backbone oligonucleotides: proof of principle, in vitro and in vivo activities, and mechanisms. *Current cancer drug targets* 5, 43-49.

Zhang, Z., Wang, H., Li, M., Agrawal, S., Chen, X., and Zhang, R. (2004). MDM2 is a negative regulator of p21WAF1/CIP1, independent of p53. *The Journal of biological chemistry* 279, 16000-16006.

Zhang, Z., Wang, H., Li, M., Rayburn, E.R., Agrawal, S., and Zhang, R. (2005b). Stabilization of E2F1 protein by MDM2 through the E2F1 ubiquitination pathway. *Oncogene* 24, 7238-7247.

Zhong, L., Arner, E.S., Ljung, J., Aslund, F., and Holmgren, A. (1998). Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. *The Journal of biological chemistry* 273, 8581-8591.

Zindy, F., Eischen, C.M., Randle, D.H., Kamijo, T., Cleveland, J.L., Sherr, C.J., and Roussel, M.F. (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes & development* 12, 2424-2433.