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MODULATION OF P53´S TRANSCRIPTIONAL FUNCTION BY SMALL MOLECULES

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Cover Illustration. “Galaxy of p53 transcription factor”

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To my beloved Diana and Family

ABSTRACT

p53 tumour suppressor is a transcriptional factor which induces apoptosis or growth arrest in response to stress thus eliminating damaged cells. p53 function is frequently abrogated in tumours either via inactivation mutations in the *TP53* gene or by elevated activity of p53 negative regulators HDM2 and HDMX. Therefore application of small molecules that reactivate p53 function is a promising strategy for anti-cancer therapy. In addition, small molecules can serve as valuable research tool to study p53 biology.

This thesis is focused on the studies of p53 transcriptional response induced by small molecules and the molecular mechanisms contributing to the induction of apoptosis by p53. Using chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) we compared genome-wide DNA binding profile of p53 activated by three different small molecules RITA, 5-FU and Nutlin-3a, causing various biological outcomes in breast carcinoma MCF7 cell line. We found that the major pattern of p53 binding to DNA does not depend on the mechanism of p53 activation or p53-induced cell fate. Surprisingly, we demonstrated that the majority of sites bound by p53 are located far away from transcription starting sites (TSS), thus making unclear their functional role. Comparison of p53 DNA binding sites in vicinity to TSS with changes in gene expression using microarray analysis revealed 280 novel p53 target genes. While the majority of p53 transactivated genes shared classical p53 consensus motif, we found it only in a few repressed genes, suggesting different mechanism of p53 transrepression. We validated several novel p53 target genes, including *AURKA* gene which is negatively regulated by p53. In addition, we showed that STAT3 transcription factor antagonizes p53-mediated regulation of several target genes, including *AURKA*. We demonstrated that the expression level of novel p53 target genes correlates with p53 status, tumour grade and survival in 265 breast cancer patients.

Investigation of molecular mechanisms of p53-mediated apoptosis upon RITA treatment revealed that in addition to activation of pro-apoptotic targets, p53 inhibited the expression of several crucial oncogenes. Thus, we showed that inhibition of several oncogenic and pro-survival factors, including c-Myc and Mcl-1, on mRNA and protein levels critically contributes to robust induction of apoptosis. We found that in contrast to p53-mediated transactivation, transrepression is more tightly regulated by HDM2 and depends on the ratio of p53 and HDM2 bound to gene promoters.

We found that RITA-activated p53 mediates a decrease in expression and protein stability of its negative regulator HDMX. Impaired stability of HDMX is caused by ATM-mediated phosphorylation of HDMX. In turn, the elevated activity of ATM correlates with depletion of p53 target gene Wip1 phosphatase that inhibits ATM. We demonstrated that the depletion of either HDMX or Wip1 enhances growth suppressive effects of p53-reactivating molecules RITA and Nutlin3a.

Our data showed that RITA inhibits glycolytic enzymes in p53-dependent manner. We found that p53 binds to DNA in vicinity from TSS of the metabolic genes and represses their transcription. Our data suggests that SP1 is a p53 transcriptional cofactor contributing to p53-mediated transrepression of several metabolic genes. Importantly, we showed that the block of glycolysis amplifies induction of apoptosis in cancer cells upon RITA treatment.

In conclusion, our data contribute to a deeper understanding of transcriptional response induced by p53, along with the identification of novel p53 target genes. Our studies revealed new targets of pharmacologically activated p53 which significantly increase the robustness of p53-mediated apoptosis.

LIST OF PUBLICATIONS

I. Fedor Nikulenkov, Clemens Spinnler, Claudia Tonelli, Mikko Turunen, Hai Li, Teemu Kivioja, Alexander Kel, Jussi Taipale and Galina Selivanova
Insights into p53 Transcriptional Function via Genome-Wide Chromatin Occupancy and Gene Expression Analysis
Submitted munuscript

II. Grinkevich VV, Nikulenkov F*, Shi Y*, Enge M, Bao W, Maljukova A, Gluch A, Kel A, Sangfelt O, Selivanova G.
Ablation of key oncogenic pathways by RITA-reactivated p53 is required for efficient apoptosis.
Cancer Cell, 2009, May 5;15(5):441-53.

III. Joanna Zawacka-Pankau*, Vera V. Grinkevich*, Sabine Hüenten[§], Fedor Nikulenkov[§], Angela Gluch, Hai Li, Martin Enge, Alexander Kel, Galina Selivanova
Inhibition of glycolytic enzymes mediated by pharmacologically activated p53: targeting Warburg effect to fight cancer.
JBC, 2011, August 23, [Published ahead of the print]

IV. C. Spinnler*, E. Hedström*, H Li, J de Lange, F Nikulenkov, AFAS Teunisse, M Verlaan-de Vries, V Grinkevich, A Jochemsen and G Selivanova
Abrogation of Wip1 expression by RITA-activated p53 potentiates apoptosis induction via activation of ATM and inhibition of HdmX
Cell Death Differ, 2011, May 6, [Published ahead of the print]

*, [§] equal contribution

ASSOCIATED PUBLICATIONS

- Zhao CY, Grinkevich VV, Nikulenkov F, Bao W, Selivanova G.
  Rescue of the apoptotic-inducing function of mutant p53 by small molecule RITA.
  Cell Cycle. 2010, May;9(9):1847-55.
- Wenjie Bao, Fedor Nikulenkov[§], Ying Zhao[§], Clemens Spinnler, Jiri Bartek, and Galina Selivanova
  53BP1 contributes to tumour suppression by wild-type p53 and to gain-of-function of mutant p53 via regulation of their stability in an oncogene-dependent manner
  Submitted manuscript
- Yao Shi, Fedor Nikulenkov, Hai Li, Natalia Issaeva, Galina Selivanova
  Induction of the DNA damage signalling by RITA-reactivated p53 via depletion of Wip1 and p21
  Manuscript
- V.V.Ginkevich*, A. Warnecke*, F. Nikulenkov, C.Spinnler, A.Gluch, A. Kel, K. Pokrovskaja and G.Selivanova
  Abrogation of STAT3 signalling in tumour cells by pharmacologically activated p53
  Manuscript

*, [§] equal contribution

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5-FU	5-flourouracil
5-FUTP	5-fluorouridine 5’-triphosphate
5-FdUMP	5-fluoro-2’-deoxyuridine 5’-monophosphate
ARF	alternative reading frame
ATM	ataxia telangiectasia mutated
ATP	adenosine-5'-triphosphate
CDK	cyclin-dependent kinase
ChIP-chip	chromatin immunoprecipitation with microarray technology
ChIP-seq	chromatin immunoprecipitation with massively parallel DNA sequencing
CTD	C-terminal unstructured basic domain
DBD	DNA-binding domain
E3	enzyme 3
GTF	general transcription factor
HAT	histone acetyltransferase
HDAC	histone deacetylase
mRNA	messenger ribonucleic acid
NCI	national cancer institute
NK	natural killer
NTD	N-terminal transactivation domain
ONYX-015	oncolytic virus 015
$PRIMA1^{MET}$	p53 reactivation and induction of massive apoptosis (methylated)
PXXP	proline-rich motif
qPCR	quantitative polymerase chain reaction
RE	response element
RING	really interesting new gene
RITA	reactivation of p53 and induction of tumour cell apoptosis
ROS	reactive oxygen species
TAD1, 2	transactivation subdomains 1 and 2
TSS	transcription starting site
UV	ultraviolet

Note: gene name abbreviations are skipped

1 CANCER

What is cancer? Cancer is one of the major causes of death across the globe; according to WHO it accounted for 7.6 million deaths (around 13% of all deaths) in 2008 (IARC, 2011).

Generally, cancer is characterised by excessive and uncontrolled proliferation of the cells, their immortality and the ability to metastasise into other tissues, to seed and grow there, and therefore affect the normal functions of the tissues. Hanahan and Weinberg proposed in their popular review "The hallmarks of cancer" that the transition of normal cells into cancer cells is a complex and multistep process of stepwise acquisition of six hallmarks of cancer: self-sufficiency in growth signals, unresponsiveness to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000). In their recent review in 2011 Hanahan and Weinberg added four new hallmarks that enable cells to become carcinogenic and highly malignant, which are: deregulated cellular energetics, tumour-promoting inflammation, genome instability and, finally, escape from immune destruction (Hanahan and Weinberg, 2011).

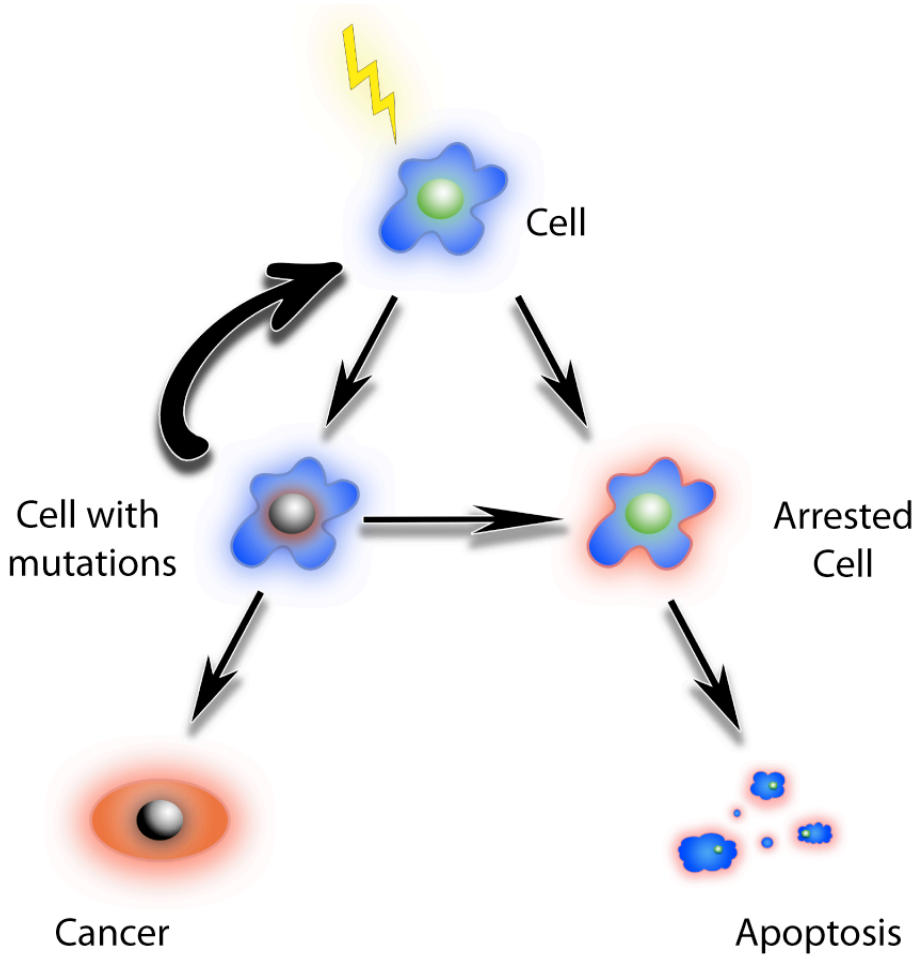

Figure 1. The model of tumourigenesis
Various types of carcinogens cause DNA lessions in normal cells that can be repaired during cell cycle arrest. In severe cases, however, the cell is subjected to programmed cell death (apoptosis). Aberrations in DNA repair and cell cycle control lead to accumulation of mutations in cells until they become tumourigenic.

It is widely accepted that the cells obtain these features by gradual accumulation of alterations in DNA of somatic cells exposed to carcinogenic factors (Weinberg book, chapter 7). Both physical (X-rays or UV light) and chemical (aflatoxin, benzopyrene, ethidium bromide) carcinogens are able to induce mutations in a cellular genome (Figure 1). There are on average 50-100 mutated genes in tumour cells, but some of these mutations are not required for cancer development. These are called "passenger" mutations in contrast to "driver" mutations which are essential for cancer growth and survival (Frohling et al., 2007; Greenman et al., 2007).

There are two different types of the genes that are absolutely crucial for tumour development: proto-oncogenes and tumour suppressors. Proto-oncogenes are precursors of oncogenes expressed in normal cells, which, due to mutations or elevated expression, gain growth-promoting or anti-apoptotic functions thus leading to hyperproliferation and cancer. Oncogenes can be divided into six functional groups: transcription factors (i.e. c-Myc, estrogene receptor), chromatin remodelers (ALL1), growth factors (PDGF), growth factor receptors (EGFR, PDGFR, VEGFR), signal transducers (SRC, Ras) and apoptosis regulators (Bcl-2, Bcl-xL) (Croce, 2008). At least a few of these genes are always found to be mutated in any type of cancer. Increased activity of oncogenes is essential, but usually not sufficient to induce a malignant tumour. A second event that has to occur during tumourigenesis is inactivation of another group of genes - the so called tumour suppressors.

1.1 TUMOUR SUPPRESSOR FACTORS

Tumour suppressors act to prevent the accumulation of mutations, restrict or suppress cell proliferation or to induce cell death. Abrogated function of tumour suppressors, either via loss of their expression or via mutations abolishing their activity, combined with oncogene activation leads to tumour development.

The first identified tumour suppressor gene was the *RB1* frequently altered in retinoblastoma. It encodes the retinoblastoma protein (pRb) that negatively regulates cell cycle progression through inactivation of E2F family of transcription factors. Discovery of the tumour suppressive activity of the pRb nicely illustrated the two-hit model proposing that in contrast to oncogenes where a mutation in only one allele is sufficient to stimulate oncogene activity, both alleles of the tumour suppressor gene have to be mutated to alter its function and induce cancer. At the same time it is now known that even partial loss of activity of a tumour suppressor can be crucial for promotion of cancer (Berger et al., 2011). Thus, deletion of only one allele of the tumour suppressor gene *TP53* can contribute to tumourigenesis (Venkatachalam et al., 1998). *TP53* was discovered more then thirty years ago and is now one of the most intensively studied genes, as evidenced by more then 60,000 publications in the PubMed database up to date. Interestingly, it was originally thought to be an oncogene, until it was discovered that it was mutated in tumour cells which were used for its cloning (Eliyahu et al., 1988; Eliyahu et al., 1989; Eliyahu et al., 1984; Finlay et al., 1988).

1.2 P53 FAMILY OF TRANSCRIPTION FACTORS

The p53 family of transcription factors consists of three members: p53, p63 and p73. All three members have a high similarity in DNA-binding domain and therefore

can bind similar DNA sequences and transactivate a similar group of genes. While both p63 and p73 might have a role in tumour suppression, they possess distinct functions (Collavin et al., 2010). Thus, p63 activity is essential for ectoderm development and prevention invasiveness and metastasis (Adorno et al., 2009; Levrero et al., 2000), whereas p73 plays a role in differentiation and neuronal development (Pozniak et al., 2000).

In the absence of stress p53 level and activity are kept low. The basal activity of p53 is sufficient to activate genes like TP53-inducible glycolysis and apoptosis regulator (*TIGAR* or *C12orf5*), sestrins 1 and 2 (*SESN1, SESN2*), glutathione peroxidises 1 and 2 (*GPX1*, *GPX2*), and aldehyde dehydrogenase-4 (*ALDH4*). These genes are involved in negative regulation of reactive oxygen species (ROS) and therefore can decrease the amount of DNA damage occurred spontaneously during proliferation of normal cells (Bensaad and Vousden, 2007). At the same time, exposure to a variety of genotoxic stresses (DNA damage, oncogene activation, hypoxia, nucleotide depletion) stabilizes and therefore activates p53 (Vousden and Lu, 2002). Depending on the severity of the stress and cell or tissue type p53 can induce cell cycle arrest, senescence, DNA repair or apoptosis (Figure 2). In other words, p53 acts as a sensor that can eliminate cells which carry irreversible DNA lesions or, if the damage is reparable, can promote DNA repair and survival of cells (Vousden and Prives, 2009).

Implementation of the p53-mediated cell cycle arrest program is predominantly controlled through transcriptional activation of the inhibitor of cyclin-dependent kinases (CDKs) $p21^{waf1}$, which induces both G1 and G2 arrest, whereas regulation of apoptotic function of p53 is more sophisticated and involve activation of pro-apoptotic genes (*BAX*, *PMAIP1*, *BBC3*), as well as repression of oncogenic factors (*BCL2*, *BIRC5*, *MCL1, IGF1R*).

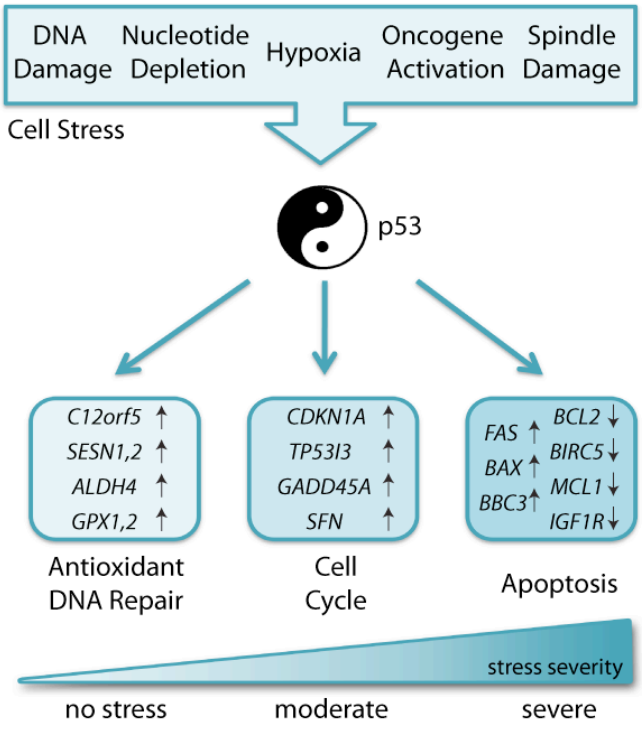

Figure 2. Severity and type of stress define p53 transcriptional response and cell fate.

p53 comprises 393 amino acid residues, which form six functional domains, that have important structural features for transcriptional regulation: 1: the N-terminal transactivation domain (NTD; including two transactivation subdomains: TAD1 (residues 1-42) and TAD2 (residues 43-92), containing five proline-rich motifs (PXXP)), 2: the most conserved central (core, or DNA-binding domain; DBD, residues 92-300) region, 3: a linker domain (residues 301-324; comprising nuclear localisation signal), 4: a tetramerization domain (residues 325-363) and 5: a C-terminal unstructured basic domain (residues 364-393; CTD) (Figure 5) (Mckinney and Prives, 2002; Scoumanne et al., 2005).

95.1% of point mutations occur in the DNA-binding domain of p53 suggesting that the sequence specific DNA binding of p53 plays a major role in its tumour suppressor function (Vousden and Lu, 2002). Although originally p53 was thought to act exclusively via regulating transcription, now it is known that p53 also mediates apoptosis in transcription–independent manner (Haupt et al., 1995), e.g. by binding to the anti-apoptotic proteins Bcl-xL and Bcl-2 and neutralizing their inhibitory effects against pro-apoptotic Bax and Bak proteins (Vaseva and Moll, 2009). Furthermore, upon an apoptotic stimulus p53 binds to Bax and Bak stimulating their ability to form homooligomers, leading to Cytochrome C release and caspase activation (Erster et al., 2004; Mihara et al., 2003).

Several factors influence the fine-tuned p53 transcriptional program upon various types of the stress. These comprise diverse post-translational modifications of p53, p53 interaction with different partner proteins, and cooperation in *cis* between p53 and other transcriptional factors (Beckerman and Prives, 2010). In addition, p53 response elements (RE) have different affinity (see below for more details). All these features dynamically affect the complex stress-mediated p53 transcriptional response that eventually defines whether the cell will be subjected to apoptosis or to cell cycle arrest.

1.3 THE MAJOR PRINCIPLES OF TRANSCRIPTIONAL REGULATION

Transcription of genes in eukaryotic cells is regulated through the binding of proteins to regulatory DNA elements, such as promoters and enhancers (Lee and Young, 2000). Nearly one quarter of the eukaryotic genes contain TATA box in their promoters. A TATA box is a consensus sequence enriched with thymine and adenine that is important for binding of general transcription factors (GTF). Promoters also contain an initiator sequence (consensus motif that serves to recruit general transcription machinery, YYANWYY, where Y is a pyrimidine, W is either A or T (adenine or thymine) and N is any nucleotide) and additional binding sites for other transcription factors, that together recruit GTFs to the transcription starting site (TSS). GTFs are essential for the life of a cell as they initiate and coordinate the basal RNA polymerase II-mediated transcription; in the absence of binding of site-specific transcription factors to proximal promoters the level of transcription in a cell is low (Figure 3). Such transcription factors recognise specific DNA structures, defined by specific DNA sequences (so-called response elements) that determine the selectivity of these factors. In addition, transcriptional activity can be stimulated through the binding of site-specific transcription factors to enhancer regions. Notably, in some cases the binding of a transcription factors *per se* is not sufficient to strongly activate promoters but requires the presence of cell-type-specific factors that recruit coactivators and consequently activate transcription of the genes. Enhancers promote gene transcription

through the recruitment of chromatin remodelling factors that open chromatin and/or recruit kinases that stimulate the elongation of transcription. On the other hand, there are transcription repressors that bind to repressing sequences and attenuate the activity of transcription factors or recruit factors that form repressive conformation of chromatin.

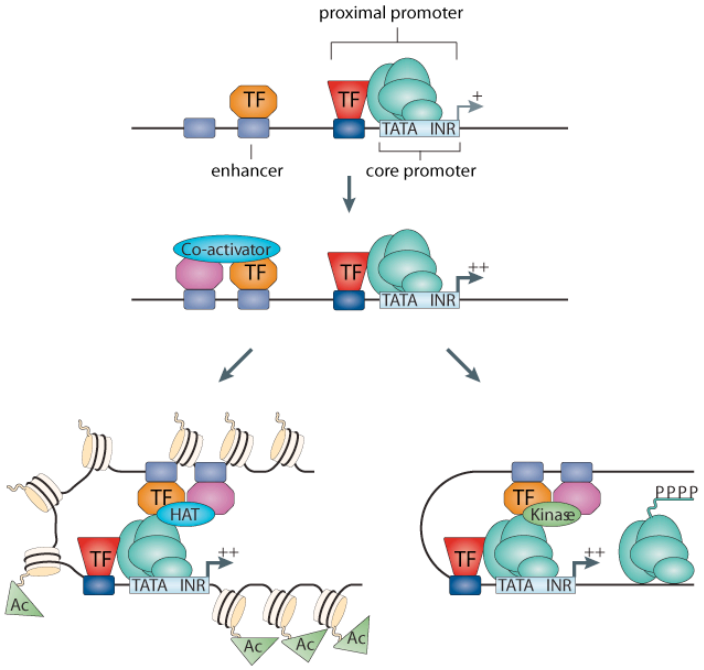

Figure 3. Basic principles of transcriptinal regulation by promoters and enhancers. General transcription factors (green ovals) recognize gene core promoters (TATA box+initiators(INR)), but form a stable transcription machinery only in combination with site-specific DNA-binding factors (orange and red) that interact with cis elements in the proximal promoter and enhancers (light and dark blue, respectively). Additionally, transcription of cell-type-specific genes is controlled by the cell-type-specific partner proteins (purple) that recruit scaffold coactivators, which in turn regulate the activity of site-specific factor in cell-type specific manner. The enhancer factors may stimulate transcription either via attracting chromatin remodeling proteins (e.g. HATs), to open up chromatin for transcription or by recruiting a kinase that phosphorylates RNA polymerase II and therefore promotes elongation. Adopted from: Farnham, 2009

Thus, the fine-tuned regulation of gene transcription requires in most cases unique combinations of transcription activators and repressors (Farnham, 2009). Therefore, identification of transcription factor response elements and its protein coregulators are the primary aims in investigation of mechanisms of transcription mediated by site-specific transcription factors.

1.4 THE MECHANISMS OF P53-MEDIATED TRANSCRIPTION

1.4.1 Binding of p53 to DNA

It is widely accepted that p53 recognises and binds with high-affinity to a consensus p53 RE, which contains two decameric half-sites 5'-RRRCWWGYYY-3' (R = purine; Y = pyrimidine; W = A or T) separated by a spacer (el-Deiry et al., 1992; Funk et al., 1992; Zauberman et al., 1993). p53 binds to DNA as a dimer of dimers,

where each p53 chain interacts with three nucleotides of the RRRCW or WGYYY quarter–sites. The spacer between the half-sites is 0-13 nucleotides, while some functional assays have proposed that a spacer <3 is optimal (Menendez et al., 2009; Riley et al., 2008; Wei et al., 2006a). Although the canonical p53 binding site has been identified and experimentally validated, it has been found that nearly 95% of the validated direct p53 target genes have at least one mismatch. Furthermore, around 10% of confirmed targets have motifs that are not clearly related to consensus sequence (Menendez et al., 2009). For example, the *PIG3* gene (p53-inducible gene 3) has a non-canonical binding site, containing a microsatellite response element $(TGYCC)_n$ (Contente et al., 2002) or the *AQP3* gene has triplet pairs of pentameric p53 elements (Zheng and Chen, 2001).

p53 REs can be found essentially anywhere within a target gene; a recent genome-wide studies show that a fraction of p53 binding sites can be found at relatively long distances from the 5' and 3' ends of a gene (more than 5 kbp) (Wei et al., 2006a). Although, the impact of such p53 sites on gene transcription remains unclear, the position of a response element relative to the TSS has a big impact on the strength of p53 transactivation and generally is lower for sites located at longer distance from the TSS. Thus, insertion of a 200 bp DNA fragment between the p53 binding site and the TATA box significantly attenuates the ability of p53 to activate transcription (Cook et al., 1995).

In addition to the p53 binding sequence and its genomic location, several other features of p53 and local spatial structure of DNA influence the binding of p53 to the DNA sites. Thus, three p53 cystein residues, namely C176, C238, and C242 play an important role for DNA binding activity via coordination of a Zn^{++} ion (Hainaut and Milner, 1993) or/and changing p53's redox status (Jayaraman et al., 1997; Seo et al., 2002). Investigation of the mechanisms that are involved in recognition of RE by p53 revealed that acetylation of CTD by p300/CBP acetyltransferase is capable to induce nonspecific binding to DNA (Avantaggiati et al., 1997; Gu and Roeder, 1997; Sang et al., 1997) but it is essential for *in vivo* occupation of p53 promoters (McKinney et al., 2004). Interestingly, the DBD of p53 bends the DNA into a conformation that promotes the binding of the p53 tetramer (Balagurumoorthy et al., 1995; Nagaich et al., 1997a; Nagaich et al., 1997b).

1.4.2 p53 regulates initiation and elongation of transcription

As mentioned above, covalent modifications of histones which open chromatin structure for binding by GTF are often needed for initiation of transcription. Transactivation of p53 target genes upon DNA damage requires cooperation of several chromatin remodelling factors that methylate/acetylate histones in the vicinity of p53 RE, e.g. histone methyltransferases PRMT1, CARM1 (An et al., 2004) and especially histone acetyltransferases (HATs) such as p300/CBP (Avantaggiati et al., 1997; Gu and Roeder, 1997; Scolnick et al., 1997), pCAF (Scolnick et al., 1997), GCN5 (Candau et al., 1997) and TIP60 (Gevry et al., 2007). Opening of DNA at p53 target promoters results in p53-mediated recruitment of several GTFs (TBP, TFIIA, TFIIH, and TAF1) to the TATA region, that allow initiation of transcription by RNA polymerase II (Ko and Prives, 1996; Li et al., 2007; Seto et al., 1992). In addition to initiation, p53 also controls elongation of transcription via its interaction with several elongation factors including Cdk9 (Claudio et al., 2006; Radhakrishnan and Gartel, 2006), FACT (Keller

and Lemberg, 2001), various components of the mediator complex (Gu et al., 1999) and ELL (Shinobu et al., 1999).

1.4.3 Transcriptional repression by p53

Apart from the transcriptional activation of the genes, p53 acts as a transrepressor (Ginsberg et al., 1991). While nearly 15% of p53 targets are known to be repressed by p53, it is still not clear whether their promoters share a consensus DNA motif defining p53 repression activity. It is possible that there are several different mechanisms of transcriptional repression by p53. For example, repression of *AFP* (alpha-fetoprotein), *BCL2* (B-cell lymphoma-2) and *HBV* (hepatitis B virus) occurs due to p53 binding to REs which overlap with binding sites of other transcription factors, like FOXA1, POU4F1, RFX1, ABL1, thus competing them out (Budhram-Mahadeo et al., 1999; Lee et al., 1999; Ori et al., 1998). On the other hand, *CCNB1* (Cyclin B1), *TERT* (telomerase reverse transcriptase), *IGF1R* (insulin-like growth factor 1 receptor), *ALB* (albumin) and *MMP1* (matrix metallopeptidase-1) genes are repressed by p53 via sequestering and squelching the function of transcriptional activators SP1, CEBPβ and AP1 (Innocente and Lee, 2005; Kanaya et al., 2000; Kubicka et al., 1999; Sun et al., 2004). Yet, another mechanism of p53-mediated repression is the recruitment of histone deacetylase HDAC1 via interaction of p53 with the mSin3a/HDAC1 complex, which is important for the repression of *MAP4* (microtubule-associated protein-4), *STMN1* (stathmin-1) and the *HSP90AB1* (heat-shock protein 90) genes (Murphy et al., 1999). p53-mediated transcriptional repression of some genes is indirect and occurs due to the activation of CDK inhibitor $p21^{Wafl}$, which promotes pRb-mediated inhibition of transcription of the E2F-regulated genes (Lohr et al., 2003).

1.4.4 Direct p53 target genes

To date 162 direct p53 target genes that regulate various cellular processes (cell cycle arrest, apoptosis, transcription, metabolism, DNA repair) have been validated (Wang et al., 2009). The application of modern high-throughput methods such as ChIP-seq and ChIP-chip revealed a much longer list of possible targets. However, the validation of these new targets requires more thorough evaluation. Three major criteria have to be fulfilled in order for a gene to be accepted as p53 target: the presence of the p53 consensus binding site in the promoter, p53 binding to this site, location in the vicinity of the TSS, and differential gene expression upon p53 activation. While this evaluation decreases the amount of false p53 target genes, it also has several serious drawbacks. It is hard to validate the target gene if it either does not have a canonical p53 RE or the RE is located at a significant distance from gene's TSS. In the latter case the regulation might occur via DNA looping. In addition, the occupancy of the p53 binding sites may occur in cell-type-specific manner depending on chromatin organisation around the p53 RE (Lidor Nili et al., 2010).

1.5 PROMOTER SELECTIVITY DEFINES P53-MEDIATED TRANSCRIPTIONAL PROGRAM

One of the major question that remains to be answered is how the differential induction of distinct transcriptional programs by p53 in response to different stimuli is achieved. Several factors could be involved in the regulation of a differential transcriptional response: the structure of p53 REs, p53 post-translational modifications, p53 binding partners, and the presence of other transcription factors in the vicinity of p53 binding sites.

1.5.1 p53 response elements

A variety of p53 binding sequences in the genome imply the existence of several DNA conformations of REs that certainly can affect the affinity of p53 to these sites. Indeed, computational and *semi-in vitro* studies (i.e., using p53 isolated from nuclear cell extracts exposed to REs attached to beads) show that p53 has a differential affinity to REs, depending on the sequence. The CATG sequence in the CWWG core especially favours p53 binding. But how do the differences in RE sequences influence the transactivation ability of p53? *In vivo* functional analysis, which allows to investigate the effect of a single nucleotide substitution in a consensus motif on transcriptional activity of p53, revealed that among all possible combinations within the CWWG decamer's core the CATG motif is the strongest one in comparison with the other three (CAAG, CTAG or CTTG) (Jordan et al., 2008). Interestingly, the alignment of all experimentally validated p53 binding sites reveal that the CWWG motifs are the most conserved in p53 RE (Figure 4).

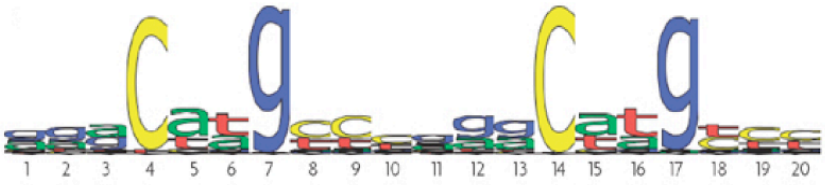

Figure 4. Sequence logo for the p53 motif based on the experimentally validated binding sites .
The height of each letter corresponds to its frequency at the particular position. The higher cummulative height at each position correlates with more significant prevalence of one letter over the other. Thus, the nucleotides at position 4,7,14 and 17 are the most conserved.
Adopted from: Riley, 2008

Incorporation of a spacer and variations in the p53 decamer's core nucleotides and adjacent nucleotides can also dramatically change the functionality of the RE. Interestingly, a comparison of several natural REs using a yeast functional assay with adjustable p53 level uncovered up to a 200-fold difference in the amount of p53 needed to transactivate reporters with inserted p53 RE. Thus, the *CDKN1A* gene (encoding $p21^{Waf1}$, crucial for cell cycle arrest) has a strong RE (requires low level of p53), whereas *BAX* (Bcl-2 associated X) and *PMAIP1* (NOXA, phorbol-12-myristate-13-acetate-induced protein 1) (important for apoptosis) have weak ones (Jordan et al., 2008). Probably the degeneracy of p53 RE in the promoters of weak genes makes their expression more dependent on the level of p53 and thus can provide precise fine-tuning which is very important for the genes deciding the fate of the cell. Thus, the level of p53 could be a decisive factor for activation of certain p53 regulated promoters that are

involved in different transcriptional programs. At the same time genome-wide studies have shown that in some cases the binding of p53 to its RE does not influence the activity of the promoter, suggesting that p53's post-translation modifications or/and interaction with cofactors are needed to induce activation of these genes (Wei et al., 2006a).

1.5.2 Post-translational modifications

Activity of p53 is regulated by various post-translational modifications that reflect activation of different cellular pathways (Bode and Dong, 2004; Sakaguchi et al., 1998; Tepel et al., 2004). A number of these modifications are involved in direct regulation of transcriptional activity of p53 (Figure 5). Thus, upon stress several kinases, like HIPK2 (D'Orazi et al., 2002), AMPK (Okoshi et al., 2008), PKC-δ (Yoshida et al., 2006), p38 (Perfettini et al., 2005) and DYRK-2 (Taira et al., 2007) phosphorylate p53 at S46 and promote selective activation of pro-apoptotic genes, e.g. the p53-regulated apoptosis-inducing protein 1 (AIP-1) (Oda et al., 2000). Interestingly, a recent genome-wide study also has shown that the decision to induce apoptosis is made by the DNA-bound fraction of S46-p53, but not S15-p53 (Smeenk et al., 2011). Furthermore, TIP60-mediated acetylation of p53 at K120 is required for activation of pro-apoptotic genes *PMAIP1* and *BAX* (Sykes et al., 2006; Tang et al., 2006), while p300-mediated acetylation at K164 is needed for the expression of most p53 target genes (Tang et al., 2008). Interestingly, the cells containing p53 with constantly acetylated K382 are able to promote only cell cycle arrest, whereas cells expressing p53 modified at K373 are also capable to induce apoptosis (Roy et al., 2005). In addition, arginine methylation at R333, R335, R337 (Jansson et al., 2008) and monoubiquitylation at K320 (Le Cam et al., 2006) can affect specifically p53-medited induction of cell-cycle arrest genes.

Thus, the existence of such a complex and diverse code of post-translational modifications at least partially explains activation of various sets of p53 targets.

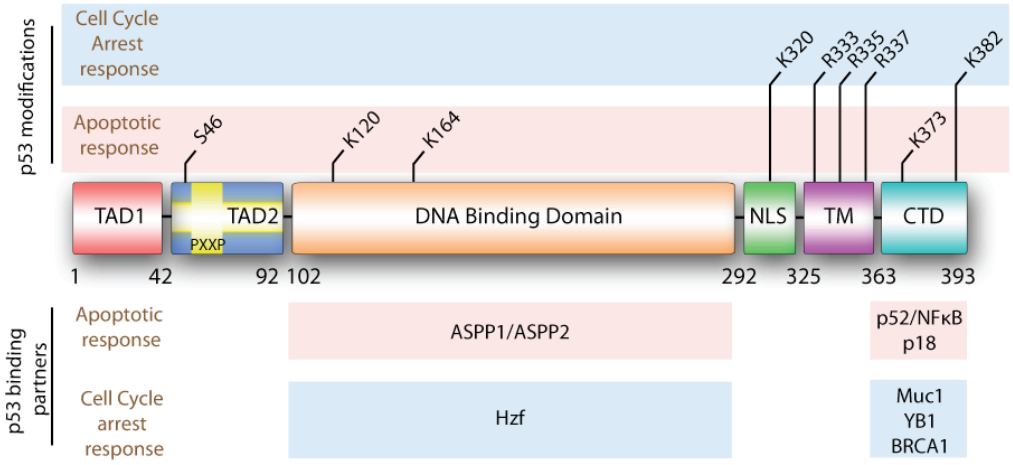

Figure 5. Post-translational modifictions and binding partners regulating p53 transactivation.
The upper part illustrates the impact of p53 modifications on transactivation of genes, preferentially inducing either apoptosis or cell cycle arrest.
The lower part shows p53 partners that affect transactivation of p53 target genes and cellular outcomes.
Modified from Beckerman and Privers , 2010

1.5.3 p53 binding cofactors

Several cofactors have been shown to modify the transcriptional activity of p53 (Figure 5). For example, the binding of two members of ASPP family proteins (apoptosis-stimulating protein of p53; ASPP1 and ASPP2) is crucial for the activation of pro-apoptotic genes such as *PIG3* and *BAX* (Bergamaschi et al., 2004), whereas the third member iASPP binds p53 and antagonizes ASPP1/ASPP2- mediated induction of apoptotic targets (Bergamaschi et al., 2006). In addition, several other proteins such as Y-box binding protein YB1 (Homer et al., 2005), p52 subunit of NFκB (Schumm et al., 2006) and p18/hamlet (Cuadrado et al., 2007; Lafarga et al., 2007) bind to p53 and promote a pro-apoptotic transcriptional program. On the other hand, p53 interaction with BRCA1 (MacLachlan et al., 2002), the membrane glycoprotein Muc1 (Wei et al., 2006b) and zinc-finger protein Hzf (Das et al., 2007) cause preferential activation of cell cycle arrest genes.

Furthermore, some p53 partners, like the E3 ubiquitin ligase HDM2, can affect p53 activity at distinct promoters, while no preference for modulation of expression of either pro- or anti-apoptotic genes has not been found (Minsky and Oren, 2004).

1.5.4 Coregulation of p53 target genes by other transcription factors

It should be noted that other master regulators could modulate the transcriptional regulation of some p53 targets genes. Thus, c-Myc in cooperation with its cofactor Miz is able to bind to the *CDKN1A* promoter, causing repression of the gene. However, such recruitment does not affect p53 binding to *CDKN1A* (Seoane et al., 2002). Additionally, a bioinformatics-based search for REs of other transcription factors revealed that Kruppel-like factor/paired box 4 (KLF/PAX4), SP1 and NFκB REs are enriched in vicinity of p53 binding sites (Smeenk et al., 2008), suggesting that these cofactors can participate in p53-mediated transcription.

1.6 HDM2 AND HDMX ARE THE MAJOR NEGATIVE REGULATORS OF P53

p53 plays a crucial role in activation of processes that affect the fate of a cell and therefore its activity has to be tightly regulated.

Two RING (really interesting gene) finger proteins HDM2 and HDMX are the main negative regulators of p53's level and activity in cells in the absence of stress (Haupt et al., 1997; Kubbutat et al., 1997).

Indeed, several elegant mouse models have shown that the regulation of p53 activity by MDM2 and MDM4 (mouse analogues of human HDM2 and HDMX) is absolutely vital during embryogenesis. Interestingly, knockout (Jones et al., 1995; Migliorini et al., 2002; Montes de Oca Luna et al., 1995; Parant et al., 2001) or conditional depletion (Francoz et al., 2006; Grier et al., 2006; Xiong et al., 2006) of either MDM2 or MDM4 during embryogenesis is embryonic lethal due to elevated activity of p53 which leads to massive apoptosis or growth arrest, respectively. The fact that p53 deletion in MDM2- or MDM4-null background can completely rescue embryonic lethality is a strong evidence of the crucial role of HDM2 or HDMX in controlling p53 function. Furthermore, even transient restoration of p53 is lethal for

adult MDM2-null mice, while MDM4-null mice survive transient p53 restoration (Garcia et al., 2011; Ringshausen et al., 2006).

Both HDM2 and HDMX have very similar structures and can interact with p53 independently of each other, however only HDM2 holds E3 ligase activity, that polyubiquitinates p53, causing its proteasomal degradation (Marine and Lozano, 2010). Binding of HDMX to p53 in turn leads to transcriptional squelching of p53 activity (Marine et al., 2007; Marine and Jochemsen, 2005).

Upon stressful conditions in normal cells the level of p53-complexes with HDM2 and HDMX is decreased through ATM-mediated phosphorylation events that lead to ubiquitination and subsequent proteasomal degradation of HDM2/HDMX (Cheng et al., 2009; Khosravi et al., 1999; Maya et al., 2001; Pereg et al., 2005). This in turn results in p53 accumulation and activation and induction of p53 target genes (Cheng and Chen, 2010), including *HDM2* thus forming a negative feedback loop (Barak et al., 1994; Barak et al., 1993; Cheng and Chen, 2010; Wu et al., 1993). The wild-type p53 induced phosphatase 1 (Wip1) is one of the p53 targets which is induced upon DNA damage. It is a functional antagonist of ATM that can dephosphorylate HDM2 and HDMX and therefore serve as an additional regulator of the p53/HDM2 negative feedback loop (Lu et al., 2007; Zhang et al., 2009).

1.7 P53 CROSS-TALK WITH SIGNALLING PATHWAYS

Activated p53 induces a global change in gene expression, although, as mentioned above, there are less than two hundred genes validated as direct p53 targets (Wang et al., 2001). This suggests that upon the differential expression of p53 targets, secondary effects on gene expression are unleashed. The ability to dramatically change gene expression makes p53 one of the key nodes of the cellular signalling which affects directly or indirectly a large number of cellular pathways. Interplay of p53 with IGF-1R/PI3K/Akt/mTOR and metabolic pathways has been investigated in my studies, and this is discussed below in details.

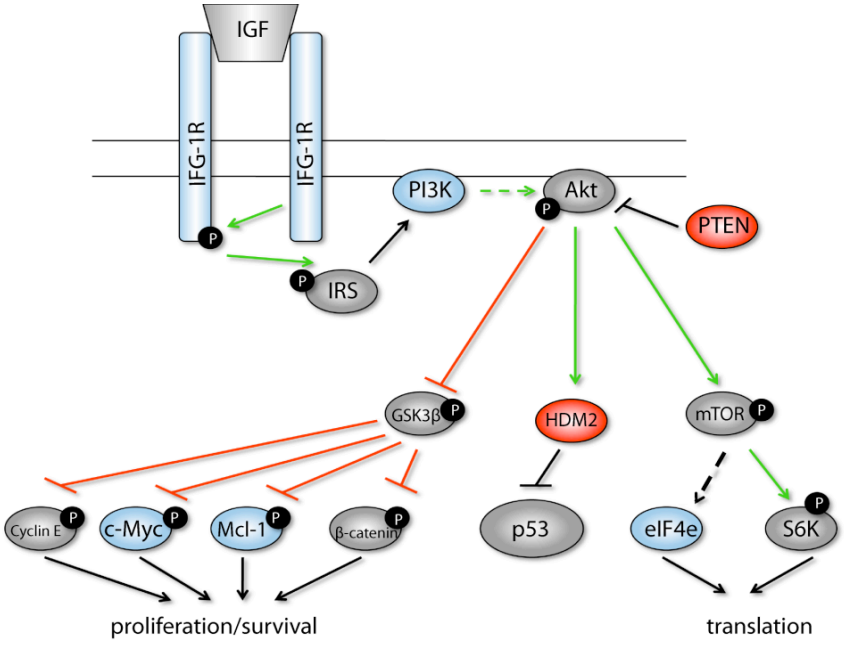

Figure 6: Crosstalk betwen p53 and IGF-1R/PI3K/Akt pathway.
Blue ovals indicate genes repressed by p53, whereas red ovals indicate induced genes. Green and red arrows point at activating or inhibitory phosphorylation events, respectively.

1.7.1 IGF-1R/PI3K/Akt pathway

Insulin-like growth factor 1 receptor (IGF-1R) is a dimeric tyrosine kinase receptor, which is involved in cell proliferation and inhibition of apoptosis (Kurmasheva and Houghton, 2006). Each monomer of the receptor contains one extracellular α-subunit and one transmembrane β-subunit that carry tyrosine kinase activity (Adams et al., 2000; Ullrich et al., 1986). Activation of IGF-1R by its ligand IGF-1 leads to the dimerization of monomers and consequently phosphorylation of insulin receptor substrates (IRS-1 and IRS-4) resulting in recruitment of p110 subunit of phosphatidylinositol 3′ kinase (PI3K), the major transducer of the signals from IGF-1R. PI3K in turn activates Akt kinase that transmits the signal to several important downstream factors, including mammalian target of rapamycin (mTOR) kinase, glycogen synthase kinase β (GSK3β) and HDM2 (Figure 6).

Activation of mTOR by Akt-mediated phosphorylation at S2448 (Nave et al., 1999; Sekulic et al., 2000) results in enhanced protein synthesis and proliferation due to the phosphorylation of S6K and inhibition of eukaryotic translation initiation factor 4E binding protein (4EBP1) (Brunn et al., 1997; Burnett et al., 1998).

Phosphorylation of GSK3β by Akt on S9 leads to its inactivation as a kinase (Frame et al., 2001). GSK3β phosphorylates and therefore modulates activity of a number of transcription factors, signalling proteins and microtubules binding proteins (Kannoji et al., 2008) via different mechanisms. In particular, it phosphorylates several oncogenes and pro-survival factors, such as c-Myc, Cyclin E, β-catenin, Notch, c-Jun and Mcl-1 thus promoting their proteasomal degradation by F-box and WD repeat domain-containing 7 (FBW7) (Inuzuka et al., 2011; Mishra, 2010).

Phosphorylation of HDM2 by Akt at S166 and S186 decreases its interaction with antagonist p14ARF and therefore promotes degradation of p53 (Gottlieb et al., 2002; Zhou et al., 2001). Therefore activation of the IGF-1R/PI3K/Akt pathway not only promotes proliferation but also restrains p53.

Interplay between the IGF-1R/Akt/mTOR pathway and p53 occurs also via other mechanisms. Thus, p53 induces the transcription of a negative regulator of PI3K *PTEN* (Stambolic et al., 2001) and at the same time represses several crucial members of this pathway, *IGF1R* (Webster et al., 1996; Werner et al., 1996), subunit of PI3K p110α (*PIK3CA*) (Astanehe et al., 2008), *EIF4E* (Zhu et al., 2005) and *MYC* (Ho et al., 2005). In turn, GSK3β regulates the transcriptional activity of p53 by phosphorylating S33 of p53 (Buss et al., 2004).

Deregulation of the components of IGF-1R/Akt/mTOR pathway is implicated in initiation of tumourigenesis (Cui, 2007) as well as in progression of various types of cancer (Belfiore, 2007). Furthermore, patients with high level of circulating IGF in blood have an increased risk to develop solid tumours in the future (Pollak et al., 2004). Essential role of this pathway in tumourigenesis along with tight interplay with p53 makes its components an important targets for anti-cancer therapy (Chitnis et al., 2008). On the other hand, repression of IGF-1R/Akt/mTOR pathway upon the pharmacological activation of p53 might be an important feature of anti-cancer therapy based on p53 activation (Gottlieb et al., 2002).

Indeed, recent studies reveal the "oncogene addiction", i.e. dependency of the tumours on driving oncogenes. Thus, inhibition of oncogenes, like c-Myc, K-Ras, β-catenin, Cyclin E, or Cyclin D1 and others significantly decreases proliferation and

survival of the several types of tumour cells, as shown by recent studies on patient samples, mouse models and cell culture experiments (Weinstein and Joe, 2006). Therefore, inhibition of several oncogenes by pharmacologically activated p53 might be an important mechanism of eliminating cancer cells carrying different driving oncogenes.

1.7.2 Metabolic pathways

Uncontrolled proliferation of cancer cells requires increased amount of energy and therefore often, if not always, tumours are characterised by deregulated metabolic pathways, which is now defined as one of the hallmarks of cancer (Hanahan and Weinberg, 2011). Under aerobic conditions normal cells produce ATP, the main source of energy, via consequent processing of glucose to pyruvate (glycolysis in the cell cytoplasm) and finally to carbon dioxide (oxidative phosphorylation in mitochondria). At the same time, anaerobic conditions shift the energy production towards eighteen times less efficient glycolysis, since this process, in contrast to oxidative phosphorylation, does not require oxygen. Notably, cancer cells are characterized by the shift to glycolysis even in the presence of oxygen and such process was named Warburg effect or aerobic glycolysis (Warburg, 1956).

The molecular basis of this paradox is partially due to the upregulation of glucose transporters, particularly GLUT1 that increases glucose uptake and compensates for the employment of inefficient glycolysis (Younes et al., 1996). At the same time, anaerobic glycolysis is also associated with oncogene activation, namely Ras and PI3K, along with hypoxia that occurs in many tumours. These two factors affect the level of HIF1α and HIF2α (hypoxia-inducible factor 1 and 2, α subunit) that are positive regulators of glucose uptake (DeBerardinis et al., 2008; Kroemer and Pouyssegur, 2008). Elevated levels of HIF1 transcription factor in turn promote the expression of several metabolic genes alone or in cooperation with c-Myc oncogene: *SLC2A1* (GLUT1), *HKII* (hexokinase II), *PDK1* (pyruvate dehydrogenase kinase 1), *PFKM* (phosphofructokinase, muscle) and *LDHA* (lactate dehydrogenase A) (Jones and Thompson, 2009; Kallio et al., 1997; Kim et al., 2006).

Interestingly, mutant status of p53 is associated with the Warburg effect in tumour cells (Jones and Thompson, 2009). Moreover, cross-talk of aerobic glycolysis with p53 is proven and occurs mainly via p53-mediated transcriptional regulation of several genes involved in glycolysis and oxidative phosphorylation. Thus, p53 transactivates TIGAR that redirects glycolysis to the penthose phosphate pathway (PPP) resulting in low ROS level and attenuated apoptosis (Bensaad et al., 2006). p53 also increases transcription of the *SCO2* gene (synthesis of cytochrome c oxidase 2), which is essential for oxidative phosphorylation induced by p53 (Matoba et al., 2006). Moreover, p53 decreases the expression level of *PGM* (phosphoglycerate mutase), a gene that enhances glycolysis (Kondoh et al., 2005). In addition to inhibition of glycolysis, p53 can directly decrease the glucose uptake by transrepression of *SLC2A1* and *SLC2A4* (GLUT4) (Schwartzenberg-Bar-Yoseph et al., 2004).

While it is clear that p53 negatively regulates glycolysis, the outcome of this inhibition is less clear and probably depends on the strength of p53 activation (Vousden and Ryan, 2009). At the same time, targeting the Warburg effect is a promising strategy to combat cancer and is under intensive investigation (Le et al., 2010; Scatena et al., 2008).

1.8 TUMOUR METASTASIS

More then 90% of all cancer-related mortalities occur due to metastatic spreading of tumours (Fidler, 2003; Hanahan and Weinberg, 2011). Moreover, in many cases the cancer is diagnosed at late stages when it is already metastatic. All this makes it important to understand the nature of metastasis formation, identification of biomarkers and the origins of metastasis in order to select a proper strategy to combat cancer, as well as to detect cancer at early stages, even before its propagation.

The theory of metastasis formation was suggested more then a hundred years ago by Steven Paget and termed "seed and soil" hypothesis (Paget, 1989). Tumour cells are compared with seeds that are constantly distributed from the primary tumours, searching for the appropriate cell environment (soil) to establish secondary tumours (metastasis). Understanding of the general concept of cancer development requires identification of the features that define metastatic phenotype of the cells. Proper understanding of the genotype of metastatic cells will offer new targets for anti-cancer strategies (Sethi and Kang, 2011). It is still unclear how the cells from primary tumours obtain metastatic features. A linear hypothesis suggests that primary tumour cells go through several rounds of mutations until they obtain necessary metastatic features (Cairns, 1975). Interestingly, only a small subset of the heterogenic primary tumour cells becomes metastatic. On the other hand, the parallel model implies that separation and migration to the secondary hearth of cells originating from the primary tumour occurs at early stages of cancer development, followed by independent accumulation of mutations and metastasis (Klein, 2009). The linear hypothesis was supported by several genomic studies that clearly have shown that metastatic cells arise as a subset of primary tumour cells, separated at a late stage of tumourigenesis (Ding et al., 2010; Yachida et al., 2010). However, a genomic pattern has been described that defines the predisposition of a fraction of the cells to form metastasis (Perou et al., 2000). Such pattern suggests that there is a complex network of signal interactions that is crucial for metastasis development. Therefore, understanding and targeting the crucial nodes of such networks can be the strategy of treating metastatic tumours.

2 P53 AS AN ANTI-CANCER TARGET

Nearly all late stage tumours have abrogated p53 function. Given the importance of p53 in tumour suppression and its cross-talk with numerous signalling pathways involved in carcinogenesis, pharmacological reactivation of p53 can be an important strategy to combat cancer. Studies in several mouse models addressed the question whether reinstatement of p53 can eliminate already established tumours and showed that the restoration of p53 activity in tumour cells leads to regression of tumours (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). On the other hand, most recent studies using mouse model of non-small-cell lung cancer (NSCLC) characterized by aberrant expression of mutant K-Ras oncogene, showed that the efficacy of p53 restoration greatly depends on level of K-Ras expression (Feldser et al., 2010; Junttila et al., 2010). p53 reactivation in early stage cancers with relatively normal level of K-Ras did not affect tumour growth. In contrast, restoration of p53 in more advanced adenocarcinomas resulted in significant tumour regression, via induction of cell-cycle arrest and apoptosis (Berns, 2010). Thus, restoration of p53 activity might be a good anti-cancer therapeutic strategy for late stage tumours, rather than for the prevention of tumour formation.

There are several known mechanisms of p53 deregulation in tumour cells. Nearly half of all tumours carry mutant p53 that is transcriptional inactive. As it was described earlier, most of these mutations occur in DNA binding domain with six hot spots at codons 175, 245, 248, 249, 273 and 282. As a result of mutations, DNA binding activity of mutant p53 towards its consensus motif is abrogated resulting in the loss of transcriptional function of p53 (Bullock et al., 2000; Epstein et al., 1998).

In the remaining 50% of the tumours *TP53* gene stays intact, while p53 activity is impaired, resulting in abrogated tumour suppressor function and enhanced formation of tumours. Such abrogation of p53 activity happens mainly due to the increased levels and/or activity of p53 negative regulators HDM2 and HDMX (Danovi et al., 2004; Momand et al., 1992; Riemenschneider et al., 1999). Enhanced activity of HDM2 occurs often via several mechanisms. Thus, gene deletion or promoter hypermethylation of the HDM2 inhibitor p14ARF elevates HDM2 level (Esteller et al., 2001; Sherr and Weber, 2000). In addition, overexpression of several p14ARF repressors including Bmi-1, Tbx-2 and TWIST results in HDM2 activation (Anim et al., 2005; Jacobs et al., 1999). On the other hand, overexpression of HDM2 in many tumours bearing wild-type p53 is achieved by *HDM2* gene amplification, increased transcription and enhanced translation (Momand et al., 1998).

Although the function of p53 is inactivated in the majority of tumours, the p53 protein is still expressed, making it possible to design strategies to “revive” the dormant tumour suppressor.

2.1 PHARMACOLOGICAL REACTIVATION OF WILD-TYPE P53

One of the most common ways to induce wild-type p53, which has been already applied for many decades, is to use chemotherapeutic drugs. It is known that chemotherapeutic drugs in many cases act as DNA damaging agents that stabilize p53 and induce p53-mediated cell cycle arrest or apoptosis. Notably, inactivation of p53 in several types of cancer cells makes them resistant to chemotherapy. However,

reintroduction of wild-type p53 to p53-null cancer cells recover their sensitivity to chemotherapeutic drugs (Wallace-Brodeur and Lowe, 1999).

While chemotherapy is efficient and widely used strategy to treat cancer and to activate p53, it is not specific for p53 and produce the severe side effects in patients. This makes the search for non-genotoxic p53 activators a very important line of research. Already more then ten years ago researchers began to design more specific means of p53 activation.

The molecular mechanisms of p53 regulation in cancer cells mentioned above suggest that inhibition of p53-HDM2/HDMX interaction might me an efficient way to fight cancer (Brown et al., 2011). While several promising chemical compounds that prevent inactivation of p53 by HDM2/HDMX have been identified in the last few years, it is still not completely clear whether they can be good drugs. Thus, mouse model studies showed that negative regulation of p53 by HDM2 or HDMX is essential for survival of mouse embryos (Jones et al., 1995; Montes de Oca Luna et al., 1995; Parant et al., 2001). At the same time a reasonable balance between p53 and its negative regulators is sufficient to induce mild level of p53 and therefore apoptosis in tumour cells without detectable side effects in normal tissues (Mendrysa et al., 2006). Milder effects of p53 restoration in HDMX-null in comparison to HDM2-null mice suggests that inhibition of HDMX might be a better therapeutic strategy for reactivating p53 (Garcia et al., 2011).

Up to date a number of small molecules that target the p53-HDM2 interaction have been found, including Nutlin-3a, Benzodiazepine, RITA, MI-219, SAH-p53 (Bernal et al., 2007; Ding et al., 2006; Garcia-Echeverria et al., 2000; Grasberger et al., 2005; Issaeva et al., 2004; Popowicz et al., 2010; Vassilev et al., 2004), while WK298 inhibits both HDM2 and HDMX interaction with p53 (Popowicz et al., 2010). There are two other specific inhibitors of HDMX/p53 interaction: SJ-172550 and a stapled p53 helix SAH-p53-8 (Bernal et al., 2010; Reed et al., 2010).

Apart of targeting interaction of p53 with HDM2/HDMX, there is a possibility to target upstream regulators of p53-HDM2 interaction. For example, tenovins which inhibit protein-deacetylating activities of SirT1 and SirT2 of the sirtuin family stabilize of p53 via promoting its acetylation, thus preventing p53 ubiquitination by HDM2. This is followed by activation of p53 target genes (Lain et al., 2008).

Different mechanisms of wild-type p53 activation by chemotherapeutic drug 5-FU and small molecules as the Nutlin-3a and RITA will be discussed in detail below.

2.1.1 5-FU

The chemotherapeutic drug 5-FU (5-fluorouracil) has been widely used for almost forty years for treatment of several types of cancers, including gastrointestinal, lung, breast and colon cancer. Despite of its efficient use, the mechanism of 5-FU is not completely clear yet. While the first metabolite of 5-FU, 5-fluorouridine 5'-triphosphate (5-FUTP), might be incorporated into RNA, the second, 5-fluoro-2'-deoxyuridine 5'-monophosphate (5-FdUMP), is a known inhibitor of thymidylate synthase (Parker and Cheng, 1990). As a consequence of such inhibition cells are arrested in S phase, followed by p53 activation. p53 activated upon 5-FU treatment induces its target genes and consequently cell growth and apoptosis (Nabeya et al., 1995; Pickard et al., 1995). While 5-FU is not a direct inducer of p53 transcriptional

activity and can even kill p53-null cells, the presence of p53 is beneficial for 5-FU action (Osaki et al., 1997).

2.1.2 Nutlins

Nutlins are a class of small molecules that inhibit p53-HDM2 interaction via direct binding to the p53-binding pocket of HDM2 (Vassilev et al., 2004). Nutlins were identified in an *in vitro* biochemical screen of a library of synthetic compounds. Nutlin-1, Nutlin-2 and Nutlin-3 are the analogs of the original compound which are more potent inhibitors of p53-HDM2 interaction. While first two molecules exist as racemic mixtures, Nutlin-3a is an active enantiomer and has the lowest IC_{50} for the disruption of p53-HDM2 complex. The disruption of the p53-HDM2 interaction by Nutlins has been shown in biophysical experiments using NMR (D'Silva et al., 2005). Furthemore, Nutlin-3a is able to induce p53 activity and growth suppression *in vivo* and *in vitro* (Vassilev et al., 2004). Interestingly, in multiple tumour-derived cell lines Nutlin-3a preferentially induces p53 target genes involved in cell cycle arrest which correlates with reduced ability of cells to undergo p53-dependent apoptosis (Tovar et al., 2006). Notably, Nutlin-3a also affects proliferation of normal cells, inducing reversible cell cycle arrest. This phenomenon lead to the idea of "cyclotherapy", i.e., to use Nutlin-3a pre-treatment as a protection of normal cells from the side effects caused by chemotherapeutic treatment or targeted drugs that would usually affect normal cells, such as the Aurora kinase A inhibitor VX-680. On the other hand, actively proliferating tumour cells with mutant p53 will be not arrested by Nutlin-3a and therefore will be sensitive to chemotherapy or VX-680 (Cheok et al., 2010).

2.1.3 RITA

RITA (reactivation of p53 and induction of tumour cell apoptosis) is a small molecule, which has been discovered via screening of a National Cancer Institute (NCI) library of compounds using a cell-based assay searching for the compounds which suppress the growth of the HCT116 cells expressing wild-type p53, but not that of the isogenic p53-null cell line (Issaeva et al., 2004). RITA binds p53 and prevents the interaction between p53 and HDM2 *in vitro* and *in vivo* (Issaeva et al., 2004).

RITA activates apoptosis in tumour cell lines of different origin, including colon, lung, skin, and breast carcinomas, osteosarcoma as well as lymphomas. In contrast, it does not affect the growth of non-transformed fibroblasts or epithelial cells (Issaeva et al., 2004). Furthermore, RITA suppressed the growth of HCT116 xenografts in mice, without noticeable side effects (Issaeva et al., 2004). Early studies attributed the growth inhibitory effects of RITA to its ability to cross-link DNA-DNA and DNA-protein complexes in human renal cancer cells, which depends on the cell uptake and metabolism of the compound (Nieves-Neira et al., 1999; Rivera et al., 1999). However, several studies have shown that RITA-mediated effects on growth suppression of cells are dependent on presence of p53 (Ahmed et al., 2011; Issaeva et al., 2004; Kazemi et al., 2011; Nahi et al., 2008). Indeed, RITA-induced DNA damage signalling occurs only in p53-positive cell lines (Yang et al., 2009a; Yang et al., 2009b).

Interestingly, in contrast to Nutlin-3a, RITA stimulates $p21^{Waf1}$ degradation that shifts the balance towards induction of apoptosis rather then cell cycle arrest upon p53 activation (Enge et al., 2009).

Recent studies also have shown new feature of RITA to induce the presence of ULBP1 and ULBP2 ligands for NK cell on the surface. These findings suggest a new anti-cancer strategy: to combine p53 activation with NK-based therapy in the wild-type p53 carrying tumours (Li et al., 2011; Textor et al., 2011).

2.2 PHARMACOLOGICAL REACTIVATION OF MUTANT P53

Since nearly fifty percent of all tumours carry mutant p53, several studies have been performed to identify small molecules that are able to reactivate mutant p53 and therefore selectively kill cancer cells expressing mutant p53. CDB3, CP31398, PRIMA-1^{MET}, PhiKan083 and SCH529074 have been found to restore the function of mutant p53 (Boeckler et al., 2008; Bykov et al., 2002; Demma et al., 2010; Foster et al., 1999; Friedler et al., 2002). Since different point mutations of p53 produce a common effect, i.e., partial unfolding of p53, most of the molecules mentioned above are active towards different mutants. The exception is PhiKan083, specifically designed to target the Y220C mutation. These compounds are currently in different stages of development. The first clinical trial for PRIMA-1^{MET}/APR-246 has been completed in 2011; hopefully a Phase II trial of APR-246 will be launched in 2012.

2.3 P53 GENE THERAPY AND ONYX-015

Another strategy to reactivate mutant p53 is to replace mutant gene with the active wild-type copy. Two different virus systems are used to achieve this.

The first strategy to integrate the *TP53* gene into the genome is to use retroviruses. Several studies shows that retrovirus-mediated incorporation of wild-type p53 in lung cancer cell lines as well as in xenografts inhibited the growth of tumour cells (Cai et al., 1993; Fujiwara et al., 1993; Runnebaum et al., 1995).

Another strategy of gene delivery is based on adenovirus. Advexin and Gendicine are two drugs that demonstrate growth suppression activity in tumours and have been tested in phase I, II and III clinical trials (Yang et al., 2010).

In contrast to *TP53* gene therapy, adenovirus-based ONYX-015 which has deleted p53 inhibitor E1B does not deliver functional p53, but selectively replicates in cells with dysfunctional p53, consequently lysing them leading to the regression of tumours (Nemunaitis et al., 2007).

3 AIMS OF THE THESIS

This thesis is focused on the studies of the molecular mechanisms of pharmacological reactivation of p53. In particular, we investigated the transcriptional activity of reactivated p53 and its role in induction of apoptosis. Deeper understanding of these issues will help to more efficiently apply the pharmacological reactivation of p53 as a therapeutic strategy to cure cancer.

Specific aims:

- To study genome-wide occupancy of p53 at its target promoters upon different means of pharmacological activation of p53 and to identify the new transcriptional cofactors of p53 (**Paper I**).

- To investigate the impact of transcriptional modulation by p53 of different sets of genes on RITA-mediated apoptosis in tumour cells (**Paper II**).

- To establish the impact of p53 reactivated by RITA on the aerobic glycolysis and genes that control this process (**Paper III**).

- To investigate the impact of pharmacologically reactivated p53 on its major negative regulators HDMX and Wip1 (**Paper IV**).

4 RESULTS AND DISCUSSION

Four papers that are discussed in this thesis focuse on different aspects of the pharmacological reactivation of p53, from phenotype to transcriptional regulation of single genes.

Paper I.

Insights into p53 Transcriptional Function via Genome-Wide Chromatin Occupancy and Gene Expression Analysis

Fedor Nikulenkov, Clemens Spinnler, Claudia Tonelli, Mikko Turunen, Hai Li, Teemu Kivioja, Alexander Kel, Jussi Taipale and Galina Selivanova

The starting point for this study was the finding that the different mechanisms of pharmacological activation of p53, i.e. by Nutlin-3a and RITA, induce very different transcriptional programs (Enge et al., 2009). More, Nutlin-3a and RITA treatment lead to diverse phenotypic responses in the same cell lines: cell cycle arrest or apoptosis, respectively. Thus we addressed a question, whether it occurs due to the differential binding of p53 to its target genes upon different stimuli, leading to the differential gene expression patterns or whether p53 binds to the same sites irrespective of the type of its induction, whereas the presence of cofactors decides whether the gene will be induced or not. Due to the technological revolution in biology, in particularly, the development of high-throughput sequencing technology it becomes possible to study global genome occupancy of transcription factors and answer such questions.

Thus, we treated breast cancer MCF7 cells with three different pharmacological activators of p53 (5-FU, Nutlin-3a and RITA) and analysed the genome-wide p53 occupancy in these cells using ChIP-seq method. Surprisingly, we found that there is a "default p53 program", comprising a set of binding sites that is occupied by p53 independently on the type of activation stimuli. Furthermore, using the p53 scan algorithm we showed that these sites are enriched for the p53 motif. However, there was also a small number of sites bound by p53 which were unique for different treatments. While these sites were not significantly enriched for the p53 consensus motif, we can not exclude the possibility that these sites, different from the "default p53 program" are important for the differential phenotypic responses.

While previous studies have shown that in most cases p53 binds to DNA in the vicinity of transcription starting sites (TSS) we found that only 34% of the "default p53 program" peaks were located within 10kbp from TSS. The relatively large number of peaks identified to be distant from TSS suggests that either a number of the p53 target genes are regulated by p53 via DNA looping or at least some of these p53-DNA binding events are irrelevant for the regulation of transcription by p53.

Combination of the analysis of global gene expression changes upon Nutlin-3a treatment and genome-wide chromatin occupancy revealed 280 novel p53 target genes. Among them the transcription of 214 genes was induced and 66 genes were repressed. Interestingly, in contrast to induced genes only a small portion of the repressed genes shared p53 consensus motif, suggesting that the mechanism of repression occurs either

through indirect p53 binding or through motifs which are significantly different from the p53 consensus.

We have chosen *AURKA* (repressed) and *SERTAD1* (induced) as the most interesting new targets of p53 and further validated them using several methods. *AURKA* encodes Aurora A kinase that is unfavourable prognostic marker in cancer and used as a target for anti-cancer therapies. Our studies suggest that STAT3 can be an important cofactor antagonizing p53-mediated repression of number of genes, including *AURKA*.

To elucidate the clinical importance of the identified p53 target genes we performed hierarchical clustering of the expression levels of p53 target genes in 265 breast tumour patient samples. We found a significant correlation between the expression of a number of p53 target genes that we identified, and the status of p53, grade of the tumours and long-term survival prognosis. These data suggested that newly identified p53 targets might be used as clinical prognostic markers in breast tumour patients.

Paper II.

Ablation of key oncogenic pathways by RITA-reactivated p53 is required for efficient apoptosis.

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[*] equal contribution

The analysis of gene expression microarrays in breast tumour MCF7 cells as well as in colon cancer HCT116 cells revealed a significant decrease in transcription of a set of oncogenes (*IGF1R*, *PIK3CA*, *PIK3CB*, *MYC*, *EIF4E*, *BCL2*, *MAP4*, *MCL1*) upon RITA treatment in comparison to untreated samples, validated further by qPCR. Additionally, the repression of these genes was totally p53-dependent, as we did not observe any changes in expression upon 1 μM RITA treatment in several p53-null cell lines (HCT116 p53-/-, H1299, SAOS2). Importantly, RITA did not affect the expression of these genes in untransformed cells.

Our data also showed that p53 activation upon RITA resulted not only in dramatic transcriptional changes. Apart from the transcriptional repression of anti-apoptotic proteins Bcl-2 and Mcl-1, as well as several proteins involved in the IGF-1R/PI3K/Akt pathway, inhibition of a number of oncogenes occurs on the level of translation and protein stability. The activity of several proteins involved in the IGF-1R/PI3K/Akt pathway was abrogated, that led to inhibition of mTOR, EIF4E and consequently c-Myc translation. The stability of already synthesized proteins c-Myc, β-catenin and Cyclin E was also decreased due to the activation of GSK3β and the E3 ligase FBXW7 (hCdc4), which play important roles in proteasomal degradation of oncoproteins. Thus, we showed that pharmacologically activated p53, apart from triggering the expression of pro-apoptotic proteins, also suppresses the survival factors via several mechanisms.

Surprisingly, we found that the inhibition of oncogenic proteins was dependent on the dose of RITA, while induction of pro-apoptotic factors was observed irrespective of a dose. At the same time, the level of p53 induction was comparable upon 0.1 μM and 1 μM RITA treatment. Notably, the absence of oncogene inhibition at lower dose (0.1 μM) of RITA in tumour cells correlated with weak apoptosis induction in comparison to that induced by 1uM RITA. Further investigation of the difference between these two doses revealed significantly higher levels of p53 on chromatin upon 1 μM RITA. Moreover, we found that HDM2 bound to p53 on chromatin might play a key role in the observed dose-dependent differences in regulation of oncogenes. Indeed, investigation of the promoter occupancy by p53-HDM2 complexes showed that treatment with 0.1 μM RITA increased the p53/HDM2 ratio on p53-induced gene (*CDKN1A*) promoters, but not on the repressed (*MCL1*), while 1 μM RITA elevated p53/HDM2 ratio on both promoters. Thus, we suggested that dose-dependent repression of oncogenes depends on the HDM2/p53 ratio on repressed promoters.

Finally, we addressed the impact of the inhibition of pro-survival genes on induction of apoptosis upon high dose of RITA. We showed that pharmacological inhibition (LY294002 treatment) of the IGF-1R/PI3K/Akt pathway or depletion of the pro-survival Mcl-1 factor in combination with low dose of RITA is sufficient to induce massive apoptosis in tumour cells. Thus, we concluded that the repression of pro-

survival and pro-proliferative factors is essential for the robust induction of p53-mediated apoptosis in tumour cells.

Paper III.

Inhibition of glycolytic enzymes mediated by pharmacologically activated p53: targeting Warburg effect to fight cancer.

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[*], [§] equal contribution

The third study was a logical continuation of second paper, where we have shown an important role of p53-mediated inhibition of oncogenes in induction of apoptosis in tumour cells.

In this study we explored whether pharmacologically activated p53 is able to inhibit aerobic glycolysis, the essential source of energy in tumour cells. Indeed, metabolic measurement of acidification status that reflects the lactate export (final product of glycolysis) showed that 1 μM RITA treatment led to p53-mediated inhibition of cell metabolism. Interestingly, glycolytic inhibition was not a consequence of apoptosis and was detected only in p53-positive cells.

Further microarray analysis of gene expression in MCF7 and HCT116 cancer cells revealed that a set of key metabolic genes, like *SLC2A1*, *HKII*, *PFKB3*, *PFK*, *PGM3*, *LDHA*, *PDK1* as well as factors regulating metabolic genes (*MYC*, *HIF1A*, *PI3CA* and *PI3CB*) were substantially repressed upon 1 μM RITA treatment. Several genes were selected for further investigation and validation by qPCR and western blot. Thus, we showed that *SLC2A1*, *HKII*, *PFKFB3*, *SLC2A12*, *PDK1* and *HIF1A* genes were repressed in a p53-dependent manner in both tumour cells and xenografts *in vivo*, although the observed downregulation of some genes was less pronounced *in vivo*. Notably, the inhibition of *HIF1A* and *HKII* expression was accompanied by a strong depletion of HIF1α and HK2 protein levels in several tumour cells (U2OS, HCT116, MCF7).

Interestingly, we found that some of these metabolic genes (*SLC2A1, HIF1A, SLC2A12*) might be direct transcriptional targets of p53, in addition to already known *C12orf5* and *MYC.*

Since many tumours face hypoxic environment and express a high level of HIF1α, we addressed the impact of HIF1α inhibition on the expression of metabolic genes under hypoxia and normoxia. Our data clearly demonstrated that p53-mediated repression of HIF1α contributes to the ablation of metabolic genes in hypoxic, but not normoxic conditions in tumour cells.

Additionally, bioinformatics promoter analysis of studied metabolic genes revealed several other cofactors that might have binding sites in promoters of these genes and therefore regulate their transcription. Key node analysis of gene expression data revealed that SP1 is tightly linked with p53. Thus, we decided to study impact of SP1on p53-mediated transcriptional regulation of metabolic genes and found that the depletion of SP1 in MCF7 cells partially, but significantly protect some metabolic genes from repression upon RITA treatment. These data implies that SP1 cooperates with p53 in transcriptional repression of metabolic genes. Furthermore, observed protection of metabolic genes from p53-mediated repression in SP1-depleted cells correlated with attenuated cell growth suppression.

On other hand, depletion of HK2 in combination with 0.1 μM RITA significantly increased apoptosis in MCF7 cells, suggesting that the repression of at least some metabolic genes contributes to apoptosis caused by RITA in tumour cells.

Paper IV.

Abrogation of Wip1 expression by RITA-activated p53 potentiates apoptosis induction via activation of ATM and inhibition of HdmX

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In the fourth study we investigated the mechanisms and impact of downregulation of p53 negative regulator HDMX upon RITA treatment in tumour cells. HDMX is known to be overexpressed in many tumours, which causes abrogation of p53 function (Ramos et al., 2001; Riemenschneider et al., 1999). Therefore, it is particularly important to investigate the molecular mechanisms of HDMX downregulation which we observed upon RITA treatment.

In this study we found that RITA induced a rapid ablation of HDMX protein level in most tumour cell lines bearing wild-type p53 (MCF7, U2OS, HCT116, HT1080, LIM1215, A549, A498) as well as in xenografts. In contrast, another pharmacological inducer of p53, Nutlin-3a did not affect the HDMX level. Depletion of HDMX upon RITA treatment was totally p53-dependent and not observed in normal cell lines. Interestingly, downregulation of HDMX correlated with RITA-induced apoptosis. All cell lines that had decreased HDMX level were sensitive to RITA treatment, while RKO and SJSA tumour cells, which did not respond to RITA, did not show any changes in HDMX. Additionally, the depletion of HDMX in HCT116 cells promoted apoptosis induced by RITA.

Further, we addressed the mechanisms underlying the downregulation of HDMX by assessing the changes in half-life of HDMX upon RITA treatment. We showed that the stability of HDMX was drastically decreased due to proteasome degradation. Since HDM2 is major negative regulator of HDMX stability we checked whether RITA-mediated downregulation was caused by HDM2. Depletion of HDM2 in HCT116 cells resulted in increased HDMX level in untreated cells, but did not prevent from RITA-induced downregulation of HDMX. Instead, we found that ATM played an essential role in HDMX degradation by RITA, since the depletion of ATM partially protected HDMX from downregulation. ATM kinase is a transducer of DNA damage signalling which phosporylates several substrates including HDMX. In line with previously published data, we showed that RITA activated ATM, which also correlated with the accumulation of HDMX phosphorylated at ATM-dependent sites S367 and S403. Further, we demonstrated that phosphorylation of HDMX on these sites is crucial for its proteasomal degradation since RITA did not affect the half-life of non-phosphorylatable triple mutant HDMX carrying S342A, S367A and S403A substitutions.

Next, we found that Wip1 phosphatase which negatively regulates the activity of ATM through its dephosphorylation, was downregulated in p53-dependent manner on transcriptional and protein levels, as accessed by qPCR and western blotting. We further showed that the depletion of Wip1 led to downregulaton of HDMX, which was even more pronounced upon RITA treatment in HCT116 cells. Additionally we showed that the depletion of Wip1 promoted growth suppression of tumour cells upon RITA treatment as well as upon Nutlin3a.

Thus, this study revealed that RITA inhibits Wip1 and HDMX oncogenes, resulting in disruption of the p53/Wip1/HDMX negative feedback loop. Such pharmacologically mediated block of p53 inhibitors might be essential for the induction of robust growth suppression, leading to regression of tumours.

5 CONCLUSIONS AND REMARKS

My way to the p53 field started seven years ago when all students at our department had to choose a laboratory for their master thesis. For most of us it was a very hard decision to select the field they wanted to work in, while I knew exactly that it should be tumour biology. Both grandparents on my mother's side died from cancer when I was very small, which kept alive in my mind how ruthless, painful and in many case hopeless this disease can be. Being idealistic, I dreamt about discovering the treatments that would help mankind to win the battle against cancer. Looking back now I understand how naive my view on cancer used to be. At the same time I appreciate now how challenging it is to unravel the mechanisms of tumourigenesis.

Although today many molecules and signalling pathways that can drive tumourigenesis are discovered, the selection of the appropriate strategy to combat cancer remains difficult. This is especially true for cancer, as each tumour is probably as individual as each patient itself and, moreover, consists of a heterogenic population of cells with various abnormalities. On the other hand, the central tumour suppressive role of p53 in cells and it's deregulation observed in nearly all tumour cells make p53 a very promising target for anti-cancer therapy. Indeed, restoration of p53 function in mice with established tumours entails growth inhibition and tumour regression, although the observed effects depend on the tumour grade (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). Several approaches are used to restore p53 in cells, while the most common is pharmacological reactivation. Thus, many chemotherapeutic drugs are the first wild-type p53 reactivators, e.g. 5-FU. In our lab several small molecules were discovered that suppress tumour growth in a p53-dependent manner, including MITA and RITA (Hedstrom et al., 2009b; Issaeva et al., 2004). Both RITA and MITA induce apoptosis in different types of tumour cell lines, such as lung, colon and breast carcinomas. In the original paper RITA was shown to bind the p53 N-terminus, thus preventing formation of p53-HDM2 complexes and, therefore, increasing stability and activity of p53. On the other hand, Nutlins activate p53 by occupying the p53-binding pocket in HDM2 (Vassilev et al., 2004), resulting in induction of cell-cycle arrest and to less extent apoptosis.

The work presented in this thesis provides insight into various mechanisms playing important roles in growth-inhibitory effects of pharmacologically reactivated p53.

Treatment with various reactivating compounds results in accumulation of p53 and induction of its transcriptional program, which differs depending on the mechanism of p53 activation. p53 regulates gene transcription via binding to REs in the vicinity to TSS and therefore promotes initiation and elongation of transcription. Thus, in the first paper we addressed the question how different pharmacological activators of p53, such as 5-FU, RITA and Nutlin-3a, influence p53's genome-wide DNA binding profile and how this leads to various cellular outcomes. In agreement with previous studies (Shaked et al., 2008; Smeenk et al., 2011), we showed that p53 occupies core binding sites irrespectively to the type of p53 activation and cell fate. Thus, we speculate that post-translational modifications or/and interaction with cofactors might redirect transcriptional activity of DNA-bound p53 from promotion of cell cycle arrest to apoptosis. Indeed, S46 phosphorylation of p53 bound to DNA results in predominant activation of pro-apoptotic genes and consequently apoptosis (Smeenk et al., 2011). In addition, we showed that many of the p53 binding sites are found at significant

distances from TSS, raising the questions whether these sites play any role in regulation of the transcription or cell fate. We also identified 280 new potential p53 targets, including *AURKA* that might serve as possible prognostic markers. Previous bioinformatic studies suggested that KLF/PAX4, SP1 and NFκB transcription factors coregulate p53 target genes (Smeenk et al., 2008). In addition to these factors, our analysis revealed STAT3 as a novel coregulator of p53 target genes that, in particular, antagonize p53 repressive activity on the *AURKA* promoter.

We investigated various aspects of p53 activity upon RITA treatment that contribute to apoptosis. A global analysis of transcriptional changes caused by RITA demonstrates that expression of more then two thousand genes is affected in a p53-dependent manner (Enge et al., 2009). Furthermore, pathway analysis reveals that pro-apoptotic genes are among the most efficiently induced. Moreover, in paper II we showed that transcription of a number of oncogenic and pro-survival factors, such as *IGF1R*, *PIK3CA*, *PIK3CB*, *MYC*, *EIF4E*, *BCL2*, *MAP4*, *MCL1,* was repressed. In addition, the pro-survival protein Mcl-1 and c-Myc oncogene were inhibited on multiple levels. For both, the block of the IGF-1R/PI3K/Akt pathway was affecting protein degradation and translation of these proteins. In conclusion, we showed that the observed repression of oncogenes significantly contributes to apoptosis and represents an additional mechanism that increases the robustness of p53-mediated tumour suppression. Interestingly, we observed differential regulation of induced pro-apoptotic genes and repressed oncogenes, which depends on the binding of HDM2 to the target gene promoters. In addition, a study by Enge et al. demonstrates that HDM2 inhibits $p21^{Waf1}$ protein level. Such depletion plays an important role in RITA-mediated growth suppression by tilting the balance between growth arrest and apoptosis (Enge et al., 2009).

On other hand, in paper IV we showed that RITA downregulates mRNA and protein level of another important HDM2-related inhibitor of p53, namely HDMX. The observed depletion of HDMX levels was mediated by DNA damage induced ATM kinase, whose activity was in turn elevated due to p53-mediated downregulation of Wip1 phosphatase. Inactivation of HDMX and Wip1 further extend the complexity of RITA action and its robust ability to induce apoptosis in tumour cells.

Previous study from our group by Hedström et al. shows that, in addition to p53, RITA also binds theoredoxin reductase 1 (TrxR1) and induces pro-oxidant genes as well as ROS levels in a p53-dependent manner (Hedstrom et al., 2009a). In addition, paper III shows that RITA represses the anti-oxidant gene *C12orf5*. Furthermore, we investigated the impact of RITA on ATP production from aerobic glycolysis in tumour cells and surprisingly found that a set of key metabolic genes was repressed. This occurred both on mRNA and protein level in a p53-dependent manner, leading to inhibition of cell metabolism. Interestingly, we found that transcription of *SLC2A1*, *HIF1A* and *SLC2A12* might be directly repressed by p53. Furthermore, we identified the SP1 transcription factor to be crucial for the p53-mediated repression of a number of metabolic genes and induction of apoptosis.

In conclusion, we identified several p53-mediated effects of RITA that impinge on several crucial factors important for tumour cell survival. These broad effects reveal RITA as potent anti-cancer compound. Taking in account that tumours are very heterogeneous and that several subpopulations of cells with various aberrations are present within the same tumour, the ability of pharmacologically activated p53 to affect

multiple signalling pathways might be favourable as it allows to induce apoptosis in many tumour cells, regardless of what mutations they carry.

Furthermore, we used the properties of p53 activating molecules to address general questions concerning the biology of p53-mediated transcription and regulation of cell fate decision. Along with the newly identified target genes, these data may contribute to the deeper understanding of tumour biology and inspire the development of future cancer therapies.

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