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SMALL MOLECULES THAT AFFECT THE P53 PATHWAY AND THEIR POTENTIAL USE IN THE TREATMENT OF CANCER

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Karolinska Institutet

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Cover illustration: 3D structure of a p53 tetramer surrounded by chemical compounds described in this thesis. The 3D structure was modeled by Chandra Verma (Bioinformatics Institute, A*STAR, Singapore) and reprinted with his permission.

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To my parents

ABSTRACT

The tumor suppressor p53 was identified 35 years ago and has since then been studied extensively, but despite all efforts, no drug or therapy directly involving it has been clinically approved - yet! A lot of potential new drugs are on their way that can reactivate p53 function by various mechanisms. Even a whole new approach called cyclotherapy has been established, during which p53 is activated in normal cells to protect patients from the adverse effects of chemotherapy while tumor cells are still being killed efficiently. In this thesis, 16 drug combinations are being described in this context (**paper I**). Four individual p53-activating compounds, i.e. tenovin-6, leptomycin B (LMB), nutlin-3 and actinomycin D at low doses (LDactD), were used prior to the addition of each one clinically approved chemotherapeutic agent, i.e vinblastine, vinorelbine, cytosine arabinoside or gemcitabine. LDactD, which is clinically approved, showed the most promising results.

Unexpectedly, we identified two compounds that can inhibit p53's ability to induce p21, i.e. the novel SirT2 inhibitor tenovin-D3 (**paper II**) and the widely used histone deacetylase inhibitor (HDACi) trichostatin A (TSA) (**paper III**). Inhibition of p21 in tumor cells might be desirable during cancer treatment to prevent tumor cells from undergoing cell cycle arrest, which would make them more vulnerable to classic chemotherapy. On the other hand, an inhibition of cell cycle arrest in normal cells might occur, which may worsen the side effects caused by chemotherapy. However, SirT2 plays a role in neurodegenerative diseases, and hence compounds like tenovin-D3 may be of use in the treatment thereof. Furthermore, the decrease in p21 levels may be a contributing factor in the previously observed increase in efficacy during the generation of induced pluripotent stem cells upon treatment with TSA; also tenovin-D3 could be useful in this context.

With the aid of a cell-based screen we identified two small molecules that can activate p53:

1) MJ05 was one of the most active hit compounds and was very selective (**paper IV**); it was highly cytotoxic in ARN8, especially when combined with nutlin-3, whereas it was cytostatic or had a very mild effect in other tumor cell lines and normal cells. It inhibited tumor growth *in vivo*, an effect that was enhanced upon co-treatment with nutlin-3. Furthermore, MJ05 selectively killed chronic myelogenous leukemia stem cells *ex vivo* while having milder effects in leukocyte stem cells derived from cord blood. Preliminary data strongly suggest that MJ05 acts by inhibition of pyrimidine (deoxy-) nucleotide synthesis.
2) Despite being a hit compound in our screen, MJ25 was not very potent at activating p53 (**paper V**). Nevertheless, its ability to inhibit thiredoxin reductase 1 (TrxR1) and its selectivity towards melanoma cell lines compared with normal cells were interesting features. We compared it with the TrxR1 inihibitor auranofin, which was very potent and selective at killing melanoma cells in cell viability assays. The insolubility of MJ25 at concentrations required for *in vivo* studies prevented us from testing it on xenografts in mice. Furthermore, MJ25 might not be specific for TrxR1, so the identification of additional targets could be investigated in the future. Auranofin, the other hand, has a more defined mechanism of action and is clinically approved for the treatment of rheumatoid arthritis. These traits combined with its potentially selective cytotoxic effect at low micromolar concentrations in melanoma cells may turn this compound into a potential drug candidate to be tested in patients suffering from malignant melanoma.

In the final study presented in this thesis (**paper VI**) we tested the small molecule tenovin-6 in zebrafish embryos The compound had been described previously by our group. The original aim of this study was to investigate if the activation of p53 in an organism could affect the ability of tumor cells to disseminate. Even though tenovin-6 did not activate wild-type p53 under the conditions tested, *in vivo* activity of the compound was still detectable, since embryos expressing mutant p53 (M214K) displayed an increase in p53 protein levels; furthermore, the compound was lethal in a dose- and time-dependent manner, and the embryos lost most of their brown/black pigmentation. The exact mechanism behind the latter observation could not be elucidated in the course of the project. However, tyrosinase, a key enzyme in melanogenesis, was not inhibited by tenovin-6, and the combination of data obtained by others on mutated or pharmacologically inhibited vacuolar H^+-ATPase (V-ATPase) and yeast mutant strains suggested that the compound may target V-ATPase.

LIST OF SCIENTIFIC PAPERS

I. Ingeborg M.M. van Leeuwen, Bhavya Rao, **Marijke C.C. Sachweh** and Sonia Laín
An evaluation of small-molecule p53 activators as chemoprotectants ameliorating adverse effects of anticancer drugs in normal cells
Cell Cycle (2012) 11(9), 1851-1861

II. Anna R. McCarthy, **Marijke C.C. Sachweh**, Maureen Higgins, Johanna Campbell, Catherine J. Drummond, Ingeborg M.M. van Leeuwen, Lisa Pirrie, Marcus J.G.W. Ladds, Nicholas J. Westwood and Sonia Laín
Tenovin-D3, a Novel Small-Molecule Inhibitor of Sirtuin SirT2, Increases p21 (CDKN1A) Expression in a p53-Independent Manner
Molecular Cancer Therapeutics (2013) 12(4), 352–60

III. **Marijke C.C. Sachweh**, Catherine J. Drummond, Maureen Higgins, Johanna Campbell and Sonia Laín
Incompatible effects of p53 and HDAC inhibition on p21 expression and cell cycle progression
Cell Death and Disease (2013) 4, e533

IV. Catherine J. Drummond, Ling Li, **Marijke C.C. Sachweh**, Su Chu, Alan R. Healy, Johanna Campbell, Maureen Higgins, Anna R. McCarthy, Ingeborg M.M. van Leeuwen, Marcus J.G.W. Ladds, Mihaela Popa, Trung Q. Ha, Emmet McCormack, Virginia Appleyard, Karen E. Murray, Alastair M. Thompson, Richard Svensson, Marcela Franco, Yan Zhao, John Lunec, Fredrik Tholander, Nicholas J. Westwood, Ravi Bhatia and Sonia Laín
Discovery and mechanism of action of a small molecule that selectively enhances therapeutically relevant effects of the p53 tumor suppressor
Manuscript

V. **Marijke C.C. Sachweh***, Catherine J. Drummond*, William C. Stafford, Anna R. McCarthy, Maureen Higgins, Johanna Campbell, Bertha Brodin, Elias S.J. Arnér and Sonia Laín
Redox Effects and Cytotoxic Profiles of MJ25 and Auranofin towards Malignant Melanoma Cells
Manuscript
*These authors contributed equally to this work

VI. **Marijke C.C. Sachweh**, Lin Guo, Chee Li Lian, David P. Lane and Sonia Laín
Tenovin-6 causes Hypopigmentation in Zebrafish Embryos
Preliminary Results

SCIENTIFIC PAPER OUTSIDE THE SCOPE OF THIS THESIS

I. Ingeborg M.M. van Leeuwen, Maureen Higgins, Johanna Campbell, Anna R. McCarthy, **Marijke C.C. Sachweh**, Ana M. Navarro and Sonia Laín
Modulation of p53 C-terminal acetylation by mdm2, p14ARF, and cytoplasmic SirT2
Molecular Cancer Therapeutics (2013) 12(4), 471-80

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LIST OF ABBREVIATIONS

53BP1	p53-binding protein 1
Aa	amino acids
Ac-K	acetyl-lysine
Ac-Lys	acetyl-lysine
actD	actinomycin D
AP-1	activator protein 1
Ara-C	cytosine arabinoside
ARF	alternative reading frame
ARF-BP1	ARF-binding protein 1
ASK1	signal-regulated kinase 1
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related kinase
BBSKE	1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]ethane
bp	base pairs
CBP	CREB-binding protein
CDA	cytidine deaminase
Cdc	cell division cycle
Cdk	cyclin-dependent kinases
CDKN1A	cyclin-dependent kinase inhibitor 1 A
Chk1	checkpoint kinase 1
Cip1	cyclin-dependent kinase-interacting protein 1
CML	chronic myelogenous leukemia
CRD	C-terminal regulatory domain
CRM1	chromosome region maintenance 1
CTD	cytidine triphosphate
CTCL	cutaneous T cell lymphoma
DBD	DNA-binding domain
dbSNP	Single Nucleotide Polymorphism Database
dCTP	deoxycytidine triphosphate
DDR	DNA damage response

DHODH	dihydroorotate dehydrogenase
DSB	DNA double-strand break
dTMP	deoxythymidine monophosphate
DTP	Developmental Therapeutics Program
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
ERK	extracellular signal-regulated kinase
FBXO11	F-box protein 11
FDA	U. S. Food and Drug Administration
FLp53	full-length p53
GMTB	gemcitabine
GSH	glutathione
HAT	histone acetyl transferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HIF-1α	hypoxia-inducible transcription factor-1 alpha
HIV	human immunodeficiency virus
hMOF	human males-absent-on-the-first
HNDF	human normal dermal fibroblast
HNEM	human normal epithelial melanocyte
HPV	human papillomavirus
iPSC	induced pluripotent stem cell
kD	kilo Dalton
LDactD	low doses of actinomycin D
LMB	leptomycin B
LSD1	lysine specific demethylase 1
LSC	leukemia stem cells
MAPK	mitogen-activated protein kinase
MCM2	minichromosome maintenance complex component 2
MDA6	melanoma-derived antigen 6
MDM	murine double minute

Msr	methionine sulfoxide reductase
Mule	Mcl-1 ubiquitin ligase E3
MW	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
NEDD8	neural precursor cell expressed developmentally downregulated protein 8
NES	nuclear export signal
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NLS	nuclear localization signal
Nrf2	NF-E2-related factor 2
Oct-4	octamer-binding transcription factor 4
OD	oligomerization domain
ORF	open reading frame
PCAF	p300/CBP-associated factor
PIAS	protein inhibitor of activated stat
PIG3	p53-inducible gene 3
PLK1	polo-like kinase 1
polyA	polyadenylation signal
PRMT5	protein arginine N-methyl transferase 5
PRR	proline-rich region
Prx	peroxiredoxin
PTCL	peripheral T-cell lymphoma
PTEN	phosphatase and tensin homolog
PTU	*N*-phenylthiourea
PUMA	p53 up-regulated modulator of apoptosis
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RBM38	RNA-binding protein 38
RE	regulatory element
RGC	ribosomal gene cluster

RITA	reactivation of p53 and induction of tumor cell apoptosis
RNR	ribonucleotide reductase
ROS	reactive oxygen species
SAR	structure-activity relationship
SDI1	senescent cell-derived growth inhibitor 1
Sir2	silencing information regulator 2
SirT	sirtuin
SNP	single-nucleotide polymorphisms
SUMO	small ubiquitin-like modifier
SV40	simian virus 40
TAD	transactivation domain
TBP2	Trx-binding protein-2
TFIIIC	transcription factor IIIC-gamma subunit
TIP60	tat-interactive protein of 60 kDa
Trx	thioredoxin
TrxR	thioredoxin reductase
TSA	trichostatin A
Topors	topoisomerase I binding, arginine/serine-rich, E3-ubiquitin protein ligase
TXNIP	thioredoxin-interacting protein
UMP	uridine monophosphate
UMPS	uridine monophosphate synthetase
V-ATPase	vacuolar H^+-ATPase
VCX1	vacuolar H^+/Ca^{2+} exchanger 1
VDUP1	vitamin D3-upregulated protein 1
VNB	vinblastine
VPA	valproic acid
VRL	vinorelbine
Waf1	wild-type p53-activated fragment 1
WB	Western blotting
Wt	wild-type
XPO1	exportin-1

1 INTRODUCTION

1.1 CANCER

Cancer is a collective term for all kinds of malignant tumors. The first cancer was described in the Edwin Smith Papyrus, which was written approximately 3000 BC (*1*). Tumorigenesis is considered to be a multistep process, in which several mutations must occur for a benign tumor to become malignant. This may also be the reason why the incidence rate of cancer increases with age (*2*). Hanahan and Weinberg defined six hallmarks of cancer in the year 2000 (*3*), a list which was extended by four additional hallmarks about a decade later (*4*): Self-sufficiency in proliferative signals, insensitivity to anti-growth signals, tissue evasion and metastasis, limitless replicative potential, sustained angiogenesis, resistance to cell death, avoidance of immune destruction, induction of tumor-promoting inflammation, genome instability and mutation, and deregulation of cellular energetics (figure 1).

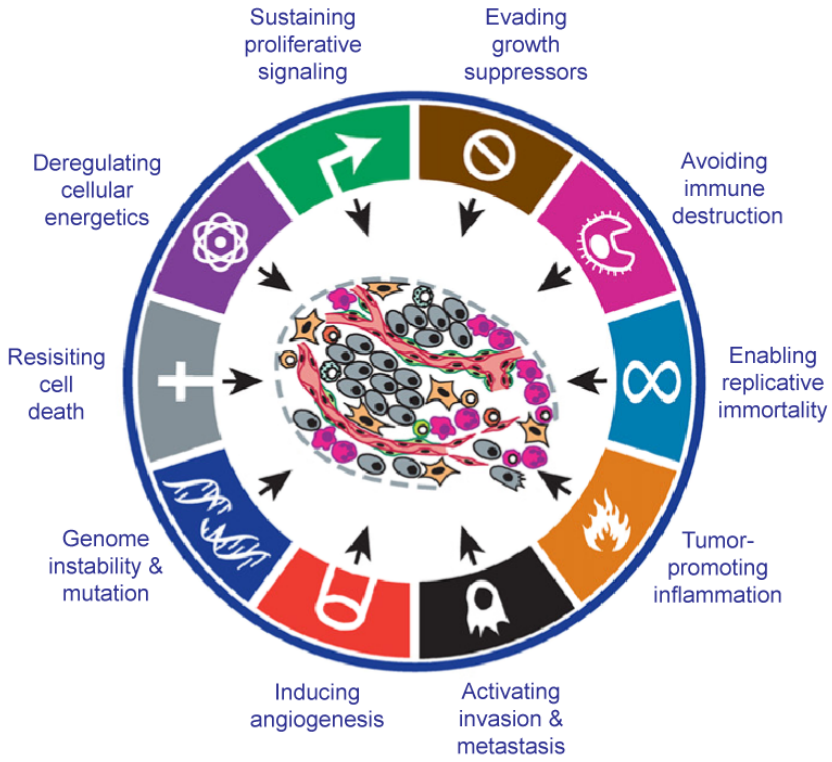

Figure 1: The ten hallmarks of cancer. Adapted from Hanahan & Weinberg (*4*) in compliance with the conditions of the Elsevier user license. Copyright © 2011 Elsevier, Inc.

1.1.1 Causes

Cancer can be caused by a large number of factors, which can be both of genetic and environmental nature. In familial types of cancer, a mutation predisposing a person to cancer is being inherited. Usually, these people develop tumors early in life (*5*). In addition, cancer syndromes exist in which a factor is mutated that is involved in the development of various types of cancer. An example is the Li-Fraumeni syndrome, in which the tumor suppressor

p53 is mutated. Subjects suffering from this syndrome often develop tumors early in life, in particular sarcomas, adrenocortical carcinomas, brain cancer and breast cancer (*6*).

On the contrary, the exposure to environmental factors typically leads to cancer later in life. About 90-95% of all cancers can be attributed to environmental factors (*7*).These can be of various natures, e.g. chemical, physical or biological. A large number of both synthetic and natural compounds has been described that can cause various types of cancer (*8, 9*). UV light, X-rays and asbestos are examples of physical damage that can cause tumor formation (*10*). Chronic inflammation might exert a tumor-promoting effect by triggering constant cell renewal at the site of inflammation. This condition may amongst other things be caused by autoimmune diseases like inflammatory bowel disease or pathogens like *Helicobacter pylori* (*11*). Viral infections, e.g. with human papillomavirus (HPV), have also been shown to cause cancer (*12*).

1.1.2 Current state of treatment possibilities

Drugs interfering with the hallmarks of cancer have been developed. Some of them have already been approved, while others are still being tested in clinical trials (*4, 13*). *Traditional chemotherapeutic drugs* aim at killing cells that divide rapidly. These drugs can be categorized into DNA intercalators, DNA alkylating agents, topoisomerase inhibitors, tubulin-binding drugs or anti-metabolites (i.e. structural analogues of naturally occurring metabolites involved in DNA and RNA synthesis). However, these drugs are not tumor-specific. In addition, a large number of these drugs is genotoxic and can therefore introduce mutations in both healthy and tumor cells. Consequently, healthy cells can die, resulting in side effects like alopecia (hair loss), anemia, thrombocytopenia and leukopenia (followed by immunosuppression). Alternatively, normal cells can accumulate mutations and hence form new tumors (*14*). On the other side, when mutations are introduced in tumor cells, these cells can become more aggressive and form metastases (*2*), which is often lethal to the patient.

More types of therapy exist. During *radiotherapy*, the affected area becomes exposed to ionizing radiation. However, as is the case for traditional chemotherapy, new mutations can be introduced and consequently lead to severe side effects, the formation of new tumors and more aggressive tumors. *Surgery* is an option in case a tumor is located where the removal of tissue would not be life-threatening, e.g in breast or prostate. *Immunotherapy* is an indirect and novel approach, during which immune cells are being activated to subsequently kill tumor cells. This can be achieved in several ways, e.g. by the use of cytokines or T-cell regulating antibodies (*15-17*). *Targeted therapy* is another novel approach that, in contrast to immunotherapy, aims at killing tumor cells directly. In this case, small molecules or antibodies are used that specifically target one protein or group of proteins (e.g. tyrosine kinase receptors). Examples are imatinib mesylate (Gleevec / Glivec), trastuzumab (Herceptin) and vemurafenib (Zelboraf) (*18*). It should be noted that therapies can be combined to achieve a more successful outcome (*15, 17*).

The success rates in oncology have been much lower compared with other fields, e.g. cardiovascular or infectious diseases (*19*). Currently, a major problem in curing patients is the resistance of cancer cells to therapy, which is often followed by relapse. It has been suggested that cancer stem cells, i.e. a fraction of cells that is supposed to drive tumor growth and progression, are responsible for this phenomenon. Targeting these cells is hence of particular interest (*4, 20, 21*).

Thus, the development of new drugs is of great importance to find cures for patients suffering from all the various types of cancer.

1.2 TUMOR SUPPRESSOR P53

p53 was the first tumor suppressor to be identified (*22-24*), although during the first ten years after its discovery it was assumed to be an oncogene (*25-27*). Currently, it is the most-studied tumor suppressor, with more than 70,000 scientific articles mentioning it on PubMed (status: July 2014). Because of its central role in cancer, it has been designated as "The Guardian of the Genome" (*28*), "The Cellular Gatekeeper for Growth and Division" (*29*) and "The Policeman of the Oncogenes" (*30*). Even though it is mutated in over 50% of all cancers (*22*), the mutation prevalence varies a lot between different types of tumors (*31*). In most of those cases, in which p53 is not mutated, the protein is still impaired in its function through one of the following mechanisms:

1) Negative regulators, in particular murine double minute 2 (MDM2) and murine double minute 4 (MDM4; also called MDMX), can be present at increased levels, e.g. due to gene amplification (*32-34*).
2) The upstream positive regulator alternative reading frame (ARF / $p14^{ARF}$) can be deleted or epigenetically inactivated (*35*).
3) Certain viral proteins can inhibit or downregulate p53, such as the simian virus 40 (SV40) large T antigen (*23, 24*), the adenovirus 5 E1b protein (*36*) and the E6 proteins of HPV 16 and 18 (*37, 38*).

More detailed information about p53 will be given in the following subchapters.

1.2.1 The genetics of p53

In this subchapter, the *TP53* gene and the different isoforms it encodes are described. The protein domains mentioned here are further described in subsection **1.2.2**.

1.2.1.1 The TP53 gene

The *TP53* gene is evolutionarily conserved (*39*) and located on human chromosome 17p13. 1. It contains eleven exons, the first of which is noncoding (*40*). A number of single-nucleotide

polymorphisms (SNPs) have been described in humans that affect p53 signaling in cells, resulting in differences in cancer risk and clinical outcome. The most studied SNP, designated rs1042522 in the Single Nucleotide Polymorphism Database (dbSNP) by the National Center for Biotechnology Information (NCBI), is located in codon 72. This SNP leads to a residue change from proline to arginine, which in turn affects the pro-apoptotic function of p53 (*39*, *41*).

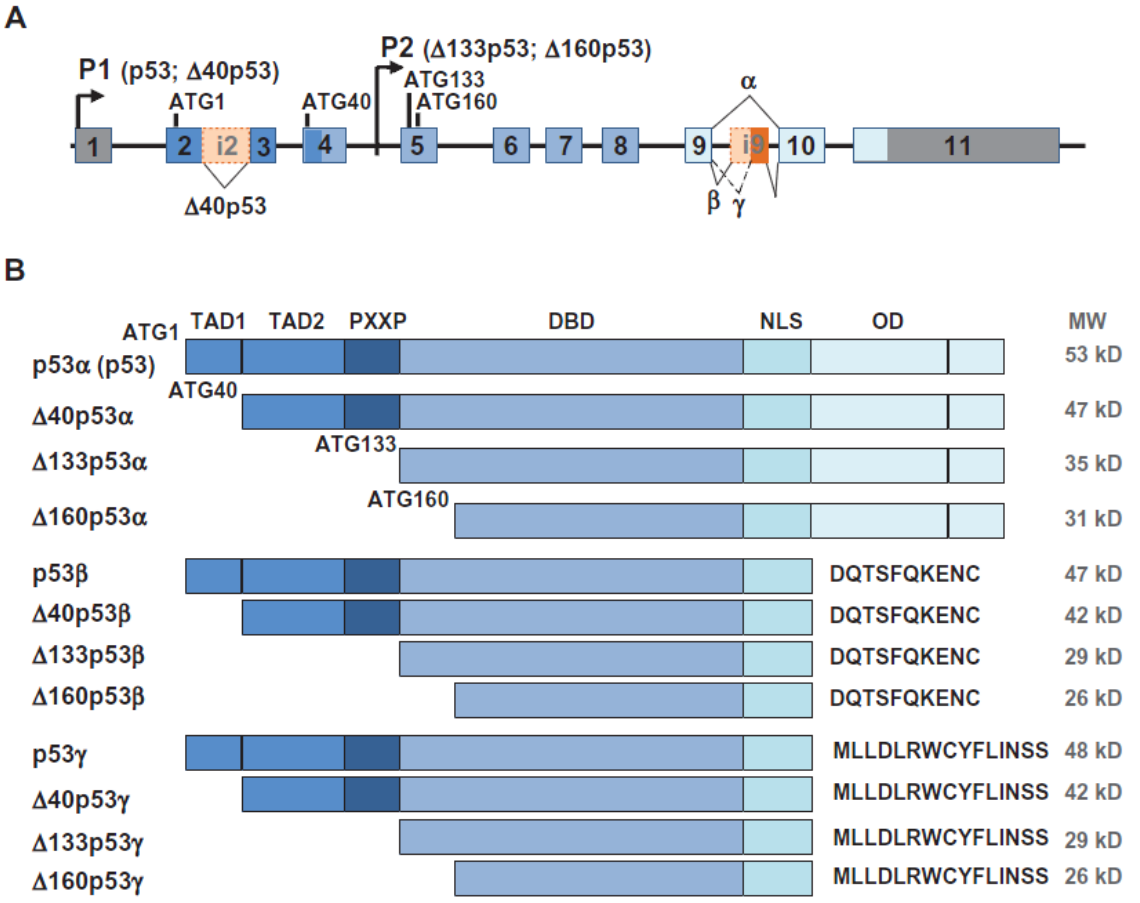

Figure 2: The isoforms of p53. (A) The human *TP53* gene structure. The *TP53* gene comprises eleven exons and encodes twelve p53 isoforms using alternative promoters (↱), splicing sites (^) or translational initiation sites (ATG). (B) Human p53 isoforms. Abbreviations: DBD, DNA-binding domain; kD, kilo Dalton; MW, molecular weight; NLS, nuclear localization signal; OD, oligomerization domain; PXXP, proline-rich domain; TAD, transactivation domain. Adapted from Surget et al. (*40*) in compliance with the conditions of the publisher's license. Copyright © 2013 Dove Medical Press Limited.

1.2.1.2 p53 isoforms

The *TP53* gene encodes at least twelve different p53 protein isoforms, which are the result of the usage of alternative promoters, initiation of translation at alternative start sites and alternative splicing as well as a combination thereof (figure 2) (*40*):

1) To date, two promoters, P1 and P2, have been identified. The proximal promoter P1 is located in front of exon 1 and encodes all isoforms that contain a complete N-terminus as well as the Δ40p53 isoforms, in which the first TAD is missing. The internal promoter P2 is situated in intron 4 and encodes all Δ133p53 and Δ160p53 isoforms, which lack both transactivation domains (TADs) and the proline-rich

region; in addition, the Δ160p53 isoforms lack parts of the DNA binding domain (DBD).

2) Four translation initiation sites have been described. The first one lies in exon 2 and initiates translation of all isoforms that contain the full-length N-terminus. The second site is in exon 4 and regulates the expression of the Δ40p53 isoforms. The remaining two translation initiation sites are situated in close proximity to each other in exon 5; translation of the Δ133p53 and Δ160p53 isoforms, respectively, starts from here.
3) Alternative splicing can occur at four different sites in the p53 transcript. Alternative splicing between exons 2 and 3 determines if isoforms containing the full-length N-terminus or Δ40p53 isoforms will be synthesized. The exclusion of intron 9 generates α isoforms, whereas partial retention thereof leads to generation of β and γ isoforms, respectively. The part of intron 9 that is included in the β isoforms is also called exon 9b, and the part included in the γ isoforms is also called exon 9g. α isoforms contain an oligomerization domain (OD) and a negative-regulation domain, both of which are lacking in the β and γ isoforms due to the presence of a stop codon in both exon 9b and exon 9g.

The canonical p53 protein (also named p53, full-length p53 (FLp53), p53α, or TAp53α) constitutes the most abundant isoform encoded by *TP53*. The different isoforms are expressed at different levels in both normal and tumor cells, and they can be expressed in different subcellular compartments. Furthermore, their functions can vary, i.e. they can inhibit or enhance p53 tumor-suppressor activity in a p53-(α) dependent and independent manner. For example, Δ40p53α has been shown to have dominant-negative effects, and Δ133p53α can promote tumor formation and aggressiveness in a p53(α)-dependent and -independent manner. On the contrary, p53β can enhance p53 transcriptional activity, resulting in a higher rate of senescence and apoptosis (*40*).

Importantly, cell lines, e.g. HCT116 and RKO, have been generated, in which p53 supposedly was knocked out. “Knock-outs” were performed by replacing the first codon present in exon 2 with a resistance marker gene (*42, 43*). However, this modification resulted in a gene that still encodes all isoforms containing a truncated N-terminus, i.e. all Δ40p53, Δ133p53 and Δ160p53 isoforms. Indeed, expression of Δ40p53 protein has been detected in HCT116 p53-deficient cells (*44*). This should be borne in mind when using these cell lines.

1.2.2 The functional domains of the p53 protein

The canonical p53 protein is 393 aa long (*45*) and contains the domains described in the following subsections. Figure 3 illustrates the domains. It should be noted that the exact number of amino acid residues assigned to the different domains can vary depending on the source of information.

Figure 3: p53 protein domains. Numbers indicate amino acid residues. Orange bars indicate NLSs; the black bar shows where the NES is located. The protein and its domains are drawn to scale. Abbreviations: CRD, C-terminal regulatory domain; DBD, DNA-binding domain; NLS, nuclear localization signal; OD, oligomerization domain; PRR, proline-rich domain; TAD, transactivation domain.

1.2.2.1 Transactivation domain (TAD)

The TAD, which is required for p53 to act as a transcription factor, is located at the N-terminus. This domain can be further subdivided into TAD 1 at amino acids (aa) 1–40 and TAD2 at aa 41–61. The TAD is a binding site for a multitude of interacting proteins, such as components of the transcription machinery, the transcriptional co-activators p300/CBP (CREB-binding protein), and the negative regulators MDM2/MDM4 (*45, 46*). Despite the earlier assumption that TAD1 played a more important role in transactivation than TAD2 (*47*), it has been shown that the transactivating function of p53 depends on four residues, each two of which are present in each TAD (*46*).

1.2.2.2 Proline-rich domain (PRR)

In close proximity to the TADs is a proline-rich domain (PRR) (aa 64–92), which contains five repeats of the aa sequence PXXP (where P represents proline and X any amino acid). It is involved in growth suppression and protein–protein interactions through binding to Src homology 3 domains (*45, 48*).

1.2.2.3 DNA-binding domain (DBD)

The DBD is located in the center of the protein (aa 94–292). It is crucial for p53 to function as a transcription factor, since it facilitates binding to response elements (REs). (*49*). Six mutation hotspots have been identified in the *TP53* gene, and they are all located in this domain (*31*). All of these mutations result in the loss of p53 binding to REs and eventually altered target gene expression. Two of the mutants harboring hotspot mutations are so-called "contact mutants". They have mutations at Arg-248 and Arg-273, which are residues that make direct contact to DNA. The other four hotspot mutants, i.e. Arg-175, Gly-245, Arg-249 and Arg-282, are "conformational mutants" that have undergone a conformational change in the 3D structure of the DNA-binding surface and hence lose their ability to bind REs (*50, 51*). It should be noted, however, that mutant p53 can have pro-oncogenic functions (i.e. a gain-of-function), which in part may be due to the binding of genes other than those targeted by wt p53 (*46*).

1.2.2.4 Nuclear localization signals (NLSs)

p53 contains three nuclear localization signals (NLSs) that facilitate translocation from the cytoplasm into the nucleus, a pre-requisite for p53 to become transcriptionally active. NLSI is located in closest proximity to the DBD, i.e. at aa 318-322, and is regarded as being the most

essential NLS for nuclear import. NLSII (aa 378-382) and NLSIII (aa 386-390) are weaker than NLSI and contribute less to p53 nuclear import (*52-54*).

1.2.2.5 Oligomerization domain (OD)

p53 is most active as a tetramer. Its oligomerization domain (OD) facilitating tetramerization is located at aa 325–355. A leucine-rich nuclear export signal (NES) (aa 340-351) is located inside this domain. It facilitates export from the nucleus into the cytoplasm and is also required for tetramerization. Tetramer formation masks the NES and prevents access to the nuclear export machinery, which further enhances p53's transcritional activity (*55*).

1.2.2.6 C-terminal regulatory domain (CRD)

Also the C-terminus of p53 has a regulatory function. Next to the nuclear localization signals NLSII and NLSIII mentioned above it contains residues that can be post-translationally modified. More information about this topic can be found in subsection 1.2.3.

1.2.3 Post-translational modifications

Various post-translational modifications have been described in connection with the p53 protein. It can be phosphorylated, ubiquitinated, neddylated, sumoylated, acetylated and methylated (figure 4) (*56*). Phosphorylation and acetylation generally activate p53, whereas ubiquitination targets p53 for nuclear export and degradation by the proteasome.

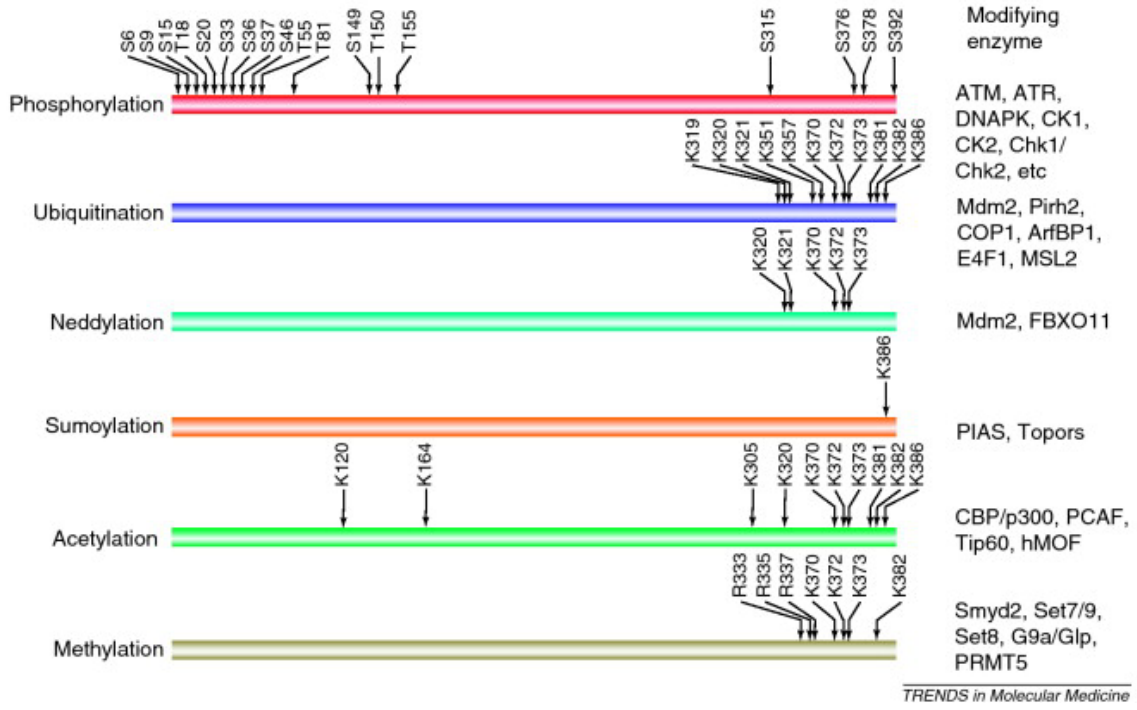

Figure 4: Post-translational modifications of p53. Amino acid residues that can be post-translationally modified are each indicated on top of the corresponding bar. A selection of enzymes catalyzing these modifications are shown on the right. Please refer to figure 5 for an updated version of the sites that can be acetylated. Adapted from Dai & Gu (*56*) with permission from the publisher. Copyright © 2010 Elsevier, Inc.

1.2.3.1 Phosphorylation

Phosphorylation was the first post-translational modification of p53 to be identified (*57*). A number of serine and threonine residues, which are mainly located at the N-terminus, can be phosphorylated by several kinases, including ATM/ATR/DNAPK and Chk1/Chk2, that become activated upon DNA damage. In particular, phosphorylation at Ser-15 and Ser-20 has been studied extensively. Phosphorylation at these residues stabilizes p53 by disrupting its interaction with MDM2 and promotes the recruitment of transcriptional coactivators (*56, 58*).

1.2.3.2 Ubiquitination

During the process of ubiquitination, either one or more ubiquitin molecules, about ~8 kDa in size, are conjugated to lysine residues of a target protein. An E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin-ligating enzyme are required for this process. Ubiquitination has been detected at eleven lysine residues at the C-terminus of p53, six of which (i.e. Lys-370, Lys-372, Lys-373, Lys-381, Lys-382 and Lys-386) can be ubiquitinated by MDM2. The latter is the principal E3 ubiquitin-ligase for p53 next to approximately 20 other E3 ubiquitin-ligases. MDM2 can catalyze both multi-mono- and polyubiquitination. Multi-monoubiquitination, i.e. the simultaneous monoubiquitination of several lysine residues, results in inhibition of acetylation of p53, which is crucial for p53 to function normally, as well as nuclear export of p53. Polyubiquitination leads to proteasomal degradation of p53. Next to ubiquitinases, a comparatively small number of deubiquitinases (DUBs) have been identified. They aid in stabilizing the p53 protein and removal of the ubiquitin tag for nuclear export (*56, 59*).

1.2.3.3 Neddylation

A ubiquitin-like protein called neural precursor cell expressed developmentally downregulated protein 8 (NEDD8) can be conjugated to p53 as well. It resembles ubiquitin in both its 3D structure and its mechanism of conjugation through lysines. MDM2 can neddylate p53 at residues Lys-370, Lys-372 and Lys-373, whereas F-box protein 11 (FBXO11) neddylates Lys-320 and Lys-321. In contrast to ubiquitination, neddylation does not induce changes in p53 localization or stability, but it affects its transactivational activity (*56, 58*).

1.2.3.4 Sumoylation

Small ubiquitin-like modifier (SUMO) is another ubiquitin-like protein. Only one residue, i.e. Lys-386, has been identified to date that can be sumoylated. Enzymes facilitating sumoylation of p53 are sumoylated by members of the protein inhibitor of activated stat (PIAS) family and topoisomerase I binding, arginine/serine-rich, E3-ubiquitin protein ligase (Topors). The function of sumoylation is unclear. Both promotion and inhibition of p53 transcriptional activity as well as translocation of p53 to the cytoplasm have been suggested (*56, 58, 59*)

1.2.3.5 Acetylation

During the process of acetylation, acetyl groups are enzymatically placed onto the ε-amino group of lysine residues of target proteins. p53 was the first non-histone substrate shown to be acetylated by histone acetyl transferases (HATs) (*60, 61*). Acetylation occurs in response to cellular stress, e.g. DNA damage, and leads to stabilization and activation of p53 as well as recruitment of transcriptional cofactors. 13 lysine residues can be acetylated in the p53 protein (figure 5), three of which are located in the DBD (Lys-120, Lys-164 and Lys-292), one in the linker region between DBD and OD (Lys-305), three in the OD (Lys-320, Lys-351 and Lys-357) and the remaining six in the CRD (Lys-370, Lys-372, Lys-373, Lys-381, Lys-382 and Lys-386).

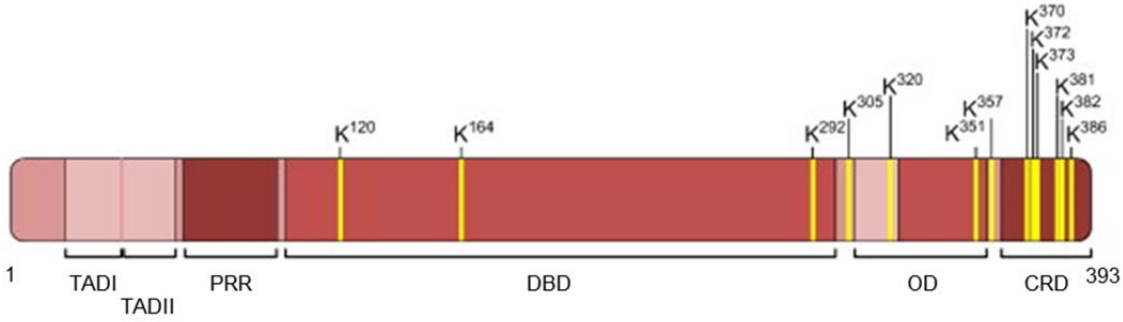

Figure 5: Acetylation sites of p53. Acetylation sites are indicated by yellow bars and the respective aa residue number within the p53 protein. Abbreviations: CRD, C-terminal regulatory domain; DBD, DNA-binding domain; K, lysine; OD, oligomerization domain; PRR, proline-rich domain; TAD, transactivation domain. Adapted from Wagner et al. (*62*) with permission from the publisher. Copyright © 2014 Elsevier, Inc.

The HATs p300 and CREB-binding protein (CBP) acetylate p53 at positions 164, 305, 370, 372, 373, 381, 382 and 386; the HAT p300/CBP-associated factor (PCAF) acetylates p53 at Lys-320. Human males-absent-on-the-first (hMOF) and tat-interactive protein of 60 kDa (TIP60) can acetylate p53 at Lys-120. Ac-Lys-120 was shown to be critical for induction of apoptosis, but to have no effect on DNA binding and protein stability (*56, 61-63*). Simultaneous acetylation of eight lysine residues, i.e. Lys-120, Lys-164, Lys-370, Lys-372, Lys-373, v381, Lys-382 and Lys-386, was shown to be required for interruption of the physical interaction between p53 and MDM2 at target gene promoters. This is a pre-requisite for p53 to activate transcription of the pro-apoptotic genes *BAX*, p53 up-regulated modulator of apoptosis (*PUMA*) and p53-inducible gene 3 (*PIG3*). Furthermore, when these eight residues are acetylated, phosphorylation of p53 becomes dispensable for transcription of these target genes upon treatment with the DNA damage-inducing compound actinomycin D (actD). Interruption of the MDM2-p53 interaction by mutation of the above-mentioned eight lysine residues also abolished p21 expression (*64*). However, in contrast to pro-apoptotic proteins, p21 can be expressed at lower levels in the presence of MDM2-p53 complexes, suggesting that its transcription is less sensitive to the physical interaction between p53 and MDM2. Interestingly, transcription of the *MDM2* gene was shown to be unaffected by the binding of MDM2 to p53 (*58, 61*).

Acetylation is a reversible process. Acetyl-groups can be removed by histone deacetylases (HDACs), a more detailed description of which will follow in chapter 3. HDAC1 present in a

protein complex was shown to deacetylate p53. HDAC1 may not be able to directly interact with p53, but might depend on other proteins to reach it (*65*). Also HDAC2 has been shown to deacetylate p53, i.e. at residues Lys-320, Lys-373 and Lys-382. Which residues are targeted might be cell-type specific, though (*62*). In addition, the class III HDACs sirtuin 1 (SirT1) and SirT2 have been shown to deacetylate p53 at Lys-382 (*66-69*).

1.2.3.6 Methylation

p53 can be methylated at arginine and lysine residues at its C-terminus. A number of methyltransferases have been described that can mono- or dimethylate p53. Protein arginine N-methyl transferase 5 (PRMT5) interacts with p53 *via* the protein Strap and methylates Arg-333, Arg-335 and Arg-337. Methylation of these residues might be required for p21 transcription and a subsequent induction of cell cycle arrest (*70*). Smyd2 and Set8/PR-Set7 monomethylate p53 at K370 and K382, respectively, which leads to repression of p53 activity. Set7/9 monomethylate p53 at Lys-372, which facilitates acetylation of p53 by Tip60 and prevents methylation of K370 by Smyd2; therefore, monomethylation of Lys-372 results in enhanced p53 activity (*71, 72*). In addition, p53 can be dimethylated on Lys-370 and Lys-382, which facilitates its interaction with p53-binding protein 1 (53BP1), an important mediator of the DNA damage response (DDR) upon occurrence of DNA double-strand breaks (DSBs) (*73, 74*). Demethylation of Lys-370me2 to Lys-370me1 by lysine specific demethylase 1 (LSD1) represses p53 function through the inhibition of the interaction between p53 and 53BP1 (*56, 74*).

1.2.4 The p53 pathway

p53 can be activated by various types of stress, e.g. DNA damage, oncogene activation, nutrient deprivation, ribonucleotide depletion, telomerase shortening or hypoxia (figure 6) (*27*). Once activated, it mainly acts as a transcription factor. In addition, p53 has been shown to induce cell death via non-transcriptional mechanisms (*75*):

1) It can promote translocation of the pro-apoptotic protein Bax from the cytoplasm to mitochondria. In turn, Bax forms pores in the mitochondrial outer membrane, which results in cytochrome c release and eventually apoptosis.
2) It can facilitate the release of the pro-apoptotic protein Bak from inhibitors in the mitochondrial outer membrane to facilitate pore formation and subsequent cytochrome c release, which results in apoptosis.
3) p53 can induce necrosis upon reactive oxygen species (ROS) formation.

p53 has a large number of transcriptional targets, through which it exerts its effects like cell cycle arrest, apoptosis, DNA repair, senescence or the induction of an anti-oxidant response (*27*). Important target genes and key players in the p53 pathway are described in the following subsections.

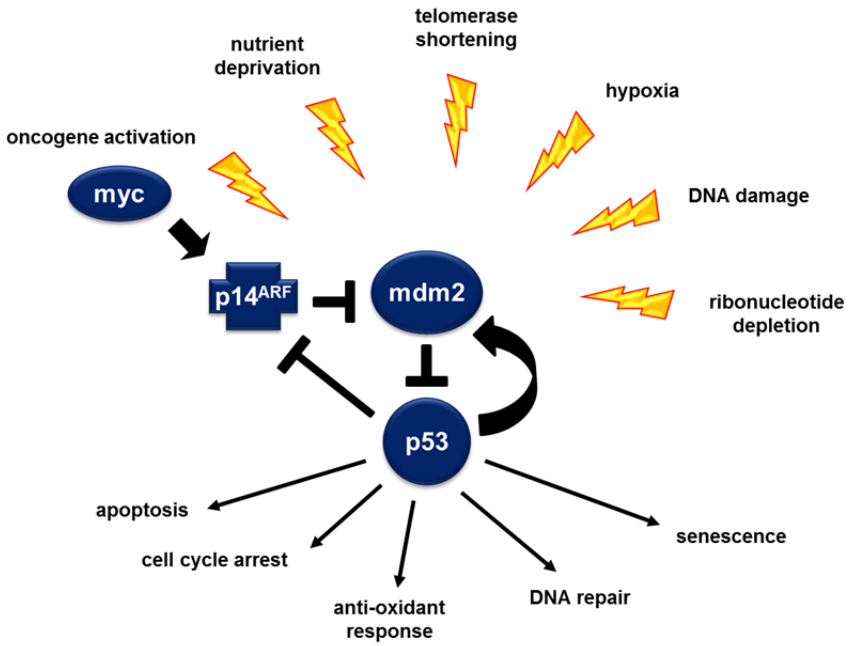

Figure 6: Regulation and action of p53.

1.2.4.1 CDKN1A (p21)

p21 is also known as wild-type p53-activated fragment 1 (Waf1), cyclin-dependent kinase-interacting protein 1 (Cip1), senescent cell-derived growth inhibitor 1 (SDI1), melanoma-derived antigen 6 (MDA6) and cyclin-dependent kinase inhibitor 1 A (CDKN1A), and is encoded by the *CDKN1A* gene. p21 has many functions, the most studied of which is it ability to inhibit cyclin-dependent kinases (cdks), in particular cdk1 and cdk2, and their interaction with cyclins. This inhibition eventually leads to cell cycle arrest and/or senescence (*76*).

1.2.4.2 Pro-apoptotic genes

Target genes like *BAX*, *PUMA, PIG3, NOXA, FAS* (also known as e.g. CD95) and death-receptor 5 (*DR5*; also known as e.g. *KILLER* or *TRAILR2*) encode proteins that exert pro-apoptotic functions (*22*).

1.2.4.3 MDM2

MDM2 encodes the main negative regulator of p53. In humans, it is called *HDM2*.As mentioned in subsection *1.2.3.2*, it is an E3-ubiquitin ligase that can both multi-mono- and polyubiquitinate p53. Crystallographic data showed that the N-terminal domain of MDM2 forms a deep hydrophobic cleft into which the TAD (aa 18-23) of p53 binds (*77-79*). This physical interaction interferes with p53's transactivational abilities (*33, 61, 77, 80*).

1.2.4.4 MDMX

The *MDMX* gene (also called *MDM4*; *HDMX* in humans) can be transcribed from two promoters, i.e. the constitutive P1 promoter and the alternative P2 promoter. The latter gives rise to a protein that has a longer N-terminus than its constitutively expressed counterpart and is called HDMX-L ("L" standing for "long"). p53 can only transactivate transcription of

HDMX-L and does so only under certain circumstances (*81*). In general, MDM4 is thought to play a dual role in p53 regulation. It can form heterodimers with its homolog MDM2 via its RING domain and, depending on its abundance, it can subsequently enhance or decrease the ubiquitin-ligase function of MDM2 (*33*).

1.2.4.5 p14^{ARF}

p14^{ARF} (called p19^{ARF} in mice) is one of the three products of the *INK4b-ARF-INK4a* locus, also called *CDKN2A*, that encodes p15^{INK4B}, p14^{ARF} and p16^{INK4a}. Due to alternative splicing the open reading frames (ORFs) differ between the two possible transcripts, eventually resulting in one of the protein products. p14^{ARF} becomes induced upon activation of certain oncogenes, e.g. myc, upon which it exerts its function as an MDM2 inhibitor (*82, 83*). p14^{ARF} can physically interact with MDM2 (*84*). The literature suggests that this can lead to either MDM2 degradation or stabilization (*84, 85*). Furthermore, this binding may lead to inhibition of MDM2's ubiquitin-ligase activity on p53 as well as sequestration of MDM2 in the nucleolus, where p14^{ARF} is mainly located. Furthermore, p14ARF may inhibit the export of p53/mdm2 complexes from the nucleus (*86-88*). Next to MDM2, p14^{ARF} can inhibit ARF-binding protein 1 (ARF-BP1; also known as Mcl-1 ubiquitin ligase E3 (Mule)). Like MDM2, ARF-BP1 acts as specific E3 ubiquitin- ligase for p53. However, ARF-BP1 may not be transcriptionally regulated by p53 (*89*). Thus, p14^{ARF} can increase p53 levels through the inhibition of at least two negative regulators. Interestingly, p53 negatively regulates p14^{ARF} expression (*85*), which contributes to the negative feedback loop of p53 (figure 6).

1.2.5 Pharmacological reactivation of wt p53

p53 is mutated in over 50% of all cancers, and in the remaining cases it is usually inactive due to overexpression of MDM2 or MDMX (*32-34*), deletion or epigenetic silencing of p14^{ARF} (*35*) or the presence of viral proteins (*23, 24, 36-38*). Reactivation of p53's tumor-suppressive function in cancer has been approached by many scientists in academia and industry. Several strategies have been developed to reactivate wt p53, such as inhibition of the interaction between p53 and its negative regulators MDM2 and MDMX or inhibition of other factors that directly or indirectly affect p53 activity. Also strategies for reactivation in tumors expressing mutant p53 have been established, such as p53 gene therapies and restoration of the 3D structure of conformational p53 mutants (*90*). Since the studies presented in this thesis focus on pharmacological reactivation of wt p53, the following subsections will highlight this topic.

1.2.5.1 Inhibition of MDM2/MDMX-p53 interactions via binding to p53

p53 interacts with MDM2 and MDMX via its TAD. Only one small molecule, called reactivation of p53 and induction of tumor cell apoptosis (RITA), has been described so far that reactivates wt p53 by interruption of MDM2-p53 and MDMX-p53 interactions through

direct binding to p53. The compound induces apoptosis in tumor cells, but not in normal cells (*91, 92*), which is the desirable effect in cancer therapy.

1.2.5.2 Inhibition of MDM2-p53 interactions via binding to MDM2

A number of compounds have been identified that interfere with the interaction of MDM2 and p53 by competitive binding to MDM2. Some of these are structural analogs, e.g. nutlin-3, RG7112 (RO5045337) and RG7388 (RO5503781); RO-2443 and RO-5963; and MI-219, MI-773 (SAR405838) and MI-888 (*90*). In addition, new compounds (RO2468, RO5353 and RO8994) have recently been designed by combining functionally and pharmacokinetically important chemical groups of RG7388 and MI-888 in one molecule (*93, 94*). Nutlins were the first small-molecule inhibitors of the p53–MDM2 interaction to be identified, with nutlin-3a being the most potent structural analog amongst those tested in the initial study, which was published ten years ago (*95*). Following up on that trend, the nutlin analog RG7112 was the first inhibitor of p53–MDM2 binding being tested in clinical trials (*96*). Since then, also RG7388, MI-773 and DS‑3032b have entered phase I clinical trials (*90*). It should be noted, however, that nutlins have a pro-apoptotic response in some tumor types, whereas in others they induce cell cycle arrest (*90*). The latter is an undesired effect in the treatment of cancer, since relapse may occur after termination of the therapy. Careful choice of patients and further studies will therefore be required.

1.2.5.3 Inhibition of p53-MDM2/MDMX interactions by binding to MDM2 and/or MDMX

RO-2443 and its more water-soluble structural analog RO-5963 do not only bind to MDM2, but also MDMX with high affinity. This has been shown to be of great advantage in those tumors that express high levels of MDMX, since these can be resistant to compounds that target MDM2 only (like nutlins and compounds from the MI series). RO-2443 and RO-5963 homo- and/or heterodimerize MDM2 and MDMX proteins, thereby preventing both of them from interacting with p53. This leads to p53 activation and eventually cell cycle arrest and cell death (*97*).

Stapled peptides are a new class of molecules that entered the p53 field less than five years ago. Several stapled peptides have been developed to inhibit p53-MDM binding. Interestingly, most stapled peptides developed in this context so far have been shown to inhibit both MDM2 and MDMX, suggesting that resistance to these molecules due to elevated MDMX levels may not be a problem (*90*).

1.2.5.4 Other approaches of p53 reactivation

Since p53 can be regulated by a large number of upstream factors and events to fulfill its function as a tumor suppressor, there are a many additional ways to reactivate it. A few examples are the following ones:

1) As mentioned in subsection *1.2.3.5*, the sirtuins SirT1 and SirT2 can deacetylate p53 at Lys-382, which leads to inactivation of the protein (*66-69*). Sirtuin inhibitors, e.g.

tenovin-6, salermide or sirtinol, can activate p53 by simultaneous inhibition of SirT1 and SirT2 (*68, 98*).

2) Roscovitine can, next to its CDK-inhibitory function, reduce MDM2 levels inside cells, which in turn activates p53 (*99*).
3) ActD has also been shown to induce p53. At low doses (i.e. ≤ 20 nM) (LDactD) it does not induce DSBs and induces p53 by inhibition of RNA polymerases by DNA intercalation in guanosine-rich regions (*100-102*). Ribosomal DNA is particularly rich in guanosine, and hence a decrease in rRNA levels upon inhibition of RNA polymerases I and/or III by LDactD may lead to increased levels of free ribosomal proteins, such as L11. The latter inhibits MDM2 (*101-103*). Further, inhibition of RNA polymerase II by LDactD followed by a decrease in mRNA levels has been described to activate p53 (*101*).
4) p53 becomes exported from the nucleus by exportin-1 (XPO1; also called chromosome region maintenance 1 (CRM1)), which binds to the NES at the p53 C-terminus. Inhibition of XPO1 by leptomycin B (LMB) or the compounds KPT-185, KPT-276 and KPT-330, respectively, leads to accumulation of p53 in the nucleus where it acts as a trancription factor. However, both leptomycin B and the KPT compounds were shown to have a strong cytostatic effect, which may be undesirable in cancer therapy (*104-106*).

1.2.5.5 Cyclotherapy

Chemotherapeutic drugs like tubulin poisons and DNA damaging compounds are widely used in the clinic to treat cancer. However, since they target any type of cell that divides frequently, side effects like alopecia, anemia, thrombocytopenia, leucopenia and the formation of new tumors as well as further dedifferentiation of existing tumor cells are often the consequence (*14*). The concept of p53-dependent cyclotherapy was first suggested by David P. Lane in 1992 (*28*), but it took about another 20 years, until it was tested in cell culture for the first time (*107*) and the term "cyclotherapy" was coined (*108*). During cyclotherapy, patients carrying p53-mutant tumors would initially be treated with a non-genotoxic drug that activates wt p53 and primarily has a cytostatic effect. Since the tumors would express mutant p53, only the patients' healthy cells would react to this drug. Afterwards, a second drug that has a p53-independent cytotoxic effect would be given to the patients, such as a classical chemotherapeutic agent. Since these drugs primarily kill dividing cells, only tumor cells would be affected. After tumor clearance, drug treatment would be terminated. Healthy cells would have survived and would recover from potential temporary damage caused by the p53-activating drug (figure 7) (*109, 110*).

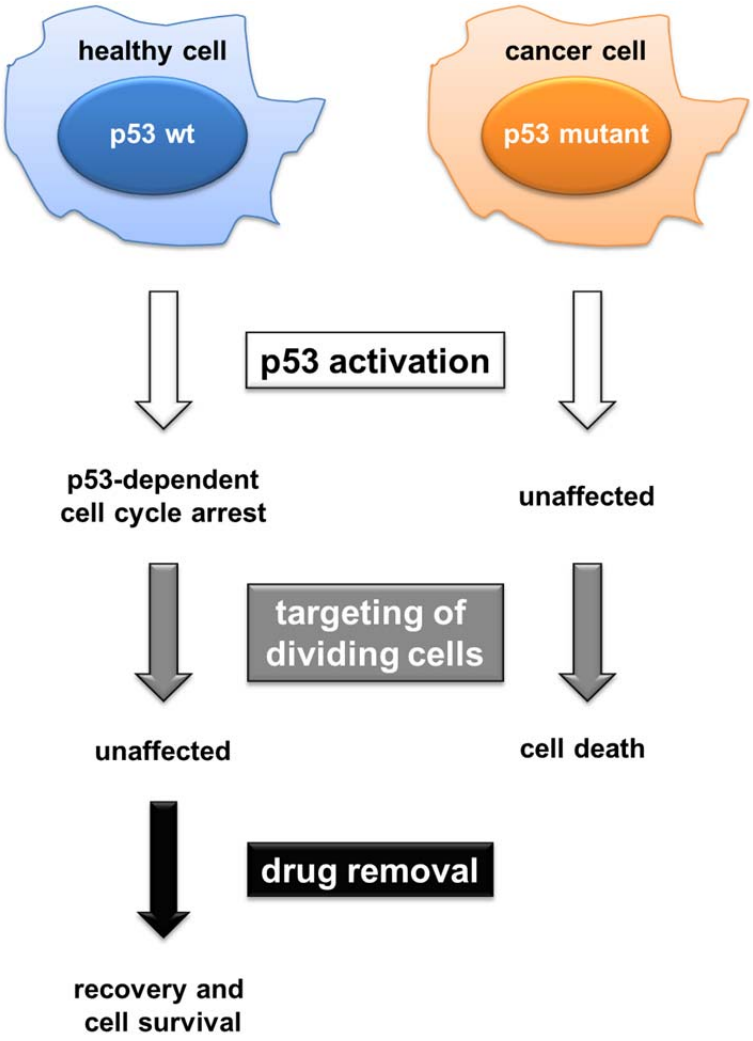

Figure 7: The concept of cyclotherapy.

Several drug combinations have been tested in this context in cell culture (*109, 110*). A xenograft study showed that neutropenia caused by the polo-like kinase 1 (PLK1) inhibitor BI-2536 could be prevented, if mice were co-treated with nutlin-3 (*43*). This may be the only study that suggests that the cyclotherapy concept could work *in vivo*. Thus, further *in vivo* studies will be required for cyclotherapy to be tested in clinical trials. Furthermore, the majority of the cytostatic compounds that have been tested in cell culture in a cyclotherapy context have not been clinically approved yet. Some possible candidates are currently being tested in phase I clinical trials, e.g. RG7112, RG7388, MI−773 and DS−3032b, whereas others, like tenovin-6, have not reached this stage yet. Thus, time will tell if cyclotherapy eventually will enter the clinic.

1.2.5.6 Cell-based assay to screen for activators of wt p53

Several screens using a cell-based assay have been performed in Sonia Laín's laboratory to identify small molecules that can reactivate wt p53. Structural analogs or the actual hit compounds identified *via* these screens form the basis of five out of six studies presented in this thesis, i.e. tenovin-6 (**papers I and IV**), tenovin-D3 (**paper II**), MJ05 (**paper IV**) and MJ25 (**paper VI**). The cell-based assay used for screening purposes was developed by Frebourg and colleagues in 1992 and was originally intended as a method for the

identification of germline mutations that lead to transcriptional inactivation of p53 (*111*). In 2005, Berkson and colleagues were the first ones who applied this assay for the purpose of screening small molecules to identify wt p53 reactivators (*112*).

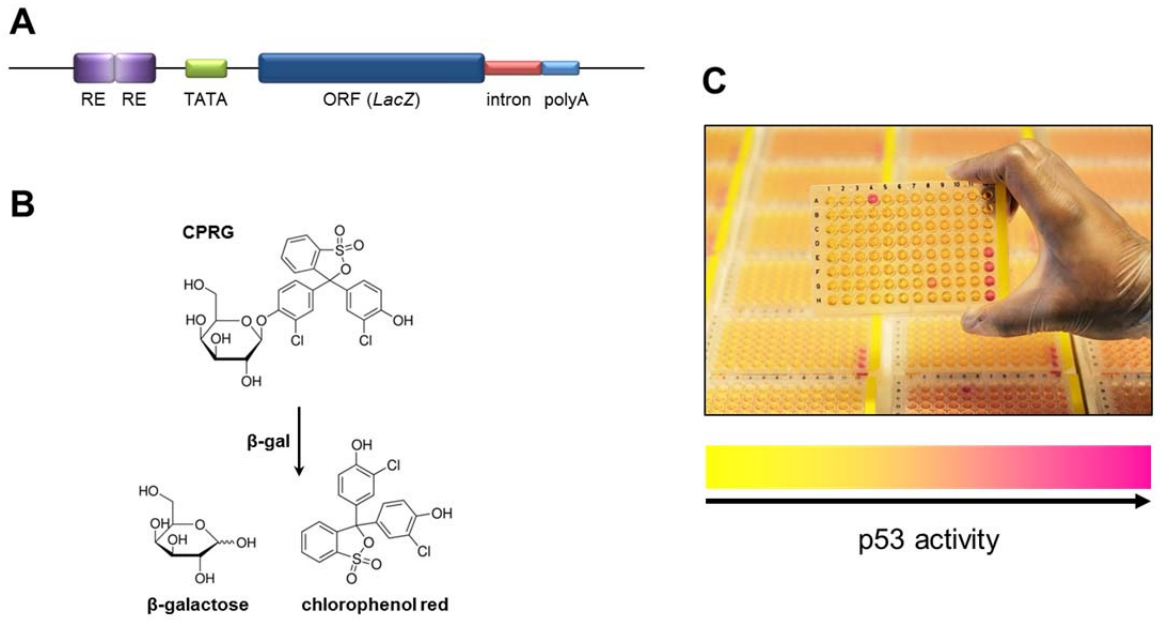

Figure 8: Cell-based reporter assay for the determination of p53 activity. (A) The reporter construct pRGCΔFosLacZ contains the following elements: Two p53 response elements (REs) derived from the ribosomal gene cluster (RGC) in a head-to-head orientation, a promoter containing a TATA box derived from a truncated promoter of the murine *fos* gene, the open reading frame (ORF) of the *LacZ* gene encoding β-galactosidase, an intron derived from the SV40 small-t antigen and the SV40 polyadenylation signal (polyA). (B) p53 activity can be measured by detection of chlorophenol red, a red chromophore produced by β-galactosidase (β-gal) in the presence of chlorophenol red-β-D-galactopyranoside (CPRG). (C) Illustration of the assay in 96-well format. A yellow-to-magenta gradient is proportional to the presence of chlorophenol red and hence an indirect indicator of p53 activity. Panel C was adapted from van Leeuwen and colleagues (*113*) in compliance with the conditions of the publisher's license.

Two cell lines, i.e. the murine prostate-derived cell line T22 (*112, 114*) and the human melanoma cell line ARN8 (*115*) (a subclone derived from the A375 cell line), have been generated by stable transfection with the plasmid pRGCΔFosLacZ (figure 8A). This plasmid contains two copies of the p53-binding site in the ribosomal gene cluster (RGC) in a head-to-head orientation (*116*), which was cloned upstream of a deletion mutant of the murine *fos* promoter. The latter consists of a fragment that includes 56 base pairs (bp) located upstream of the transcription start site of the *fos* gene; this fragment contains a TATA box, but is devoid of other regulatory elements (REs) (*117*). The Δ*fos* promoter is followed by the ORF of the *LacZ* gene (encoding β-galactosidase), the SV40 small-t antigen intron, which may increase the efficiency of transcription and the stability of the mRNA product, and the polyadenylation signals of SV40 (*111*).

T22 and/or ARN8 cells are treated with compound of interest, e.g. small molecules derived from chemical libraries as in Sonia Laín's screens, for the desired period of time (e.g.

overnight). Afterwards, they are lysed and a substrate of β-galactosidase, i.e. chlorophenol red-β-D-galactopyranoside (CPRG), is added to the lysates. CPRG is yellow in color and becomes converted to β-galactose and chlorophenol red (figures 8B and 8C). The latter is a red chromophore that can be quanitified spectrophotometrically, e.g. with a microplate reader.

1.3 HISTONE DEACETYLASES

Histone deacetylases (HDACs) were identified due to their ability to deacetylate acetyl-lysine (Ac-K / Ac-Lys) residues in histones, but are nowadays sometimes referred to as KDACs due to their ability to target Ac-Lys in non-histone proteins (figure 9). The first mammalian HDAC (HDAC1, back then called HD1) was identified in 1996 (*118*) and since then 17 additional HDACs have been identified (*119*).

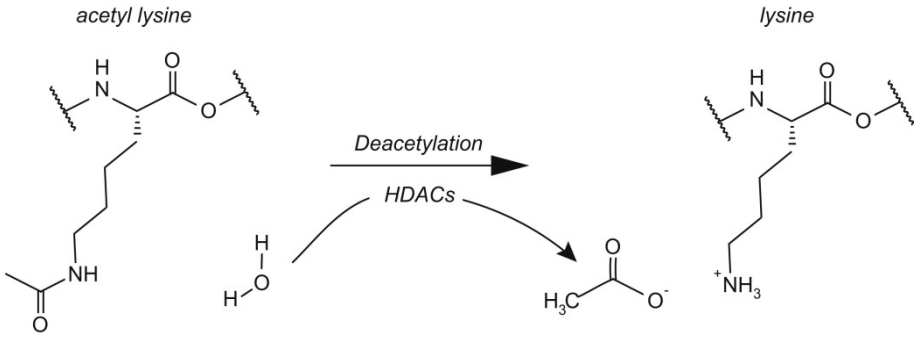

Figure 9: Deacetylation of lysine residues present in histones and non-histone proteins by histone deacetylases (HDACs). An acetyl group present at the ε-amino group of a lysine residue is removed in the presence of water (H_2O) and an HDAC, resulting in the formation of an acetate molecule and lysine. Adapted from Barneda-Zahonero & Parra (*119*) in compliance with the conditions of the Elsevier user license. Copyright © 2012 Elsevier, Inc.

1.3.1 Classification

HDACs have been classified according to their homology with yeast proteins (*119*):

- Class I HDACs, i.e. HDAC1, HDAC2, HDAC3 and HDAC8, are highly homologous to the yeast transcriptional regulator Rpd3p. They are ubiquitously expressed in all tissues.
- Class II HDACs, i.e. HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10, are homologs of Hda1. This class can be further subdivided into class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and class IIb (HDAC6 and HDAC10) based on similarities and differences in their protein structure. Class II HDACs are expressed in a tissue-specific manner.

- Class III is comprised of the sirtuins, i.e. SirT1 to SirT7, and they are homologous to silencing information regulator 2 (SIR2). Their expression is not restricted to certain tissues, but their subcellular localization can differ (see below).
- Class IV consists of HDAC11, the catalytic core region of which is homologous to that in class I and II HDACs. This HDAC is also expressed in a tissue-specific manner (*120*).

1.3.1.1 "Classical" HDACs

Class I, II and IV HDACs are also referred to as "classical" HDACs and they are Zn^{2+}-dependent. They exert partially overlapping and partially individual functions. Overall, HDACs are considered to be able to deacetylate histones, which results in chromatin condensation (i.e. the formation of heterochromatin) and therefore a decreased access of transcription factors to their target genes. Depending on their individual target proteins and expression in different tissues, HDACs can be involved in the regulation of proliferation, apoptosis, DNA damage responses and cell differentiation as well as the development and physiology of organisms (*119*).

1.3.1.2 Sirtuins

Sirtuins, on the other hand, are dependent on the cofactor NAD^+ for their catalytic action. They can act as deacetylases (SirT1, SirT2, SirT3 and SirT5) or ADP-ribosyltransferases (SirT4 and SirT6), although none of these functions has been assigned to SirT7, which has been shown to regulate rRNA transcription by RNA polymerase I through a yet unknown mechanism (*121*). Due to their NAD^+ dependence, sirtuins have been linked to metabolism. Indeed, they sense the cell's energy and redox status. They can deacetylate metabolic enzymes, regulate metabolic gene transcription, regulate autophagy and, besides, regulate DNA repair (*122*). The subcellular localization varies between the different sirtuins. SirT1, SirT6 and SirT7 are primarily localized in the nucleus, with SirT1 being present in the nucleoplasm, SirT6 being associated with heterochromatin and SirT7 with nucleoli; SirT2 is mainly present in the cytoplasm, and SirT3, SirT4 and SirT5 in mitochondria (*121, 123*). However, the precise subcellular localization may vary in different cell types and upon changes in stress and molecular interactions. In particular, SirT1 and SirT2 have been shown to shuttle back and forth between nucleus and cytoplasm where they interact with different proteins (*124*). Despite the ability of SIR2 or its respective ortholog to increase lifespan in yeast (*S. cerevisiae*), worm (*C. elegans*) and fruit fly (*Drosophila*) (*124*), overexpression of single sirtuins did not have that effect in cultured HNDFs or prostate epithelial cells (*123*). Nevertheless, sirtuins have been shown to play a role in several ageing-related diseases, such as type II diabetes mellitus, a number of neurodegenerative diseases, cancer and cardiovascular disease, and also in inflammation and the infection with human immunodeficiency virus (HIV) (*124*).

1.3.2 HDAC inhibitors

A large number of HDAC inhibitors (HDACis) have been characterized that are derived from both natural and chemical sources. Some of them are pan-HADCis, targeting several HDACs at the same time, whereas others are highly specific (*125*). An example of a specific HDACi is the chemically synthesized small molecule tubacin, which targets HDAC6 and thereby promotes acetylation of α-tubulin (*126*). A widely used pan-HDACi is trichostatin A (TSA), a natural compound produced by several strains of the Actinobacteria genus *Streptomyces* (*127*). TSA targets classical HDACs except for those of class IIa with an IC_{50} in the nanomolar range (*128*). In general, HDACis can be subdivided based on their chemical structure as follows (*125*):

1) Hydroxamic acids, e.g. TSA, M-344, suberoylanilide hydroxamic acid (SAHA; also known as vorinostat or Zolinza), PXD101 (belinostat; also called Beleodaq), LBH589 (panobinostat) and PCI-24781 (abexinostat hydrochloride)
2) Aliphatic acids, including valproic acid (VPA), butyric acid and phenylbutyric acid
3) Benzamides, including MS-275 (entinostat) and MGCD0103 (mocetinostat)
4) Tetrapeptides/depsipeptides, including apicidin and FK228 (FR901228, depsipeptide, romidepsin, Istodax)
5) Sirtuin inhibitors, such as the pan-inhibitor nicotinamide and the specific SirT1 and SirT2 inhibitors tenovin-6, sirtinol, cambinol and EX-527.

1.3.2.1 Clinically approved HDACis

So far, the following three HDACis have been approved by the U. S. Food and Drug Administration (FDA):

1) The first HDACi to be clinically approved was SAHA (vorinostat, Zolinza), namely in October 2006 for the treatment of cutaneous manifestations of cutaneous T-cell lymphoma (CTCL) in patients with progressive, persistent, or recurrent disease on or following two systemic therapies (*129*).
2) In November 2009, romidepsin (Istodax) was approved for the treatment of CTCL in patients who have received at least one prior systemic therapy. In addition, the drug was approved in June 2011 for the treatment of peripheral T-cell lymphoma (PTCL) in patients who have received at least one prior therapy (*130*).
3) Very recently, on 3rd July 2014, the FDA granted accelerated approval to belinostat (Beleodaq) for the treatment of patients with relapsed or refractory PTCL (*131*).

1.3.2.2 Improvement of cellular reprogramming efficiency by HDACis

Next to the use as therapeutic drugs, HDACis have been used in another context, i.e. the generation of induced pluripotent stem cells (iPSCs). The classical pan-HDACis SAHA and in particular TSA and VPA were shown to promote reprogramming efficiency during iPSC

generation (*132*). The mechanism of action has been attributed to their broad effect on HDACs, although we propose a model (**paper III**) suggesting that repression of p53 activity by TSA, in particular with regards to p21 expression, may contribute to a decreased frequency of senescence, which is a limiting event during the generation of iPSCs (*132, 133*).

1.4 THE THIOREDOXIN SYSTEM

The thioredoxin (Trx) system consists of Trx, the selenoprotein thioredoxin reductase (TrxR) as well as nicotinamide adenine dinucleotide phosphate (NADPH). The system is involved in the regulation of redox signaling and maintenance of a balanced cellular redox status, thereby protecting cells from oxidative stress caused by ROS and consequently from cell death. Furthermore, it can protect cells from nitrosative stress caused by reactive nitrogen species (*134, 135*). Trx and TrxR can be either in an oxidized (Trx_{ox} / $TrxR_{ox}$) or reduced state (Trx_{red} / $TrxR_{red}$). $NADPH+H^+$, which are formed during the pentose phosphate pathway, can reduce a disulfide group in $TrxR_{ox}$, resulting in $TrxR_{red}$. In turn, the latter can reduce a disulfide present in the active site of Trx_{ox} to a dithiol (resulting in Trx_{red}), thereby activating the latter (figure 10) (*136*).

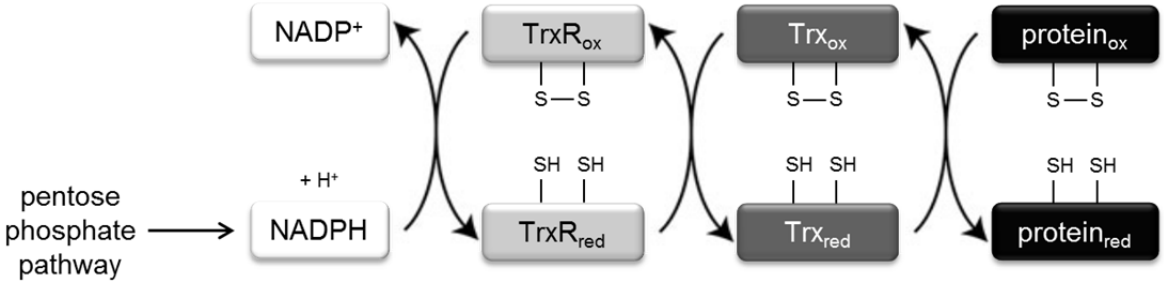

Figure 10: The Trx system and the regulation of the oxidation status of its components. Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; ox, oxidized; red, reduced; Trx, thioredoxin; TrxR, thioredoxin reductase. Adapted from Lee et al. (*136*) with permission from the publisher. Copyright © 2013, Mary Ann Liebert, Inc.

1.4.1 Target proteins and functions

Trx_{red} can reduce a large number of target protein. A few examples are given below (*134, 136*).

1) Reductive enzymes such as peroxiredoxin (Prx), ribonucleotide reductase (RNR) and methionine sulfoxide reductase (Msr), which in turn catalyze the reduction of peroxides, ribonucleotides, and methionine sulfoxides, respectively.
2) Redox-sensitive molecules, including apoptosis signal-regulated kinase 1 (ASK1), thioredoxin-interacting protein (TXNIP) and phosphatase and tensin homolog (PTEN).

3) Redox-regulated transcription factors which contain redox-sensitive cysteines in their DNA binding domain, e.g. activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p21, p53, hypoxia-inducible transcription factor-1 alpha (HIF-1α), the glucocorticoid receptor, the estrogen receptor, NF-E2-related factor 2 (Nrf2), octamer-binding transcription factor 4 (Oct-4), and transcription factor IIIC-gamma subunit (TFIIIC).

Due to its large spectrum of targets, Trx is involved in the regulation of many processes on the cellular and organismal level, such as proliferation, apoptosis, cell migration, inflammation and immune function, metabolism, development and neuroprotection. Furthermore, dysregulation of the Trx system can result in various diseases and disorders, such as cardiovascular diseases, inflammation, metabolic syndrome, type 1 and 2 diabetes mellitus, neurodegenerative diseases, arthritis and cancer (*134, 136, 137*).

1.4.2 Isoforms of Trx and TrxR

Four isoforms of Trx encoded by three separate genes have been identified in humans (*134, 136*):

1) Trx1, which is primarily located in the cytoplasm, but can be translocated into the nucleus as well as secreted from the cell under certain circumstances.
2) Trx2, which is located in mitochondria.
3) Trx3 is localized in the Golgi apparatus of spermatocytes and spermatids; hence it is also called SpTrx.
4) A truncated form of Trx1 (Trx80) is formed upon cleavage of Trx1 by α-secretase. This isoform lacks oxidoreductive properties and is not reduced by Trx reductase. It can prevent the aggregation of β-amyloid, thereby reducing toxicity caused by the latter.

The following isoforms of the selenoprotein TrxR have been described, which are encoded by three separate genes:

1) TrxR1, which is located in the cytoplasm. Its pre-mRNA can undergo alternative splicing at the 5'-end, resulting in two additional isoforms.
2) TrxR2, which is located in mitochondria. Two additional isoforms of TrxR2 can be formed by alternatively splicing, and these isoforms are cytosolic.
3) TrxR3, also called Trx glutathione (GSH) reductase, which is primarily expressed in male germ cells.

1.4.3 Inhibitors of Trx and TrxR

1.4.3.1 Trx inhibitor TXNIP

TXNIP is an endogenous inhibitor of Trx1 and Trx2. It is also called human Trx-binding protein-2 (TBP-2) and vitamin D3-upregulated protein 1 (VDUP1). It interacts with the active center of Trx1, which leads to inhibition of the reducing activity of the latter (*137*).

1.4.3.2 TrxR inhibitors

TrxR is contains a selenocystein (i.e. the 21st "naturally occurring" amino acid in the genetic code) in its C-terminus, which makes it easily accessible to electrophilic compounds (*135, 138*). A large number of TrxR inhibitors has been identified and can be subdivided into the following four classes (*135*):

1) Type I, comprising metal or metalloid containing compounds, e.g. auranofin, cisplatin, arsenic trioxide or lead ions.
2) Type II are Michael acceptors, e.g. quercetin, juglone or curcumin.
3) Type III consists of compounds that contain sufur, selenium or telluride, including 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]ethane (BBSKE); inhibition with the latter is reversible, which is unusual compared with the majority of TrxR inhibitors.
4) Alkylating agents belong to type IV, such as carmustine or dinitrochlorobenzene.

2 AIMS OF THIS THESIS

The following aims were addressed in this thesis:

- Further validation of the cyclotherapy approach
- Determination of new drug combinations to be used in cyclotherapy
- Identification of structural analogs of former hit compounds and attribution of their mechanism of action
- Investigation of the combination of HDACi TSA with p53-activator nutlin-3 regarding the expression of p53 and its target genes as well as tumor cell survival
- Discovery of p53-activating small molecules through a cell-based screen which exert a selective cytotoxic effect towards tumor cells, and determination of the mechanism of action thereof
- Description of a study in which the use of tenovin-6 in zebrafish embryos led to an unexpected discovery

3 RESULTS AND DISCUSSION

3.1 IDENTIFICATION OF NEW DRUG COMBINATIONS FOR CYCLOTHERAPY AND VALIDATION OF THE CYCLOTHERAPY CONCEPT (PAPER I)

Cyclotherapy is an alternative approach to currently applied cancer therapies that was tested on cells for the first time almost 15 years ago (*107*). Even though studies testing nutlin-3 in this context showed very promising results (*95, 139-143*), this compound may not be the best-suited one for this approach. It has not been clinically approved yet, its *in vivo* efficacy is low and very careful dosing would be required, since it has a very narrow working range, i.e. doses above 10 μM lead to DNA damage and doses below 2 μM have no detectable effects in cultured cells (*43, 95, 144, 145*). Therefore, we tested new drug combinations in comparison with nutlin-3 (**paper I**). To activate wt p53, we used tenovin-6, LMB, nutlin-3 and LDactD, which were shown to be non-genotoxic (*102*). The chemotherapeutics we subsequently applied were the M-phase (mitotic) poisons vinblastine (VNB) and vinorelbine (VRL), and the S-phase poisons cytosine arabinoside (Ara-C) and gemcitabine (GMTB), all of which are clinically approved.

We first determined if tenovin-6, LMB, nutlin-3 and LDactD could induce cell cycle arrest in human normal dermal fibroblasts (HNDFs) without causing cell death, and if the effect on the cell cycle was reversible after removal of the compounds. Indeed, flow cytometric analyses indicated that each compound induced arrest in G1- and G2-phases of the cell cycle, an effect that was reversible after removal of the compounds. These data were confirmed by light microscopy (tenovin-6) and clonogenic assays (LMB).

Next, we investigated whether the transient cell cycle arrest induced by p53 activators in normal cells could protect them from damage caused by the nucleoside analogs GMTB and Ara-C, which exert their toxic effects during S-phase of the cell cycle (*146-148*), or the vinca alkaloids VNB and VRL, which prevent microtubule formation and therefore inhibit mitosis (*149*). HNDFs were treated with p53-activating compounds for 24 hours prior to the addition of the chemotherapeutic drugs. Three days after treatment with the latter the cells were rinsed and given the chance to recover for several days. Following chemotherapy, i.e. treatment with S- or M-phase poisons alone, cells displayed nuclear aberrations, abnormal morphologies and impaired growth. In strong contrast, cells pre-incubated with p53 activators resembled untreated fibroblasts with normal nuclei, morphology, size and viability.

The breast adenocarcinoma-derived cell lines MDA-MB-231 and MDA-MB-468 were used to investigate if the above-mentioned drug combinations have a cytotoxic effect in tumor cells expressing mutant p53 or if these would be protected from chemotherapeutic agents as well. Interestingly, the p53-activating compounds had varying effects on these cell lines, and can hence be classified into two groups, namely (1) tenovin-6 and LMB and (2) nutlin-3 and LDactD. As determined by flow cytometry and clonogenic assays, MDA-MB-231 cells neither induced cell cycle arrest nor became protected by nutlin-3 and LDactD. Interestingly,

LDactD made both p53-mutant cell lines more sensitive to the chemotherapeutic agents tested her. In contrast, tenovin-6 and LMB induced cell cycle arrest in G1-phase in MDA-MB-231 cells and protected both p53-mutant cell lines from the cytotoxic effects of vinca alkaloids. In addition, tenovin-6 conferred protection from GMTB-induced damage in MDA-MB-231 cells. This suggests that the combination of tenovin-6 with S-phase poisons might be safer than a combination with M-phase poisons.

To further investigate the p53-dependence of tenovin-6, LMB, nutlin-3 and LDactD with regards to protection from chemotherapeutic agents, we performed experiments in HCT116 cells following the same schedule as the one mentioned above, comparing p53-wt and p53-deficient cells. Strikingly, only nutlin-3 specifically protected p53-wt expressing cells, whereas the other three compounds also protected p53-deficient cells. In some cases, the protective effect in p53-deficient cells was even stronger than in p53-wt cells. This may be due to the fact that nutlin-3 might be the most p53-specific compound used here, since it inhibits the direct interaction between p53 and its negative regulator MDM2 by binding to the p53 binding pocket in the latter (*95*). Targeting the deacetylases SirT and SirT2 (with tenovin-6 (*98*)), CRM1 (with LMB (*104, 105*)) and RNA polymerases (with LDactD (*101, 102*)) might result in a number of additional effects besides p53 activation, which may explain the protection seen in p53-deficient cells.

Furthermore, HCT116 p53-deficient cells might still able to express p53 isoforms which have N-terminal truncations (*43, 44*). Some of these isoforms may be tumor-promoting, but it should also be noted that they may in part be p53-wt dependent with regards to their expression or mode of action (*40*). Strikingly, nutlin-3 may not be able to affect these isoforms, since they may not be under control of MDM2 due to the lack of aa 18-23, i.e. the major binding site of MDM2 (*79*). Also LDactD eventually may exert its effects on p53 via inhibition of MDM2 (*101, 102*), but HCT116 p53-deficient cells were affected by LDactD. This discrepancy between nutlin-3 and LDactD may be explained by the notion that inhibition of RNA polymerases might have a much broader effect on cells than blockage of the p53 binding site in MDM2.

Since the p53 activators used in this study caused polyploidy (8n DNA content) in HNDFs, we tested if this was due to *de novo* induction of endoreduplication or simply an accumulation in G2-phase of the cell cycle of those cells that had already been polyploid before treatment start. Therefore, we serum starved otherwise untreated HNDFs, which might be a quite mild technique to induce cell cycle arrest. Flow cytometric analyses indicated that also serum starvation led to polyploidy, suggesting that the four p53 activators tested here did not actively promote endoreduplication.

In conclusion, LMB may be of use in cyclotherapy, but should not be combined with mitotic poisons, such as vinca alkaloids. Tenovin-6 only provided protection from treatment with one out of four chemotherapeutic agents, so its use in cyclotherapy would be restricted. Nutlin-3 conferred protection of HNDFs, whereas p53-mutant cells were unaffected by this

compound. Therefore, it may be a better choice than tenovin-6 or LMB. However, all three compounds have not been clinically approved yet, so further alternatives are needed. LDactD may be a good candidate, since it is being used in the clinic and efficiently protected HNDFs but not p53-mutant cells against M- and S-phase poisons. Furthermore, it even had an additive effect together with the chemotherapeutics tested here in p53-mutant cells, which on one hand suggests that it does not act in a p53-dependent manner, which is supported by the data obtained using HCT116 p53-deficient cells, but also that LDactD may exert a double-positive effect during cyclotherapy, i.e. protection of normal cells and active killing of tumor cells. Thus, LDactD might be the best choice of the compounds tested here.

3.2 IDENTIFICATON OF A NOVEL TENOVIN WITH SIRT2-INHIBITORY FUNCTION (PAPER II)

Paper II describes tenovin-D3, a structural analog of tenovin-1, which was a hit compound in one of the cell-based screens performed previously in our lab (*98*). Since tenovin-1 and its more water-soluble analog tenovin-6 display inhibitory activity towards the sirtuins SirT1 and SirT2, the inhibition of the latter two by tenovin-D3 was investigated. An *in vitro* assay using fluorescently labeled peptide substrates indicated that tenovin-D3 specifically inhibits SirT2, with an IC_{50} of 21.8 ± 2 µmol/L, but not SirT1 (IC_{50} of > 90 µmol/L). Inhibitory activity towards SirT2 was confirmed using α-tubulin derived from cell lysates as a substrate. In addition, deacetylation of α-tubulin derived from tenovin-D3-treated cells was detected by Western blotting (WB), an effect that was reversed upon overexpression of SirT2.

In line with the model that simultaneous inhibition of SirT1 and SirT2 might be required for activation of p53, tenovin-D3 was neither able to induce p53 protein levels nor p53-dependent transcription, as indicated by WB, CPRG assay and quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Interestingly, p21 levels were induced upon treatment with tenovin-D3, both at the mRNA and protein level. p21 was induced in cells expressing wt p53, mutant p53, no p53 or viral proteins promoting p53 degradation, but in general a stronger effect was seen in the absence of wt p53. There was a strong correlation between SirT2 inhibition and p21 induction, as suggested by the use of various structural analogs of tenovin-1 with SirT2-inhibitory or non-inhibitory function, an increase in p21 levels upon transfection of cells with a dominant-negative SirT2 mutant as well as the absence of cell cycle arrest in SirT2 knock-out cells upon treatment with tenovin-D3. However, the increase in p21 levels by tenovin-D3 was only partially reversed by SirT2 overexpression, whereas acetylation of α-tubulin upon tenovin-D3 treatment was completely abolished in SirT2-overexpressing cells. This discrepancy may be due to off-target effects of tenovin-D3. It is unlikely that "classical" HDACs are affected by this compound, since they neither resemble sirtuins in their protein structure nor their cofactor dependence. Supporting this hypothesis, an *in vitro* assay suggested that HDAC8 was not inhibited by tenovin-D3. Also, co-treatment with tenovin-D3 and TSA, which is an inhibitor

of most "classical" HDACs that was shown to induce p21 levels in a similar fashion to tenovin-D3 (**papers II and III**), had an additive effect on the increase in p21 levels. Furthermore, SirT3 was not inhibited by tenovin-D3. If other sirtuins were affected was difficult to investigate due to the lack of assays.

Acetylation of α-tubulin has been suggested to play a role in cell migration, since deacetylation of α-tubulin upon inhibition of HDAC6 by tubacin can inhibit this cellular process (*126*). Acetylated α-tubulin is also a target of SirT2 and, indeed, short-term treatment with tenovin-D3 affected cell migration in a transwell assay. This reflected another parallel between tenovin-D3 and inhibitors of "classical" HDACs in addition to the above-mentioned effect of p21 expression (**papers II and III**).

Two "classical" HDACi are in clinical use against cutaneous T-cell lymphoma (CTCL), i.e. vorinostat and romidepsin (*150, 151*). Since tenovin-D3 showed some similarities with the "classical" HDACi TSA, which in turn is structurally and functionally highly similar to SAHA (*150*), a potential future use of tenovin-D3 or other SirT2 inhibitors in CTCL or other malignancies could be an option for further investigation. Furthermore, SirT2 might play a role in neurodegenerative diseases (*152*), so tenovin-D3 or functionally similar compounds may be of potential use in this field as well.

3.3 TSA INHIBITS P21 INDUCTION BY P53 AND *VICE VERSA* (PAPER III)

The literature describing studies on the effects of TSA on p53 are partially contradictory. On one hand, TSA has been shown to stabilize the p53 protein, potentially by inhibition of HDAC1 and HDAC2, two deacetylases of p53 (*62, 65, 128*). On the other hand, the compound has also been shown to reduce p53 expression by decreasing p53 promoter activity or even destabilization of p53 mRNA (*153, 154*).

Since nutlin-3 may exert a cytostatic effect in tumor cells, and induces and stabilizes HDM2, this compound may not lead to eradication of tumors (*95, 155, 156*). Here (**paper III**), we combined TSA and nutlin-3 to investigate if that combination could have a positive effect on p53 activation or if it even may lead to an enhanced cytostatic effect due to the ability of nutlin-3 and TSA to induce p21 in different ways.

As shown by CPRG assay, WB and qRT-PCR, TSA was able to inhibit p53 transcriptional activity induced by nutlin-3 in tumor cells. This effect was specific for some p53 target genes, i.e. *CDKN1A* (p21), *PIG3* and – to a weaker extent – *HDM2*, whereas NOXA was unaffected. This suggested that TSA, despite being a pan-HDACi, can have specific effects in cells. Strikingly, TSA treatment led to an induction of p21 and – again to a weaker extent – HDM2 mRNA and protein levels if used as a single agent, suggesting an incompatible effect with nutlin-3-induced p53. Similar results were obtained in HNDFs with the exception of HDM2, which was unaffected by TSA, both when used as single agent and in combination

with nutlin-3. The lack of effect on HDM2 mRNA levels in HNDFs may be related to the ability of TSA to induce the expression of $p14^{ARF}$, which may lead to stabilization of HDM2 (*85, 157*). Since *de novo* synthesized p53 levels might be reduced in TSA-treated HNDFs, as suggested by a decrease in p53 mRNA levels, there may be less transcriptionally active p53 present in the cells, which eventually may result in decreased MDM2 transcription. Thus, a decrease in *de novo* synthesized MDM2 in combination with stabilized pre-existing MDM2 protein may outbalance MDM2 protein levels and hence lead to a net equalization of MDM2 protein levels in HNDFs. The gene encoding $p14^{ARF}$ (*CDKN2A*) is deleted in MCF7 cells, and one allele is deleted in HCT116 cells whereas the other allele is mutated and hypermethylated (*158, 159*); therefore, $p14^{ARF}$ is not expressed in these cell lines.

In line with the literature, p53 mRNA levels were reduced upon TSA treatment, both in the presence and absence of nutlin-3 (*153, 154*). However, p53 protein levels were not affected and acetylation of Lys-382 was even enhanced. A change in the subcellular localization of p53 was not detected.

We noticed that the induction of p21 protein by TSA in MCF7 cells and HNDFs, both of which express wt p53, was quite weak compared with the induction detected previously in MDA-MB-468 cells, which express mutant p53 (**papers II and III**). Indeed, by comparing HCT116 p53-wt and p53-deficient isogenic cell lines as well as in transfection experiments using the p53-null cell line H1299 we found that the presence of wt p53 interfered with the induction of p21 by TSA.

Since the combination of TSA with nutlin-3 resulted in decreased p21 levels, the consequences thereof on the cell cycle were investigated. Nutlin-3 induced mainly an arrest in G1-phase of the cell cycle after 6 hours, whereas TSA led to an increase in cells entering S-phase and a slight increase in the percentage of cells in G2-phase after short-term treatment (i.e. 7 hours, since cells were always pre-treated with TSA for 1 hour prior to addition of nutlin-3). Co-treatment led to an intermediate result compared with single treatments. Also after long-term treatment this intermediate effect was detected, with the majority of cells undergoing G1- and G2-phase arrest. The high fraction of cells undergoing cell cycle arrest upon co-treatment suggested that the combination of HDACis and p53 activators may not be advantageous in a clinical setting. However, flow cytometric analyses after long-term treatment indicated that the combination of TSA and nutlin-3 led to an increase in the percentage of dead cells. In line with that, fewer colonies formed in a clonogenic assay upon co-treatment compared with single treatment of each compound, suggesting that the combination of such compounds may be safer than initially expected. It should be noted, however, that the drug combination tested here did not eradicate the cells, suggesting that it might be better to identify other combinations that are exclusively cytotoxic in cancer cells.

Furthermore, our results may explain why HDACis such as TSA may improve the generation of iPSCs (*132*). One of the major problems with iPSC generation is the induction of activation of p53 and p21, resulting in senescence. Knockout of p53 has been shown to

improve iPSC generation, but also resulted in teratoma formation (*160*). Transient pharmacological inhibition of p53 may represent a safer method to obtain stem cells. However, also with this technique the risk of teratoma formation cannot be excluded and would require further investigation to proof its safety.

3.4 IDENTIFICATION OF NOVEL P53 ACTIVATORS (PAPERS IV AND V)

We performed a cell-based screen as described in subsection *1.2.5.6* in the human melanoma-derived ARN8 cell line to test 20,000 small molecules derived from a chemical library provided by ChemBridge (San Diego, CA, USA) on their ability to activate p53. The two hit compounds MJ05 (**paper IV**) and MJ25 (**paper V**) were further studied due to different reasons: MJ05 appeared to be interesting due to its extremely high selectivity towards certain tumor cell lines with regards to the induction of cell death compared with other tumor cell lines as well as normal cells. MJ25 showed selectivity comparing melanoma cell lines and normal cells, and was furthermore selected due to its potential TrxR1-inhibitory capabilities.

3.4.1 MJ05 (Paper IV)

MJ05 was one of the top ranking compounds identified in this screen (**paper IV**). Pharmacokinetic studies suggested that this compound has a moderate to high plasma protein-binding capacity, which might be sufficient to fulfill the criteria of being a suitable drug candidate. Furthermore, MJ05 may not cause DSBs as suggested by the lack of induction of γ-H2AX and Ser-15 phosphorylated p53, suggesting that the compound may be safe with regards to genotoxicity.

MJ05 induced p53 as well as its targets p21, MDM2 and PIG3, and this effect was stronger in ARN8 cells compared with HNDFs. These data confirmed the selectivity for tumor cells.

Next, we tested if a combination of MJ05 with other p53-activating compounds would lead to an enhanced response in tumor cells. Indeed, MJ05 was highly cytotoxic in combination with nutlin-3 in ARN8 cells, even though nutlin-3 mainly induced cell cycle arrest as single agent. However, this effect was not seen when combined with tenovin-6 or LDactD. Importantly, nutlin-3 as well as the combination of nutlin-3 and MJ05 led to a cytostatic effect in HNDFs. MJ05 itself had no effect on HNDFs in that regard during the first two days of treatment, and a slight induction of cell cycle arrest could be detected after three days. These data confirmed tumor selectivity once again.

The synergism between MJ05 and nutlin-3 suggested that these compounds may work through different mechanisms of action. Indeed, no binding of MJ05 to MDM2 (or MDMX) could be detected *in vitro*. Also, treatment of a number of tumor cells with differences in p53 status indicated that a cytotoxic effect by MJ05 was not dependent on wt p53, but that there

was still selectivity within the cell lines tested, suggesting that the factor targeted by MJ05 was not part of the p53 pathway. Further tests suggested that MJ05 neither inhibits 16 CDKs nor an additional ~150 kinases.

Interestingly, MJ05 and nutlin-3 had a slightly more than additive effect on PIG3 induction in ARN8 cells at the mRNA and protein level, an effect also seen in HNDFs, but to a smaller extent and only at the mRNA level. In contrast, p21 levels were decreased upon co-treatment in ARN8 cells, an effect seen previously for tenovin-D3 (**paper II**) and TSA (**papers II and III**). However, this effect was not seen in HNDFs. These data suggest that the cytotoxic effect of MJ05 in tumor cells is the consequence of the strong induction of the pro-apoptotic protein PIG3 and a simultaneous reduction of p21. In HNDFs, the weak induction of PIG3 combined with a strong induction of p21 might explain the observed cell cycle arrest.

Since MJ05 treatment led to a slight reduction of γ-H2AX levels and failed to increase p53 Ser-15 phosphorylation, we investigated if the compound could inhibit ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related kinase (ATR) (*161*). However, this was not the case.

We further discovered that MJ05 induced a delay in S-phase of the cell cycle, but not S-phase arrest. The absence of a change in phosphorylation of ATR / checkpoint kinase 1 (Chk1) suggested that this delay was not due to complete ribonucleotide depletion (*162*). We then tested whether MJ05 could delay S-phase progression by reducing replication fork assembly or firing. Cell division cycle 7 (Cdc7) kinase activity is required for the activation of replication origin helicases such as minichromosome maintenance complex component 2 (MCM2) (*163*); however, MJ05 did not inhibit MCM2 phosphorylation. Next, we investigated if cell division cycle 6 (Cdc6) may be involved in S-phase delay upon MJ05 treatment. The ATPase Cdc6 is a key factor in the licensing of replication origins prior to their activation (*164*). MJ05 reduced the levels of Cdc6 in a p53-independent manner in ARN8 cells and to a smaller extent in HNDFs. However, this did not happen in HCT116 $p21^{-/-}$ cells, which also accumulated in S-phase upon MJ05 treatment. This finding combined with the fact that Cdc6 downregulation was quite a late event suggested that Cdc6 did not play a role in the S-phase delay seen here. Also, certain similarities between MJ05 and $p14^{ARF}$, e.g. a p53-independent delay in S-phase progression (*165, 166*) or enhanced PIG3 expression in combination with nutlin-3, were detected. It may be quite unlikely that MJ05 acts like $p14^{ARF}$, since $p14^{ARF}$ expression did not lead to a reduction in nutlin-3-induced p21 levels in tumor cells. However, it should be borne in mind that $p14^{ARF}$ was shown here to stabilize the p21 protein, which may prevent a reduction in its levels.

MJ05 has a chiral center and hence exists as two enantiomers, (*R*)-MJ05 and (*S*)-MJ05. Our studies indicate that (*R*)-MJ05 is the only active enantiomer, suggesting that MJ05 may be specific regarding target inhibition.

In vivo activity of (*R*)-MJ05 was tested in a xenograft study with ARN8 cells in SCID mice, both as a single agent and in combination with nutlin-3. MJ05 and nutlin-3 each affected

tumor growth and the combination resulted in an additive effect. In addition, MJ05 was tested alone and in combination with nilotinib, a drug that is clinically approved for the treatment of chronic myelogenous leukemia (CML) (*167*), on the ability to selectively kill leukemia stem cells (LSCs) derived from patients suffering from CML. Indeed, MJ05 efficiently induced apoptosis and inhibited growth of CML stem/progenitor cells *ex vivo*, alone and even stronger in the combination with nilotinib.

The exact mechanism of action of MJ05 still needs to be elucidated. According to our data it is likely that MJ05 inhibits enzymes involved in the *de novo* synthesis of UMP and hence reduces pyrimidine ribonucleotide and pyrimidine deoxyribonucleotides levels, as uridine supplementation rescued ARN8 cells from the cytotoxic effects of this compound. Strikingly, MJ05's cytotoxic effect occurred selectively in ARN8 cells but not in other tumor cell lines, not even those expressing wt p53. Instead, MJ05 induced cell cycle arrest or had a very mild effect in all other tumor cell lines as well as normal cells that express wt p53 and p21. This suggested that p53, which becomes activated upon MJ05 treatment, may detect the hypothesized reduction in (deoxy)ribonucleotide levels before all of them have vanished, upon which the compound halts the cell cycle until new (deoxy)ribonucleotides, in particular UMP and its derivatives UTP, CTP, dCTP and dTTP (figure 11, blue box), become available. In the less sensitive cell lines, activation of salvage pathways for pyrimidine nucleotide production may prevent MJ05's effect. In addition, cytidine in combination with a UMP synthesis inhibitor (pyrazofurin) has been previously described to be cytotoxic due to a lack of expression of the salvage pathway enzyme cytidine deaminase (CDA) (*168*). Indeed, cytidine supplementation in combination with MJ05 killed more rapidly than MJ05 on its own. Confirming this hypothesis, A375 cells, which ARN8 cells are derived from (*115*), express low levels of CDA, suggesting that the salvage pathway rescuing cells from UMP depletion may not be fully functional in these cells. In line with that, other tumor cell lines tested in this study that did not die upon treatment with MJ05 – some of which even continued to proliferate – had been reported previously to express high levels of CDA. Thus, the salvage pathway might be functional in these cells, so that inhibition of *de novo* synthesis of UMP would not affect these cells tremendously.

A question that remains is how p53 levels increase upon inhibition of an enzyme involved in UMP synthesis. Our data indicate that p53 mRNA levels did not change strongly upon MJ05 treatment, although a slight increase was detectable in ARN8 cells. However, this increase may not be sufficient to explain the strong increase at the protein level. In addition, MJ05 did not increase p53 protein stability. Thus, another explanation would be an enhanced rate of translation. This seems illogical at first sight, since ribonucleotides are required for the synthesis of rRNA and tRNA, which in turn are needed for translation to take place, besides the mRNA serving as a template. However, based on the literature we propose the following model (figure 11, yellow box): MJ05 may reduce the levels of RNA-binding protein 38 (RBM38) mRNA in ARN8 cells, since the 5' end of the RBM38 mRNA is rich in cytidines. Because RBM38 binds to p53 mRNA and subsequently inhibits its translation, a reduction in

RBM38 levels may lead to an increase in p53 translation, at least in short-term when complete ribonucleotide depletion has not occurred yet. In addition, RBM38 has a stabilizing effect on p21 mRNA (*169*), which may explain why MJ05 reduces nutlin-3-induced p21 protein levels. Furthermore, p53 induces RBM38. This would happen in case the salvage pathway is still functional, i.e. in those cells expressing high levels of CDA. Eventually, induction of RBM38 by MJ05 would reduce p53 levels after pyrimidine (deoxy)ribonucleotides levels are restored allowing cells to proliferate normally.

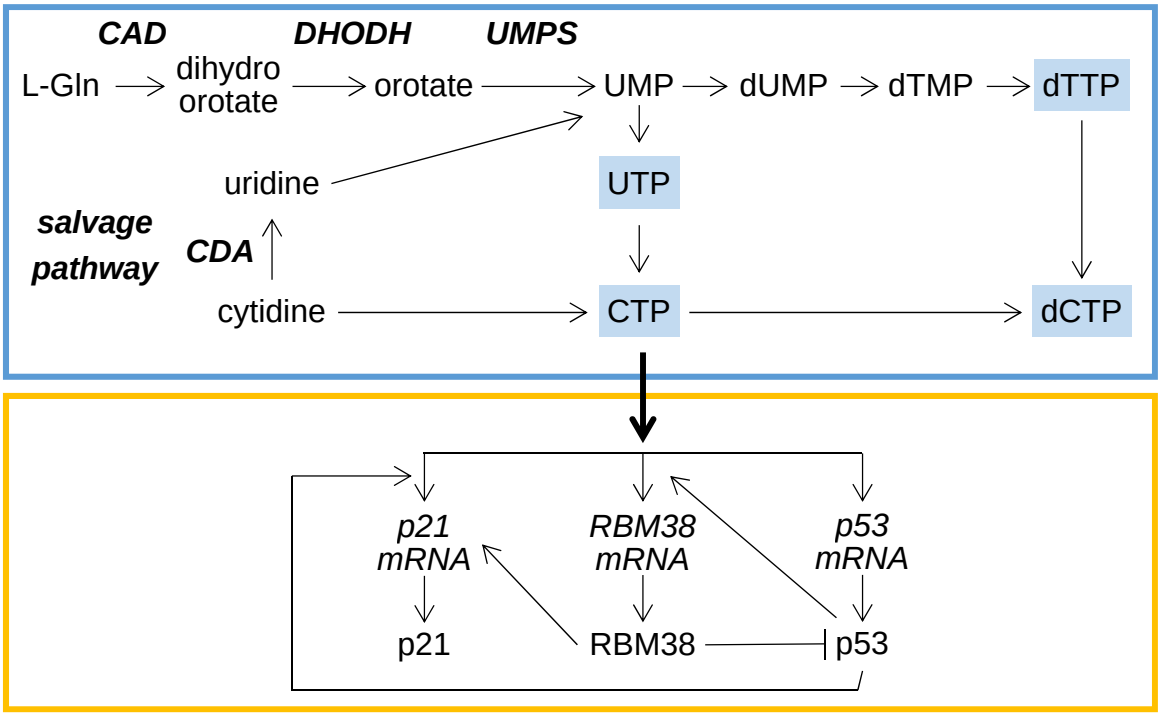

Figure 11: *de novo* synthesis of UMP and its derivatives UTP, CTP, dCTP and dTTP (blue box) and a possible model on how p53 translation can take place in the absence thereof (yellow box). Abbreviations: CDA, cytidine deaminase; CTP, cytidine triphosphate; dCTP, deoxycytidine triphosphate; DHODH, dihydroorotate dehydrogenase; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate; L-Gln, L-glutathione; RBM38, RNA-binding protein 38; UMP, uridine monophosphate; UMPS, uridine monophosphate synthetase.

Altogether, MJ05 is a novel p53 activator that acts selectively in tumor cells. Furthermore, only those tumor cells may undergo cell death that have lost the salvage pathway for synthesis of pyrimidine (deoxy)ribonucleotides. The compound might have very few off-target effects. *In vivo* and *ex vivo* data suggest that MJ05 may be a potential drug candidate worth studying further.

3.4.2 MJ25 and the identification of auranofin as a potential drug against melanoma (Paper V)

The small molecule MJ25 is described in **paper V**. Even though it was a hit compound in our screen, it was not very potent at activating p53 as a single agent, albeit co-treatment with nutlin-3 enhanced p53 activation to some extent. Nevertheless, we considered this compound to be worth studying, since it had been found to be a potent inhibitor of the selenoenzyme TrxR1 in an *in vitro* screen (*170*). The combination of p53 activation and TrxR1 inhibition had previously been shown to be a successful strategy to kill tumor cells (*171*). Indeed, MJ25 killed various melanoma cell lines after 72 hours of treatment, whilst being less toxic to HNDFs and human normal epithelial melanocytes (HNEMs).

Importantly, the compound did not show signs of genotoxicity, as suggested by data on induction of γ-H2AX (representing DSBs) obtained by WB and an alkylation assay.

A slight p53-dependence regarding execution of a cytotoxic effect and impairment of clonogenic potential was observed when comparing the wt-expressing tumor cell lines HCT116 and RKO with their p53-deficient counterparts. Interestingly, data obtained by WB suggested that MJ25 induced p21 in HNDFs and p53-mutant cell lines but not p53-wt expressing ARN8 cells. This further supported the data obtained by clonogenic assays and viability assays, suggesting that MJ25 may induce a cytostatic effect in cells that do not express wt p53, and therefore MJ25 would not be a suitable drug in the treatment of p53-mutant cells. Furthermore, this finding combined with the fact that MJ25 is a weak p53 activator suggests that this compound would not be a suitable candidate for cyclotherapy.

All of the melanoma cell lines tested in this study harbor a mutation in the serine/threonine kinase BRAF ($BRAF^{V600E}$) that leads to constitutive activation of the mitogen-activated protein kinase (MAPK) / extracellular signal-regulated kinase (ERK) signaling pathway (*172*). Since MJ25 was selective at killing cells expressing $BRAF^{V600E}$, we investigated if the presence thereof played a role in this context. Indeed, MJ25 was slightly more potent at killing tumor cells expressing $BRAF^{V600E}$ than isogenic cells lacking this mutant protein. However, clonogenic assays, in which ARN8 cells were co-treated with the clinically approved $BRAF^{V600E}$ inhibitor vemurafenib (*172, 173*), as well as a WB analysis indicating upregulation of the downstream kinases ERK1/2 upon treatment with MJ25 suggested that the compound might affect the BRAF pathway indirectly.

Next, we studied the ability of MJ25 to inhibit the selenoenzyme TrxR1 in further detail. MJ25 could inhibit the enzyme obtained through protein synthesis from recombinant cDNA *in vitro*. Furthermore, it inhibited TrxR1 present in ARN8 cell lysates. However, MJ25 was less potent than auranofin (Ridaura), a TrxR1 inhibitor clinically approved for the treatment of rheumatoid arthritis (*174-181*). Both compounds were affected by supplementation of cells with sodium selenite prior and during treatment, which further suggested that these compounds act through TrxR1. Auranofin was more sensitive to this supplementation, suggesting once again that this drug might be more potent at inhibiting TrxR1 than MJ25. In

addition, both compounds induced increased levels of reactive oxygen species (ROS) and the expression of factors involved in the anti-oxidative response of the cell, further suggesting that TrxR1 was affected by MJ25. In line with the results mentioned above, auranofin was more potent at inducing some of these anti-oxidative factors and had more of a long-lasting effect than MJ25. Strikingly, when intracellular glutathione levels were nearly depleted, which leads to a stronger dependence of cells on the Trx system for an anti-oxidative response, only auranofin showed a marked increase in potency with regards to impaired cell viability. This suggested that MJ25 may have additional targets through which it exerts its cytotoxic effects towards melanoma cells. Further studies would be required in this respect.

Interestingly, also auranofin showed some dependence on the MAPK/ERK signaling pathway with regards to impairment of melanoma cell clonogenic potential, and had, like MJ25, an additive effect when combined with vemurafenib. Similar to MJ25, auranofin induced phosphorylation (i.e. activation) of ERK1/2. This might be an indirect consequence of increased ROS levels upon treatment, as it has been reported previously that ROS can induce higher levels of phosphorylated ERK1/2 (*182*). Furthermore, a previous study showed that constitutively active BRAF activates the anti-oxidative protein Nrf2 (*183*), which in turn suggests that high ROS levels are present in cells expressing constitutively active BRAF. Thus, a further increase upon inhibition of TrxR1 might be lethal to cells expressing $BRAF^{V600E}$.

Strikingly, auranofin was able to eradicate whole melanoma cell populations at low micromolar concentrations. Vemurafenib, which is in clinical use, cannot achieve this. Also MJ25 was able to kill significantly more cells than vemurafenib, with almost no cells recovering from treatment. It should be noted, however, that MJ25 showed selectivity towards melanoma cells both in cell viability assays as well as clonogenic assays, whereas auranofin only did so in viability assays.

The p53-dependence of auranofin was less clear than that of MJ25. First of all, even though auranofin induced p53 at the protein level, p53's transactivational function was inhibited by auranofin, which became particularly clear when combined with nutlin-3. Furthermore, clonogenic potential of HCT116 cells was affected more strongly in the presence of full-length p53, whereas RKO cells deficient in p53 were more sensitive to the drug than their p53-wt counterparts. These data suggest that auranofin may have off-target effects, and that these differ from the ones of MJ25.

In contrast to MJ25, auranofin was not able to induce p21 protein levels in HNDFs. However, p21 was induced in p53-mutant cell lines, suggesting that this drug would not be suitable for the treatment of p53-mutant tumors.

WB analyses of γ-H2AX suggested that auranofin may not be genotoxic. Furthermore, our data indicated that this drug was potent at killing melanoma cells. This was confirmed by a study in connection with the Developmental Therapeutics Program (DTP) by the National Cancer Institute (NCI) at the National Institutes of Health (NIH)

(http://dtp.nci.nih.gov/index.html) (*184*). Here, 60 tumor cell lines were tested on the ability of auranofin to affect cell viability. Nine melanoma cell lines were included, only one of which had been tested in our studies; hence the data from the DTP extended our data set. Importantly, all melanoma cell lines tested appeared to be highly sensitive to auranofin. Even though auranofin appeared to be cytotoxic towards HNDFs in clonogenic assays, this was not seen in another experimental setup, i.e. cell viability assays. Furthermore, auranofin is clinically approved for the treatment of rheumatoid arthritis and might therefore be tolerated by patients. If the doses required for cancer therapy would be in a similar range to those used against rheumatoid arthritis would, however, need to be investigated.

Unfortunately, MJ25 is not very water-soluble at concentrations required for *in vivo* studies. Therefore, it could not be tested if this compound reduces the growth of xenografts. Chemical modification of the compound would be required to perform such studies. Simultaneously, one could perform structure-activity relationship (SAR) studies to identify structural analogs with enhanced potency.

3.5 THE UNEXPECTED FINDING OF TENOVIN-6 CAUSING HYPOPIGMENTATION IN ZEBRAFISH EMBRYOS (PAPER VI)

The study described in **paper VI** originally aimed at investigating if tenovin-6 was able to induce wt p53 in zebrafish (*Danio rerio*) embryos and what consequences that would have on metastasis formation. However, neither induction of p53 protein nor of mRNA of selected transcriptional targets could be detected in wt embryos after 10 hours of treatment, which was shown to be optimal for the CDK inhibitor roscovitine to induce p53 at the protein level (*185, 186*). In contrast, an increase in the protein levels of mutant p53 (M214K) could be detected after 10 hours of treatment. Mutant p53 is more stable than wt p53 protein; hence this induction suggested that tenovin-6 essentially was able to induce p53. The lack of effect seen in wt embryos may be due to low stability of the tenovin-6 compound, so it may be possible that wt p53 only became induced for a very short time in zebrafish embryos upon treatment, and this time may not have been long enough for target gene transcription to occur.

Next to the induction of mutant p53, there were further indications that tenovin-6 was bioactive in the embryos: First, treatment was lethal, and this effect was dose- and time-dependent; in line with the lack of wt p53 induction, no strong correlation between p53 status and lethality was detected. Second, the p53 target gene *PUMA* was induced by tenovin-6, both in wt and p53-mutant embryos. This suggested that proteins other than p53 were responsible for this effect. The p53 paralog p73 may be a candidate, since it transactivates *PUMA* and was shown to be regulated by SirT1, i.e. a target of tenovin-6 (*187-189*). Third, tenovin-6 caused long-lasting hypopigmentation in zebrafish embryos.

The exact mechanism behind the observed hypopigmentation after tenovin-6 treatment has not been elucidated yet. The compound might inhibit a factor / factors directly or indirectly

involved in pigmentation. Tenovin-6 did not inhibit tyrosinase, a key enzyme in melanogenesis, in an assay using cell lysate derived from murine pigmented melanoma cells and human melanocytes. This was unexpected, since tenovin-6 is structurally related to *N*-phenylthiourea (PTU), which inhibits tyrosinase through copper chelation and is widely used to prevent pigmentation in zebrafish embryos (*190*). PTU did not activate p53 in a CPRG assay, which again was different from the action of tenovin-6. This might be a very positive finding, though, since PTU is commonly used in zebrafish experiments, and constitutive activation of p53 otherwise could have affected the outcome of a large number of them.

A potential tenovin-6 target candidate could be vacuolar H^+-ATPase (V-ATPase), a proton pump required for the establishment of the acidic environment present in lysosomes and lysosome-like organelles such as melanosomes. Zebrafish embryos in which V-ATPase is mutated (called *Mustard*) show a phenotype resembling that of tenovin-6, and so does the phenotype of embryos treated with the V-ATPase inhibitor bafilomycin A1 (bafA1) (*191, 192*). The latter compound has been shown to inhibit autophagy, a cellular process in which unnecessary and dysfunctional cellular components (e.g. damaged organelles) are degraded (*193*). Lysosomal enzymes are needed for this process and hence requires the fusion of autophagosomes with lysosomes (*194*). Tenovin-6 is thought to block autophagy after fusion of autophagosomes and lysosomes has occurred, as suggested by accumulation of early (non-acidic) and late (acidic) autophagosomes as well as accumulation of the autophagy-marker LC3B-II (*192, 195*) (Ladds et al., unpublished data). Tenovin-6 is thought to accumulate in the acidic environment of late autophagosomes and lysosomes due to the presence of a tertiary amine in its chemical structure; in addition, co-treatment with bafA1 did not lead to an enhanced level of LC3B-II, and this has been suggested to be due to different functions of bafA1 and tenovin-6 with regards to autophagy (*192, 195*). However, it cannot be excluded that tenovin-6 and bafA1 fulfill exactly the same function. The presence of tenovin-6 in late autophagosomes / lysosomes has not been shown yet, but would answer the question which step of autophagy is affected by this compound. An additional argument for the potential inhibition of V-ATPase by tenovin-6 is that a yeast strain hemizygous for vacuolar H^+/Ca^{2+} exchanger 1 (*VCX1*) was hypersensitive to tenovin-6 (*98*). Vcx1p is a vacuolar H^+/Ca^{2+} antiporter that transports Ca^{2+} into vacuoles to maintain the cytoplasmic Ca^{2+} homeostasis and is dependent on the proton gradient established by V-ATPase (*196-198*). Thus, hemizygosity for VCX1 might lead to hypersensitivity of the yeast strain to further interference with the machinery that regulates proton levels inside vacuoles, eventually leading to constitutively elevated Ca^{2+} levels in the cytoplasm, which might be a toxic condition.

In conclusion, our studies in zebrafish embryos led to the unexpected discovery that tenovin-6 causes hypopigmentation. Furthermore, a new target of the compound may have been identified.

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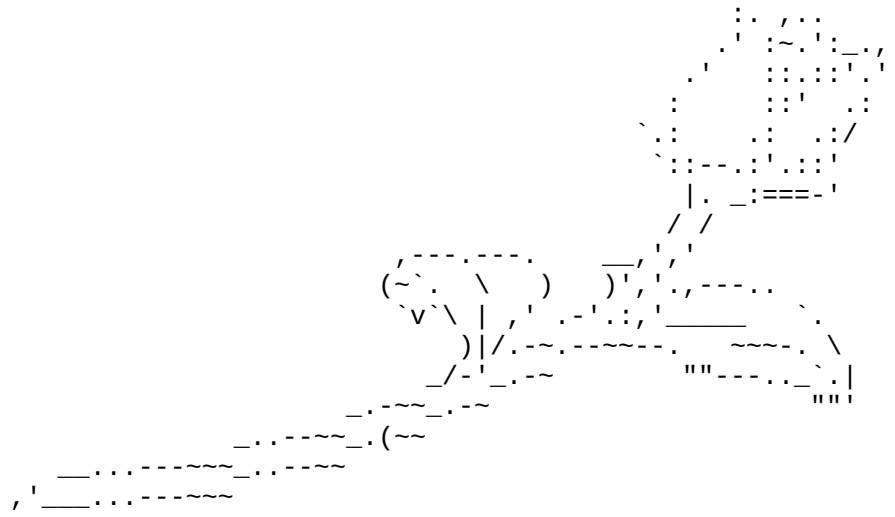

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