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P53-DEPENDENT AND -INDEPENDENT MECHANISMS OF P53-TARGETING SMALL MOLECULES

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p53-dependent and -independent mechanisms of p53-targeting small molecules

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Tumor suppressor p53 (*Tp53*) is mutated in around half of human cancers, while in wild type p53 cells its activity is continuously inhibited by MDM2 through proteasome degradation resulting in the loss of its function. Currently, cancer treatments with small molecules based on reactivation of wild type p53 and restoration of mutant p53 have moved to clinical trials and exhibited promising anti-cancer effects. Our lab previously found a small molecule RITA which reactivates p53 and has strong anti-cancer effect without affecting normal cells. However, small molecules always have multiple targets and those should be validated for either predicting potential side effects or evaluating their efficacy in different types of cancers.

In this thesis, we addressed a p53-independent mechanism of RITA along with two other anti-cancer compounds Aminoflavone and Oncrasin-1. Using thermal proteome profiling (TPP) approach, we found that transcription machinery is commonly inhibited by these three compounds in a reactive oxidative species (ROS)-dependent manner. Global transcription inhibition results in massive downregulation of the majority of oncogenes as well as genes that are involved in homologous recombination (HR). By taking advantage of that, we performed combination treatments of these three compounds with PARP-1 inhibitors Olaparib and talazoparib. The combination treatments displayed clear synergistic anti-cancer effects in several cancer lines as well as in primary ovarian and breast cancer patient samples.

Moreover, we found that mRNA translation is also inhibited by RITA through activation of eIF2α phosphorylation, in a p53-independent manner. Complementary to these findings, we discovered a potent downregulation of MDM2 by RITA. Using different approaches, we confirmed that MDM2 is not inhibited by RITA through proteasome degradation, autophagy or microRNAs-mediated translation inhibition. In addition, the inhibition of MDM2 is not the cause of cell death since both MDM2 overexpression and MDM2 KO could not rescue RITA killing effect. We conclude that, RITA dramatically inhibits RNA processing in cancer cells, leading to inhibition of transcription and translation, resulting in cell death.

Reactivation of p53 also has dark sides which are related to p53-mediated growth arrest or apoptosis in normal tissues. We investigated the mechanism of action of the well-known p53 inhibitor PFT-α and found that PFT-α cannot prevent p53 activation-induced growth repression in several cancer cell lines but can attenuate post-translational modifications (PTMs) of p53 and by that differentially inhibit p53 target genes. Although we found that PFT-α exhibits strong intracellular antioxidant activity through activation of AHR/NRF2 pathway, we cannot link the antioxidant activity to its capacity to attenuate PTMs of p53. Worth to note, both PFT-α and NAC can promote primary fibroblasts growth *per se*. Therefore, PFT-α rescued Nutlin-3-induced growth repression in primary fibroblasts. Our findings suggest that caution needs to be taken when using PFT-α to study p53 signaling cascade, since it is not a pan-p53 inhibitor as it is described. The phenomenon we observed with PFT-α in primary fibroblasts also indicates the clinical potential of combining p53 reactivators with PFT-α in cancer therapies.

LIST OF SCIENTIFIC PAPERS

I. Sylvain Peuget, **Jiawei Zhu**, Gema Sanz Santos, Madhurendra Singh, Massimiliano Gaetani, Xinsong Chen, Yao Shi, Amir Ata Saei, Torkild Visnes, Mikael Lindström, Ali Rihani, Lidia Moyano-Galceran, Joseph Carlson, Elisabet Hjerpe, Ulrika Joneborg, Kaisa Lehti, Johan Hartman, Thomas Helleday, Roman Zubarev, and Galina Selivanova.
Thermal proteome profiling identifies oxidative-dependent inhibition of the transcription of major oncogenes as a new therapeutic mechanism for select anticancer compounds
Cancer Research, 2020 Feb 4. pii: canres.2069.2019. doi: 10.1158/0008-5472.CAN-19-2069

II. **Jiawei Zhu**, Madhurendra Singh, Galina Selivanova and.Sylvain Peuget.
Pifithrin-α alters p53 post-translational modifications pattern and differentially inhibits p53 target genes
Scientific Reports, 2020 Jan 23;10(1):1049. doi: 10.1038/s41598-020-58051-1.

III. Johannes Ristau, Vincent van Hoef, Sylvain Peuget, **Jiawei Zhu**, Bo-Jhih Guan, Shuo Liang, Maria Hatzoglou, Ivan Topisirovic, and Galina Selivanova & Ola Larsson.
RITA requires eIF2α-dependent modulation of mRNA translation for its anti-cancer activity
Cell Death Dis. 2019 Nov 7;10(11):845. doi: 10.1038/s41419-019-2074-3.

CONTENTS

LISTF ABBREVIATIONS

ATP	Adenosine triphosphate
AHR	Aryl hydrocarbon receptor
AML	Acute myeloid leukemia
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia telangiectasia and Rad3-related
AF	Aminoflavone
BCL-2	B-cell lymphoma 2
BER	Base excision repair
BBC3 (PUMA)	p53 upregulated modulator of apoptosis
CRISPR	Clustered regularly interspaced short palindromic repeats
CDKN1A	Cyclin-dependent kinase inhibitor 1A
COX2	Prostaglandin-endoperoxide synthase 2
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CK2	Casein kinase 2
CPT	Camptothecin
DBD	DNA binding domain
DNMT1	DNA Methyltransferase 1
ERK1	Extracellularly Regulated Kinase 1
EMT	Epithelial–mesenchymal transition
EU	5-ethynyluridine
EGFR	Epidermal growth factor receptor
E3	Enzyme 3
EIF2α	Eukaryotic translation initiation factor 2 subunit alpha
FGFR3	Fibroblast growth factor receptor 3
GOF	Gain of function
HIPK2	Homeodomain interacting protein kinase 2
HATS	Histone acetyltransferases
HDACS	Histone deacetylases
HIF1-α	Hypoxia-inducible factor 1-alpha
HR	Homologous recombination
JNK	c-Jun N-terminal kinase
LRRK2	Leucine-rich repeat kinase 2
Mdm2	Mouse double minute 2
Mdmx	Mouse double minute x
Mcl-1	Myeloid cell leukemia 1
MAPK	Mitogen-activated protein kinase
NAC	N-acetyl cysteine
NRF2	Nuclear factor erythroid 2-related factor 2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PDGFR-β	Platelet derived growth factor receptor-beta
PPM1D	Protein phosphatase 1D
PTMs	Post-translational modification
PET-CT	Positron emission tomography–computed tomography
PDK1	3-Phosphoinositide-dependent kinase 1
PARP	Poly (ADP-ribose) polymerase
PLK3	Polo-like kinase 3
PKCδ	Protein kinase C delta type
RITA	Reactivation of p53 and induction of tumor cell apoptosis
ROS	Reactive oxygen species
siRNA	Small interfering RNA
shRNA	Short hairpin RNA or small hairpin RNA
SCO2	SCO cytochrome c oxidase assembly protein 2
SIRT1	Sirtuin 1
TGF-β	Transforming Growth Factor Beta
VEGF	Vascular endothelial growth factor
ZEB1	Zinc finger E-box-binding homeobox 1

1 P53 AND CANCER

1.1 HISTORY OF P53

In 1979, tumor suppressor p53 was discovered in mammalian cells which have been transformed by the SV40 DNA tumor virus [1–3]. A co-transfection of p53 cDNA and *ras* oncogene into rat embryo fibroblasts leads to cell transformation, which suggested that p53 gene was oncogene in the first place, much like the *myc* oncogene [4]. Later, it was found that the p53 cDNA used for transfection was extracted from tumor cells bearing mutant p53, while wild type p53 cDNA from normal cells turned out to suppress tumor growth [5]. Ever since, the importance of p53 as tumor suppressor is appreciated and p53 became the most studied cancer gene in the last four decades.

As a transcription factor (for p53 protein structure, see Figure 1), p53 is the central determinant of cell fate: it induces cell cycle arrest by transactivating cyclin dependent kinase inhibitor p21 that inhibits phosphorylation of Rb and prevents cells from entering into S phase. It induces apoptosis by activating BH3 domain family members such as *BBC3* (PUMA) and *PMAIP1* (NOXA) which prevent anti-apoptotic members of BCL2 family from binding to pro-apoptotic proteins Bax and Bak. Bax and Bak form channels on the outer membrane of mitochondria to release cytochrome C, this triggers caspase 9-mediated apoptosis (reviewed in [6]). Here, I will discuss the relationship between p53 and cancer hallmarks; MDM2/p53 regulation; current compounds that reactivate or inhibit p53 and their clinical potential and identification of drug targets by using novel approaches.

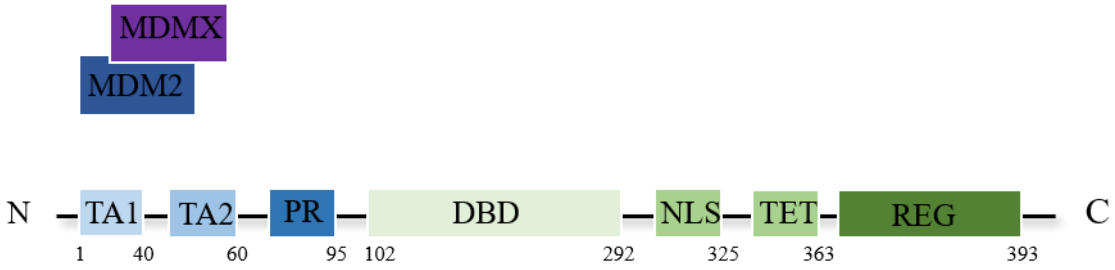

Figure 1. Human p53 protein: N-terminal transactivation domains TA1(1-40) and TA2 (40-60) are associated with transcription co-factors such as p300, CBP etc. TA1 also contains MDM2 binding site; Proline rich domain (64-95) binds to SH3 domain-proteins; DNA binding domain (102-292) is responsible for sequence-specific binding to p53's response element on the promoter of its target genes; Nuclear localization signal (316-324); Tetramerization domain (325-355); C-terminal regulatory domain (364-393).

1.2 P53 REGULATION

The activity of wild type p53 is regulated by many factors, for example, the expression of MDM2 directly affects p53 protein level [7]; p53 transcription level is regulated and can be also modulated by epigenetic factors in a complex manner; chromatin remodeling enzymes such as HATs, HDACs, DNMT1 could also affect p53 gene expression [8–10]. Early in 1997, it was found in Mass's lab by using a reporter plasmid vector that cytosine methylation of CpG

island in p53 promotor inactivates p53 gene expression. Abundant clinical results showed that aberrant promoter methylation of p53 is associated with carcinogenesis in different cancers including gliomas, epithelial ovarian cancer, breast cancer, adrenal cancer, head and neck cancer and leukemia (reviewed in [8]). p53 gene expression is also protected by the insulator CTCF from repressive histone marks, such as H3K9me3, H3K27me3 and H4K20me3 [10].

PTMs can regulate p53 activity by affecting its stability or by recruiting other transcription co-factors (activators or repressors) to the promotor of its target genes [11]. It is hypothesized that the profile of p53 PTMs induced by a specific stimulus forms a "barcode" which results in a specific cellular response [12]. For example, phosphorylation of p53 on Ser46 was found to induce apoptosis but not cell-cycle arrest [13]. p53's stability, the affinity to its target genes and ability to recruit its partners can all be modulated in a complex and stimulus-dependent manner by diverse PTMs such as ubiquitination, phosphorylation, SUMOylation and acetylation, methylation as well. Since different PTMs could coexist on p53 and lead to diverse conformational changes of p53, it remains a challenge to characterize the correlation between specific PTMs and differential p53 tasks. In general, acetylation could activate p53 which is supported by observation of enhanced p53 transcriptional activity and anti-cancer effects with HDAC inhibitor treatment. p53 function could be either activated or repressed by different states of methylation at different lysine sites of p53. Noteworthy, chromatin remodeling enzymes not only affect p53 expression but also modulate p53 activity by modulating PTMs of p53. For example, histone lysine methyltransferase KMT5 (Set9) mono-methylate p53 at K372 and stabilizes p53, but KMT3C (Smyd2) and KMT5A (Set8) mono-methylate p53 at K370 and K382, respectively , leading to prevention of p53-mediated *CDKN1A* (p21) and *BBC3* (PUMA) induction (reviewed in [14]). miRNA-125b, miRNA-504, miRNA-25 and miRNA-30d can inactivate p53 pathway by directly binding to the UTR of p53 mRNA, while some miRNAs can regulate other factors such as MDM2, AKT and SIRT1 to indirectly affect p53 signaling pathway (reviewed in [15]).

In response to a number of cellular stresses including DNA damage, oncogene activation, hypoxia, nucleotide starvation, UV radiation etc., p53 can be activated physiologically and execute its transcription factor function (Figure 2). For example: oncogene-activated p19ARF sequesters MDM2 in the cytoplasm, where MDM2 undergoes proteasome degradation; the activated kinases such as ATM, ATR and Chk1/2 can phosphorylate p53 at its transactivation domains (TADs) and disrupt MDM2/p53 complex (reviewed in [16]); it is also found that ATM can phosphorylate MDM2 at its RING domain, therefore inhibiting its E3 ligase activity (reviewed in [17]); ATR is found to phosphorylate serine 407 of MDM2 and reduces p53 export from nucleus to cytoplasm [18].

Upon pharmacological p53 reactivators such as Nutlin-3 and RITA, p53 can be stabilized by direct prevention of MDM2 binding (Figure 2), while conventional chemotherapeutic drugs such CPT, doxorubicin, cisplatin could stabilize p53 as a secondary event by activating ATM,

ATR, Chk1/2. There are many chemotherapeutic drugs used in clinic or in clinical trials that are known to activate p53 as well.

Aminoflavone (AFP464, NSC 710464) is an anti-tumor compound which has already entered phase II clinical trials to treat estrogen-positive breast cancer (ER+) patients. AF is described to cause DNA damage after it is metabolized by CYP1A1, which is transcriptionally activated by AHR [19]. In addition, AF also activates p53 and inhibits HIF1-α [20]. Recently it has been shown that AF can modulate immune response by inducing anti-tumor M1 macrophage profile [21]. Oncrasin-1(NSC743380) was identified to kill cancer cells with K-Ras mutation, but can also activate p53 [22]. Since p53 can be activated by many compounds as a secondary event, it is important to understand the primary mechanisms of action of these compounds for more efficient drug development (it is discussed further in chapter 3.2).

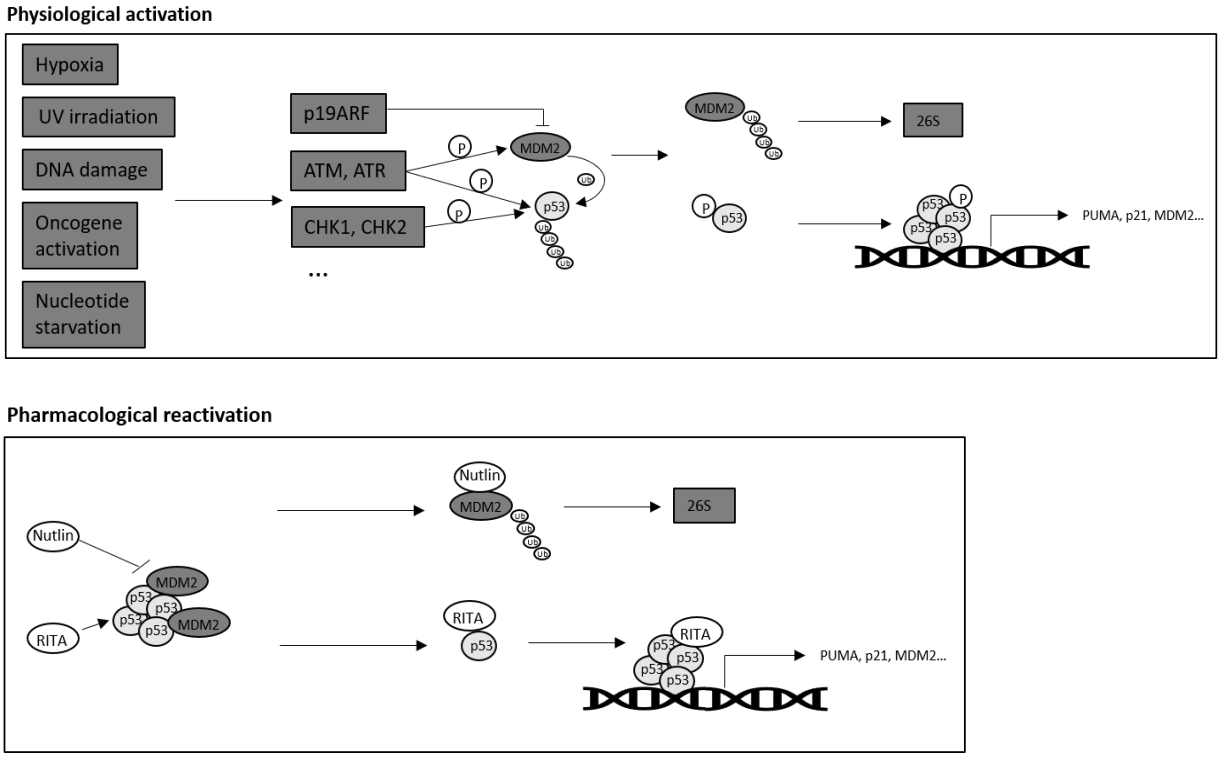

Figure 2. p53 physiological activation: stress signals including hypoxia, UV irradiation, DNA damage, oncogene activation and nucleotide starvation activate ATM, ATR and Chk1/2 which phosphorylate MDM2 and p53, or induce p19ARF which inhibit MDM2, resulting in p53 stabilization and activation. p53 pharmacological reactivation: Nutlin and RITA as examples of compounds reactivating p53 via inhibiting MDM2 binding to p53.

1.3 P53 AND MDM2 CIRCUIT

p53 is well known as a sequence specific transcription factor. It consists of N-terminal region (residues 1-101) which contains two transactivation domains, TAD1 and TAD2, a proline-rich region (PR), and nuclear export signal (NES) region; central core domain (amino acids 102-292) which contains DNA-binding domain (DBD); C-terminal region (residues 292-393) which contains tetramerization domain (Tet, residues 325-355) [23], as shown in Figure 1.

Under unstressed conditions, wild type p53 level usually remains low due to MDM2-mediated proteasome-dependent degradation [24]. MDM2 is thoroughly characterized as a negative regulator of p53 (reviewed in [15]). There are 4 well characterized domains of MDM2. At the N-terminus, residues 18-101 comprise the p53-binding region. This interaction inhibits the transcriptional activity of p53 by affecting the recruitment of canonical transcription factors (reviewed in [15]). In addition, it is reported that MDM2 N-terminus interacts with p53 C-terminus and could prevent proper p53 tetramerization [25]. In the central region there is the central acidic domain (amino acids 237-288) adjacent to a zinc finger (residues 289-331). This part of the protein is described to interact with p53 central core domain and is essential for the ubiquitination of p53. The interaction could also lead to conformational changes of p53 and therefore affects p53 DNA binding activity [26]. The C-terminus contains a RING finger (residues 436-482) that displays E3 ubiquitin ligase activity. It targets p53 to degradation but also regulates its own degradation. Loss of C-terminus RING finger results in both p53 and MDM2 stabilization [27]. Its C-terminal tail region (residues 485-491) regulates RING finger motif by forming either MDM2 homodimer or MDM2-MDMX heterodimer [28].

MDM2 is critical for maintaining p53 levels both in unstressed cells and following genotoxic stresses. In brief, once MDM2 binds to p53, it inhibits p53 transcriptional activity by binding to the TAD and DBD of p53, then p53 is translocated by MDM2 from nucleus to cytoplasm, where p53 will be degraded by the 26S proteasome after undergoing MDM2-mediated ubiquitination (reviewed in [16]). Importantly, MDM2 contains p53-responisve promoter P2 and is transactivated by cellular stress in a p53-dependent manner. This forms the components of p53-MDM2 negative feedback loop. Normally, this p53-MDM2 regulatory circuit can keep the level of wild type p53 in check to maintain cellular processes (reviewed in [15]). However, overexpression of MDM2 is observed in many types of human cancers which exhibit loss of wild-type p53 characteristics (reviewed in [15]).

1.4 MUTANT P53 IN CANCER

As the guardian of genome, p53 is the most frequently mutated gene in human cancers, among which over 80% of the mutations are missense mutations that lead to the loss of DNA binding and wild type functions of p53 and exert dominant-negative effect over wild type p53 [29]. Around 30% of missense mutations are localized at so called 6 hot-spot residues in the DNA binding domain of p53 (R175, G245, R248, R249, R273, and R282) which are highly conserved during cancer evolution. Moreover, it has been discovered that mutant p53 has gain-of-function that is independent of wild type p53 [30]. In addition to dominant-negative effects of mutant p53 which abrogate wild type p53 function, the mutant p53 gain-of function contributes to tumorigenesis, metastasis and resistance to cancer therapy [31]. Mice bearing p53 R270H or R172H developed tumors that are highly metastasized [32]. It can be related to the fact that mutant p53 has been shown to support TGF-β-mediated metastasis [33]. In the Vogelstein's model of colon cancer, p53 mutation appears to be a late event, after APC mutation and beta-catenin accumulation [34] . Other researchers believe that p53 mutations can

occur at different stages of tumorigenesis and contribute to various cellular processes including enhanced cell growth, evasion of apoptosis, migration and invasion (reviewed in [35]).

Selection pressure is considered to take place during the evolution of cancers. Some cancer cells manage to escape tumor suppression caused by wild type p53 and exhibit p53 null or p53 mutants eventually [36]. It has been shown that mutant p53 can also activate the transcription of genes which are different from wild type p53 target genes, regardless of attenuation of DNA binding affinity [37]. However, the mechanism is still not fully understood. It is known that wild type p53 needs to form a tetramer to properly function as a transcription factor. Theoretically, mutant p53 may alter the conformation of tetramer thereby being recruited by different co-factors to the promoters of different genes. p53 protein conformation is in a dynamic folded and unfolded status, which is considered being regulated by heat shock proteins such as Hsp70 or Hsp90. It is demonstrated that while Hsp70/Hdj1 system can shift wild type p53 to mutant-like conformation and enhance the unfolded state of mutant p53, Hsp90 counteracts Hsp70 to attenuate this conversion [38].

Mutant p53-associated genetic abnormality is often correlated with poor prognosis in cancer patients [39]. Li-Fraumeni patients carrying germline p53 mutations develop a wide spectrum of cancer types instead of specific tumor types [40,41]. Therefore, it appears urgent to target mutant p53 as a therapeutic strategy to combat cancer. It is discussed more in chapter 3.2.

2 P53 AND CANCER HALLMARKS

Figure 3 illustrates how p53 is involved in cancer hallmarks, which is discussed in detail below.

2.1 PROLIFERATIVE SIGNALING

Distinct from normal cells, cancer cells acquire the capability to sustain proliferative signaling through different mechanisms including autocrine signal stimulation, growth receptor overexpression or by educating neighbor cells to secret mitogens that promote tumor growth [42]. In addition, cancer cells are able to maintain their proliferative state through ligand-independent pathways. For example, cancer cells can express structure altered growth receptors that can be active without binding to mitogens [43]; cancer cells with *Ras* mutation maintain activated downstream pathways radiated from a ligand-stimulated receptor (reviewed in [44]). It has been shown that loss of p53 is correlated with amplified growth signaling such as EGFR signaling [45,46], PDGFR-β signaling [47,48], hedgehog-GLI signaling [49–51], NOTCH signaling [52–54] and NF-κB signaling [55]. Both *Ras* and *Myc* oncogenes can activate p53 expression through ARF induction [56,57], in turn, p53 inhibits *Ras* and *Myc* to repress cancer cell growth, which serves as a barrier for tumor development [58,59]. Therefore, the selection pressure for p53 mutations is very high. Consistently, it is well documented that

high expression of p53 mutants correlates with active growth receptor signaling in patients with different types of cancers [60–63].

2.2 GROWTH REPRESSION

pRb and p53 are two major tumor suppressors that could call a halt to cell proliferation. Control of R-point transition of pRb function is perturbed in most of human tumors [59] (see Table 1). It is well known that p53 can prevent tumor cells from escaping growth arrest by inducing cyclin-dependent kinase inhibitor such as P21/WAF1/CIP1, which inhibits hyper-phosphorylation of RB, therefore prevents E2F family members (mainly E2F1, E2F2 and E2F3) from transcribing genes that are needed for cells to enter S phase (reviewed in [6]). In addition to arresting cells in G1 phase, p53 negatively regulates almost all cell cycle phases by inhibiting cyclin A and cyclin B. p53-mediated cell cycle arrest is considered as an intracellular protective machinery against oncogene activation and DNA damage which leads to mutations, chromosomal aberrations and carcinogenesis (reviewed in [65]).

Alterations of pRb	Corresponding tumors
Inactivation of Rb gene by mutation	Retinoblastoma, osteosarcoma, small-lung carcinoma
Methylation of Rb gene promoter	Brain tumors, diverse others
Sequestration of Rb by Id1, Id2	Diverse carcinomas, neuroblastoma, melanoma
Sequestration of Rb by HPV E7 viral oncoprotein	Cervical carcinoma

Table 1. Alterations of Rb in human tumors from the book Biology of Cancer by Robert Weinberg.

2.3 APOPTOSIS

In response to severe stress signals, intracellular or extracellular apoptosis pathways will be activated to eliminate damaged cells. However, cancer cells evolved to acquire the ability to evade apoptosis by expressing anti-apoptotic proteins such as Bcl-2, along with its family members (Bcl-xL, Bcl-w, Mcl-1, A1) which suppress two pro-apoptotic proteins Bax and Bak, as well as sequestering BH3-only proteins such as Puma and Noxa (reviewed in [66]). As the guardian of genome, p53 can transcriptionally activate Bax and BH3-only proteins Puma and Noxa, which liberate Bax and Bak from the binding of anti-apoptotic proteins to release cytochrome C from the outer membrane of mitochondria to activate caspase 9-mediated apoptosis signaling (reviewed in [6]). Mitochondrial p53 directly activate the oligomerization of Bax and Bak to promote apoptosis (reviewed in [67]). Many studies have shown that chemotherapy- or radiation-induced tumor regression is p53-dependent, which highlights its role as a key pro-apoptotic factor (reviewed in [68,69]).

2.4 ANGIOGENESIS

Unlike normal tissues, angiogenesis in tumor tissues is continuous instead of transient. Since the sustained proliferation of cancer cells requires relatively more nutrients, cancer cells need more vessels to support their growth. The angiogenetic signaling is regulated by several factors, for instance, VEGF-A can bind to VEGFR, receptor in vascular endothelial cells. The VEGF gene can be upregulated by HIF1-α and other oncogenes (reviewed in [70]). It has been shown that p53 inhibits angiogenesis by targeting HIF1-α to degradation through physical binding [71]. p53 can also transcriptionally repress a group of pro-angiogenic genes including VEGF, COX-2 and upregulate anti-angiogenic genes including Thrombospondin-1, Brain-specific angiogenesis inhibitor 1, Ephrin receptor A2 and angiogenetic collagens (reviewed in [72]). Importantly, wild type p53 status is required for the success of anti-angiogenic therapy in mouse experiments [73,74]. The mechanism is elusive, but it implies the central role of p53 in regulating signaling pathways across different types of cells in the microenvironment.

2.5 METASTASIS

During cancer progression, cancer cells can invade neighboring tissues by breaking down extracellular matrix (ECM) and cell-to-cell adherence and metastasize to distant organs through EMT process. Metastasis is considered as the major cause of cancer-related death (reviewed in [75]). It has been shown that mutant p53 can promote metastasis by its gain-of-function, while wild type p53 could prevent metastasis. Indeed, in hepatocellular carcinoma, by activating its target gene MDM2, p53 can cause degradation of Snail, a key repressor of E-Cadherin and inducer of N-Cadherin [76]. TGF-β is the key regulator for EMT process, it has been summarized that TGF-β can activate RTK signaling, cytokine signaling, β-catenin signaling, Notch signaling and Sonic Hedgehog signaling. Many of these pathways can in turn induce the expression of ligands that comprise TGF-β family (reviewed in[77]). It is also proven that wild type p53 inhibits EMT process that is induced by TGF-β [33]. TGF-β is shown to activate Smad3/4 signaling and downregulate total endogenous p53 levels by transcriptionally upregulating MDM2 [78]. Interestingly, it has been found that MDM2 can also promote metastasis by degrading E-Cadherin independent of p53 [79]. It is possible that p53 can inhibit metastasis independent of MDM2. For example, p53 can inhibit the expression of ZEB1 by transactivating miR200 [33]. It is known that HIF1-α is also involved in metastasis by activating EMT regulators and p53 can directly bind and inhibit HIF1- α, which highlights its role as anti-metastasis factor (reviewed in [72]).

2.6 REPLICATIVE IMMORTALITY

In order to acquire unlimited replication, cancer cells need to pass through two transition barriers called senescence and apoptosis, also known as crisis. It was found that human cancer cells have high expression of telomerase which maintains length of telomeres, that is required for cell immortalization and prevention of DNA end-to-end fusion (reviewed in [80]). In mammalian cells, telomere shortening results in p53 activation leading to senescence to

prevent tumor growth [81]. In addition, it was found that p53 can suppress telomerase activity separated from its cell cycle checkpoint function and loss of p53 function is often correlated with high expression of telomerase in different types of human cancers [82].

2.7 REPROGRAMING ENERGY METABOLISM

Cancer cells prefer to utilize glycolysis to produce energy even under aerobic conditions. Tumor-associated mutant p53 has been proven to stimulate Warburg effect by enhancing GLUT1 translocation to the plasma membrane [83]. It has been shown the mutant p53 can cooperate with HIF-1 to transcriptionally activate ECM components and promote tumor progression [84]. Since the ATP production efficiency of glycolysis is around 18-fold lower than oxidative phosphorylation, cancer cells need more glucose than normal cells. This characteristic is utilized by PET-CT to detect glucose isotope to visualize tumors in clinic [85]. It is known that HIF1-α is the key factor in reprogramming energy metabolism in tumors. HIF1-α can activate glycolysis and repress TCA cycle by inducing PDK1 [86]. Hypoxia can increase the level of p53 and therefore select for cells with mutant p53 [71]. It has been shown that p53 can inhibit glycolysis not only by directly degrading HIF-1α [71] but also by activating TIGAR that degrades fructose-2,6-bisphosphate thus blocking glycolysis [87]. In addition, p53 enhances oxidative phosphorylation by activating SCO2 which is essential for the COX2 biogenesis and maintains aerobic ATP production [88].

2.8 IMMUNE SURVEILLANCE

Recently, it has been reported that p53 signaling can be activated by interferon signaling. In turn, p53 could directly activate expression of immune-response genes including CC-chemokine ligand 2, IFN regulatory factor 5, IRF9, protein-kinase RNA-activated, toll-like receptor 3 and many others (reviewed in [89]). Moreover, mutant p53 expression is correlated with high expression of PD-L1 which binds to its receptor PD-1 on T cells, thus inhibiting T cell receptor (TCR) signaling and T cell proliferation [90,91]. It has been discovered that wild type p53 can downregulate the expression of PD-L1 through miR-34 which binds to 3' UTR of PD-L1 [91]. Interestingly, restoration of wild type p53 by small molecules can activate both innate and adaptive immunity and enhance immune response in combination with anti-CTLA-4 or anti-PD1/PDL-1 therapy [92].

2.9 GENOME STABILITY

p53 is also known as the guardian of genome. It maintains genome stability through regulation of DNA repair, or elimination of cells with damaged DNA via apoptosis, cell cycle arrest and senescence. Inactivation of p53 either through MDM2-mediated degradation or hot spot mutation leads to genome instability (reviewed in [93–95]). In addition, MDM2 could promote genome instability independently of p53 [96,97]. Recently it has been found that p53 cooperates with PCNA to ensure proper progression of DNA replication [98,99]. p53 is essential to prevent transcription-replication collision and topological stress, which further supports its role in maintaining genome stability [100].

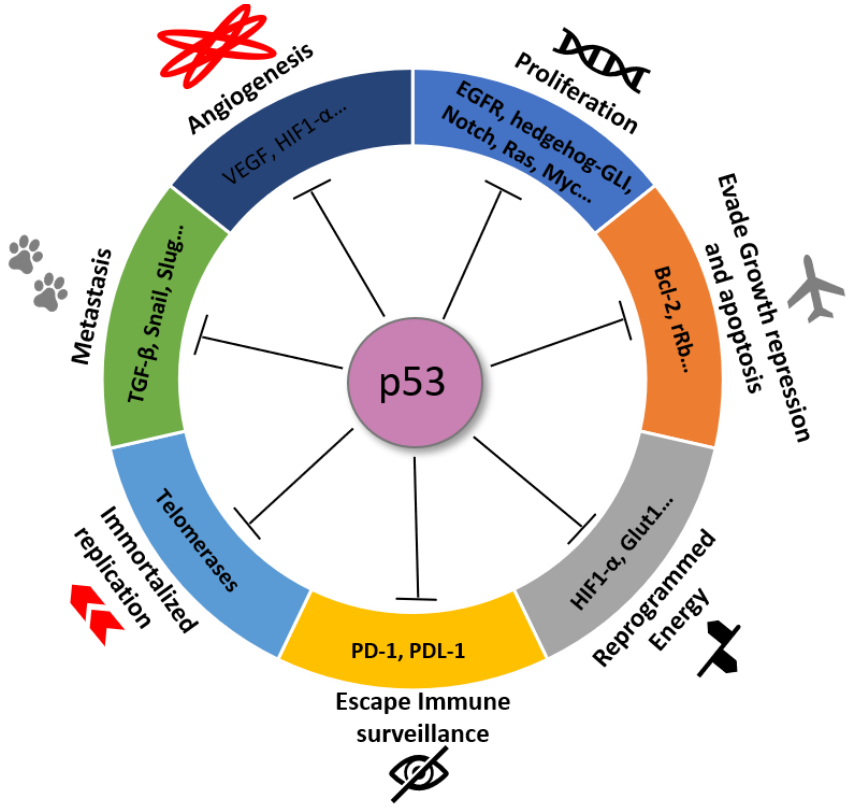

Figure 3. Schematic view of p53 and cancer hallmarks. While cancer acquired the ability to promote angiogenesis, proliferation, metastasis, immortalization and energy reprogramming, cancer can also escape immune surveillance, growth repression and apoptosis. p53 is found to repress all these cancer hallmarks directly or indirectly inhibiting genes that are critical for each process.

3 PHARMACOLOGICAL TARGETING OF P53

3.1 SMALL MOLECULES TARGETING MDM2/MDMX

Currently, a number of compounds have been invented to reactivate wild type p53 by targeting the major p53 negative regulators, MDM2 and MDMX. MDMX does not degrade p53 directly but enhances MDM2's activity via physical binding (reviewed in [101]). The crystal structure of MDM2 in complex with p53 peptide indicates three amino acid residues of p53: Phe19, Trp23 and Leu26, that are essential for MDM2 binding. Most of the MDM2 inhibitors are designed to prevent the interaction between MDM2 and p53 by mimicking these residues of p53 (reviewed in [102] , see Table 2 for some examples). Nutlin-3 is the first small molecule discovered to disrupt p53-MDM2 interaction. It has been reported that nutlin-3 can induce p53-dependent apoptosis or cell cycle arrest in cancer cells [103,104].

Induction of apoptosis or senescence by p53 depends on the balance of pro- and anti-apoptotic factors. Apoptosis is regulated by pro-apoptotic proteins Puma, Noxa and anti-apoptotic proteins such as Bcl-x_L, Bcl-2, Mcl-1. When pro-apoptotic proteins gain the ground, the activation of p53 tends to lead to apoptotic effect. Some studies suggest that Nultin-3 can

induce cell death independent of p53 transcriptional activity. They found that Nutlin-3 induces a direct translocation of p53 protein from nucleus to mitochondria, where p53 can directly activate pro-apoptotic Bcl-2 family members and induce apoptosis [105]. Currently, several advanced Nultin-3-like MDM2 inhibitors are in Phase 1 or 2 clinical trials, including RG7112, MI-773 and DS-3032b. Noteworthy, cell-penetrating stapled peptides that inhibit both MDM2 and MDMX have been developed recently, which are highly effective, able to enter every cell in a variety of cell lines and activate p53. In comparison to Nutlin-3, these stapled peptides are more stable, more permeable, more specific and have higher affinity [106]. In addition, a more efficacious MDM2 inhibitor has been developed based on the proteolysis targeting chimera (PROTAC) concept which is called PROTAC MDM2 degrader. This molecule consists of a ligand to MDM2 inhibitor (Nutlin-3), a ligand to the E3 ubiquitin ligase and a linker connecting the two ligands. This molecule not only stabilizes p53 but also targets MDM2 to degradation and effectively induce tumor regression *in vitro* and *in vivo* experiments, providing a new therapeutic strategy for the treatment of human cancers [107,108]. Other compounds such as HLI98, MEL23/24 designed to inhibit the E3 ubiquitin ligase activity of MDM2 have been shown to induce p53-dependent transcription and apoptosis, while other p53-independent effects were also observed [109,110].

3.2 SMALL MOLECULES REACTIVATING MUTANT P53

There are also compounds which target mutant p53 and restore p53 activity. PRIMA-1^{MET} (also known as APR-246) which has successfully completed a Phase 1 clinical trial and is currently being tested in Phase II and III trials in combination of carboplatin in ovarian cancer patients (NCT02098343), in combination with 5-azacitidine in myeloid malignancies, myelodysplastic syndrome and chronic/acute myeloid leukemia (NCT04214860, NCT03745716, NCT03588078, NCT03072043). PRIMA-1^{MET} induces formation of covalent adducts in mutant $p53^{R175H}$ and $p53^{R273H}$ proteins and restore wild type p53 activity [111]. However, the exact mechanism has not been fully elaborated yet. PRIMA-1^{MET} can also reactivate p53 homologues such as TAp73a, TAp73b and TAp63g8 [112].

Compounds such as PK083, PK5174, PK5196, PK7088 and various benzothiazoles seem to be able to restore p53 activity in cell lines harboring the Y220C mutation, a hotspot mutation which has been found in nearly 75,000 new cancer cases per year (reviewed in [102]). Current compounds that reactivate wild type and mutant p53 and their application status are listed in Table 2 and shown in Figure 4.

With the development of cell-based screening, more and more p53 activators have been found but their mechanisms of action are not fully understood. RITA was reported to target wild type and mutant p53 and induce apoptosis in cancer cells without killing normal cells, which makes it a promising compound for cancer treatment [113]. However, accumulating data showed that RITA has other targets in addition to p53; it also induces apoptosis in a p53-independent way [114]. In order to stratify cancer patients, the mechanism of all the compounds which reactivate p53 should be evaluated carefully in pre-clinical models.

COMPOUNDS TARGETING MDM2	STATUS
Nutlin 3a	Pre-clinical stage
RG7112 (also known as RO5045337)	Phase I trial in solid tumors, soft tissue tumor and AML (completed)
RO550381	Phase I trial in advance malignancies and AML (recruiting)
MI-773 (also known as SAR405838)	Phase I trial in malignant neoplasms (recruiting)
DS-3032b	Phase I trial in advance solid tumor lymphoma (recruiting)
COMPOUNDS TARGETING MUTANTP53	**STATUS**
PRIMA-1^{MET} (also known as APR-246)	Phase III trial in MDS with mutant p53, Phase II trial in combination with azacitidine mutant p53 AML
PK083, PK5174, benzothiazoles	Pre-clinical study
Stictic acid (also known as NSC87511)	Pre-clinical study
NSC319726	Pre-clinical study

Table 2. Examples of compounds targeting MDM2 or mutant p53 and their status in clinical trials.

3.3 SMALL MOLECULES INHIBITING P53

Although it is generally accepted that reactivation of p53 is a promising strategy to treat cancer, it also has dark sides. p53 is a key pro-apoptotic factor and it has been reported that the side effects of cancer therapy are related to p53-mediated growth arrest or apoptosis in normal tissues in mice such as spleen, thymus, lymphoid, hematopoietic organs, intestine epithelium. These tissues are drug- and irradiation-sensitive due to p53 activation. Consistently, p53-deficient mice survive high dose of irradiation that is lethal to p53 wild type mice [115]. Moreover, it is believed that p53 could serve as a resistance factor in cancer cells which undergo cell cycle arrest or senescence and therefore might be protected from chemo- and irradiation- induced apoptosis (reviewed in [116]).

Hence, it seems reasonable to inhibit p53 to avoid cancer treatment side effects in normal tissues or enhance chemo- and irradiation sensitivity in certain types of cancers under certain conditions. In 1999, PFT-α was screened as a specific p53 inhibitor by using LacZ-encoding β-Gal p53-dependent reporter system [115]. Afterward, PFT-α has been shown to protect mouse neurons against death and enhance the recovery of subventricular zone of mice after brain stroke [117]. What's more, PFT-α inhibits doxorubicin-induced cardiac cell apoptosis in mouse hearts and is protective from cisplatin-induced hair loss and toxic effects in multiple organs in mice. Interestingly, in cells lacking apoptotic signaling, inhibition of p53 by PFT-α can alter cell status from cell cycle arrest to mitosis, which could sensitize these cells to chemo- and irradiation therapy (reviewed in [116]).

However, inhibition of p53 could result in survival of genetically altered cells which are tumorigenic in a long term. Although it was demonstrated that transient inhibition of p53 is not associated with cancer initiation in mouse models and is less dangerous than p53 deficiency, some experiments indicated that cells treated by chemotherapeutic drugs followed by PFT-α display a high rate of genome abnormality which can contribute to carcinogenesis in a long term (reviewed in [116]). Thus, the idea of whether PFT-α could be used to protect cancer patients from cancer therapy side effects is still controversial. More tests in different models need to be done pre-clinically.

In 2006, another small molecule called PFT-μ was isolated by Strom E. et al. to inhibit p53. To be specific, they found that PFT-μ can prevent p53 from binding to anti-apoptotic proteins Bcl-xL and Bcl-2, therefore protect primary mouse thymocytes from p53-induced apoptosis [118], and protect mice from lethal hematopoietic syndrome upon radiation. It was confirmed that PFT-μ can protect p53 wild type cancer cell lines RKO and ML-1 from Nutlin-3-induced apoptosis, indicating that PFT-μ is a specific inhibitor of mitochondrial p53 without affecting p53 transcriptional activity [105].

3.4 CHALLENGES IN TARGETING P53

One challenge for the application of MDM2 inhibitors is that the inhibition of MDM2 or MDMX by itself could also affect cellular activity, since more and more evidence indicate that MDM2 and MDMX are involved in regulation of gene expression, DNA repair, chromatin modification and mitochondrial dynamics independent of p53 (reviewed in [119]).

Although MDM2 is well-known proto-oncogene and could serve as a bio-marker and therapeutic target for cancer patients, potential side effects due to MDM2 inhibition because of the p53 activation in normal cells, especially long-term inhibition, should also be taken into consideration. MDM2 is also involved in other potent biological processes including metabolic activity, pro-inflammation and immune response (reviewed in [120]). Therefore, more studies focused on long term side effects of MDM2 inhibitors should be done in mice. Relevant information could also be obtained from clinical trials of MDM2 inhibitors.

Worth to note, it was found that MDM2 can degrade not only wild type but also mutant p53, which brings up another challenge. One study showed that MDM2 can degrade $p53^{175H}$ and $p53^{241F}$ (reviewed in [121]). Therefore, the application of MDM2 inhibitor, for example Nutlin-3 in cancer cells harboring mutant p53 will possibly have opposite outcome. Inhibition of MDM2 could result in accumulation of mutant p53. Once stabilized, with its gain-of-function, mutant p53 promotes tumorigenesis, genome instability, resistance to chemotherapy and metastasis. In mouse models, mutant p53 correlates with more invasiveness and metastasis compared to the absence of p53 (reviewed in [122]).

While conventional chemotherapy and irradiation can stabilize wild type p53, it could also stabilize mutant p53. It has been shown in lung tumor that mutant p53 can accumulate in response to irradiation, which is unwanted effect of cancer therapy. It has been also

demonstrated in DLD-1 cells, that topoisomerase inhibitor CPT can degrade MDM2 but increase mutant p53 [123,124]. Therefore, the identification of p53 status whether it is wild type or mutant is critical for therapy determination. However, it is practically difficult because immunohistochemistry cannot distinguish between wild type and mutant p53, unless there is a very high level of p53, indicating p53 mutation [125]. The detection of mutant p53 has been improved with the advancement of DNA and cDNA sequencing, which could be used for the identification of p53 mutations in clinic [126]. In some cancer cells, mutant p53 level is low but could be increased and promote tumorigenesis when MDM2 is inhibited. Therefore, mouse models with different types and status of p53 should be tested with MDM2 inhibitors, chemotherapies and irradiation to evaluate their anti-cancer and side effects.

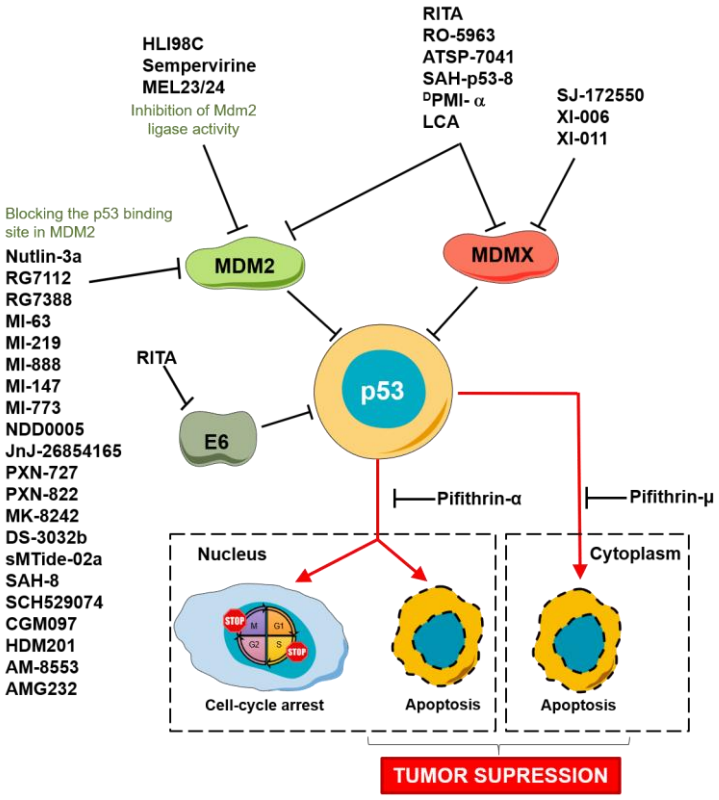

Figure 4. Summary of current small molecules targeting MDM2/MDMX to reactivate p53 by different strategies, including disrupting MDM2/p53 complex, inhibiting MDM2 E3 ligase activity, inducing MDM2/MDMX degradation; besides directly binding and reactivating p53, RITA also stabilizes p53 by inhibiting HPV E6 which degrades p53. Reactivated p53 induces cell cycle arrest or apoptosis by transcriptionally activating its target genes. Mitochondrial p53 also induces apoptosis by stabilizing Bax and Bak. Pifithrin-α is described to inhibit p53 transcriptional activity while pifithrin- μ is described to inhibit mitochondrial p53. Both have been shown to prevent p53 activity.

4 NOVEL APPROACHES TO IDENTIFY TARGETS AND MECHANISMS OF ACTION OF SMALL MOLECULES

4.1 TARGET IDENTIFICATION

From the clinical point of view, it is imperative to know the targets of drugs for better efficacy of treatment. Although a drug is originally designed to target one factor that is the major cause of a certain disease, it could have multiple targets and we should consider cell as a complexity instead of looking at a single target *in vitro*. Target identification techniques, whether it is machine learning algorithms or biological experiments, are usually based on principles of biophysics, biochemistry, genetics, chemical biology and others.

4.2 NOVEL APPROACHES

Biochemical methods are based on changes upon drug-protein physical interactions. In the last few years, a method called drug affinity responsive target stability (DARTS) has been developed, based on the principle that the protease resistance of the protein will be increased when it is bound by small molecules. This strategy is dependent on drug-protein interaction without requirement of drug modifications, which are needed, for example, for small molecule affinity chromatography. In addition, since DARTS does not require a washing step, it can be used to identify lower affinity binding of protein-drug interaction [127].

Another approach called chromatographic co-elution (TICC) to detect drug targets under physiological conditions *in vitro* is based on liquid chromatographic shift after drug-protein interaction. Other approaches such as metabolic labeling called stable-isotope labeling by amino acids in cell cultures (SILAC) and chemical labeling called isotope-coded affinity tag (ICAT) are also used in many studies.

Genetic method by using RNAi or CRISPR Cas9 KO technique can also be used to identify drug targets. For example: positive-selection screen based on cell viability can be used to confer drug resistance to identify drug targets; negative-selection screen is also based on cell viability, however, it is usually used to identify genes that can sensitize to a drug and the signal is low compared to positive-selection screen (reviewed in [128]). If the silencing of a gene has similar biological effect to a compound, it is suggesting that the protein is inhibited by this compound.

We can also use computational analysis to identify targets of new drugs by comparing them to documented profile of other compounds such as gene expression or growth suppression in different cancer cell lines (reviewed in [129]). It is recommended to combine several approaches to fully characterize any on-targets and off-targets effects to better understand the action of small molecules.

4.3 CELLULAR THERMAL SHIFT ASSAY AND THERMAL PROTEOME PROFILING

Another approach, cellular thermal shift assay (CETSA), is used based on the phenomenon that protein thermal stability will be changed after protein-drug interaction. After heating cells with or without drug treatment at several increasing temperatures, cells are lysed, then soluble and

precipitated proteins are separated by centrifugation. The soluble proteins will be detected by WB, while unfolded proteins precipitate. This allows to determine if the thermal stability of those potential target proteins is changed, thus suggesting target-drug interaction [130]. While DARTS is used to enrich target proteins that are resistant to protease and non-target proteins are digested after protease treatment, CESTA is used to identify drug-protein engagement indicated by ΔTm (melting temperature shift) (Figure 5). Since DARTS and CETSA are based on antibody readout, they are limited to a small number of proteins.

Both DARTS and CETSA can be combined with Mass spectrometry for large scale drug targets detection. Thermal proteome profiling (TPP) (for the pipeline, see Figure 6) is a proteome-wide CETSA with readout for around ~6000 to 10000 proteins. TPP has been used for unbiased identification of direct and indirect drug target proteins in several studies and has been optimized by combining several algorithms for analysis [131]. Besides drug targets investigation, TPP can also be used to study mechanism of drug action and metabolic pathways since it allows to study PTMs, protein-protein interactions and protein function upon overexpressing or knocking out a gene.

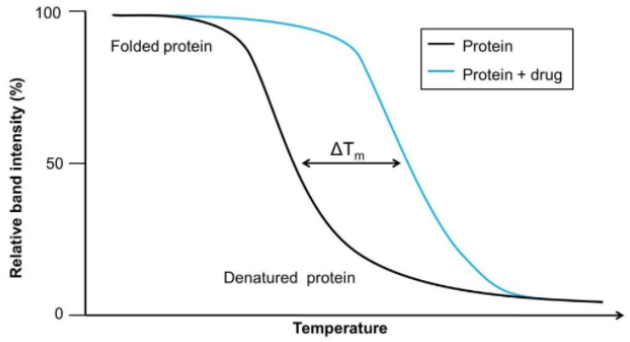

Figure 5. Melting curve from CETSA assay to study protein-drug interaction. Figure is from Sygnature Discovery: https://www.sygnaturediscovery.com/drug-discovery/bioscience/biophysical-assays/cellular-thermal-shift-assay-cetsa/

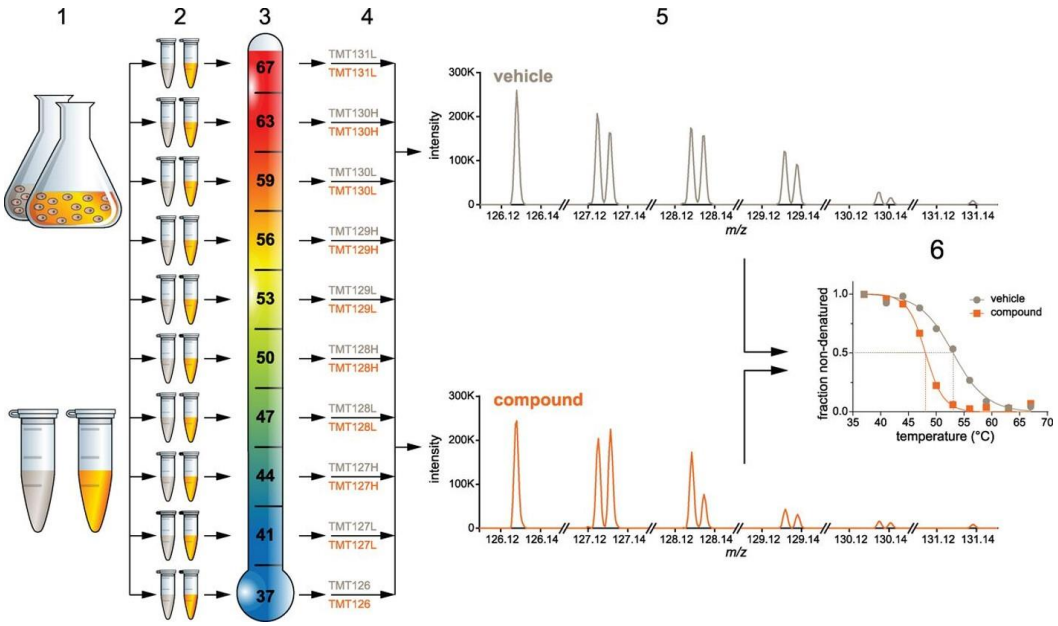

Figure 6. Pipeline of Thermal Proteome Profiling. (1) Cells are incubated with or without the compound of interest, then cell extracts will be obtained. Alternatively, compound can be added to the cell extracts. (2) Each sample will be divided into 10 aliquots. (3) Each aliquot will be heated at indicated temperature. (4) After digestion by trypsin, each sample will be labeled by different TMT10 isotope tag. (5) All samples will be mixed and analyzed by LC-MS/MS, the intensity of each labeled ion will be used to fit the melting curve. (6) The melting curve $_{Tm}$ of each protein will be separated by two conditions. *Mikhail M. Savitski et al. Science 2014;346:1255784.* Copyright © 2014, American Association for the Advancement of Science

4.4 TRANSLATOME ANALYSIS

Although transcriptome analysis with tools such as RNA extraction followed by cDNA microarray or RNA sequencing provides good indications about gene expression upon drug treatment for identifying drug targets, there is still discrepancy between mRNA level and actual protein level because of on-going variations of protein synthesis and protein degradation. Therefore, translatome analysis which can reveal mRNAs that are recruited by ribosomes for protein synthesis provides additional and important information for cell function study. Translation comprises of three steps: initiation, elongation and termination. Briefly, during initiation, mRNA will be recruited by eIFs to the ribosome complex, which recruits tRNA that contains complementary three nucleotides to the codon of mRNA. Each tRNA will be bound to a specific peptide, which usually starts with methionine corresponding to start codon AUG in eukaryotes; during elongation, polypeptide will be formed as tRNA with its specific peptide is recruited to mRNA, which leads to the shift of ribosomes on mRNA; finally, once the translation reaches to stop codons on mRNA, the polypeptide chain will be released. Herein, I will briefly describe three translatome analysis methods that are being used in my study.

Polysome profiling: Since the rate of initiation is a limiting factor of translation, a measurement of the association of cellular mRNA and ribosomes could reflect translation in general. First of all, cell extracts will be prepared in cycloheximide (CHX), used to inhibit translational elongation; translated mRNAs bound by polysomes will be separated by centrifugation through 10% to 50% density of sucrose gradient; the fraction of polysome-free RNA, small and large ribosomal subunits will be separated by monitoring the absorbance at 254 nm (A_{254}); isolated RNA from each fraction can be pooled for microarray or RNA-seq analysis.

Ribosomal profiling: Instead of using centrifugation, cell extracts will be treated with RNase I to digest the regions on mRNA that are not bound by ribosomes, the remaining ribosomes will be selected by sucrose cushion with a fragment size of approximately 30 nucleotides, protected RNA will be further used for RNA-seq analysis.

Ribosome-affinity purification: Genetically modified cells are first constructed to express affinity-tagged ribosomal proteins that are controlled by cell-specific promotor. After affinity selection, cells are collected and tagged ribosomal proteins are extracted for study. RNA isolated from captured ribosomal proteins can be measured by RNA-seq analysis.

Translatome analysis can be used to study the response to various stress signals such as hypoxia, infection, nucleotide starvation, DNA damage, inflammation, apoptosis and

endoplasmic reticulum stress. Besides, this analysis can be applied to study drug action or comparison of normal cells and cancer cells. However, since these translatome analysis methods are focused on the initiation of translation, post-translational process such as protein degradation should also be taken into consideration. Translatome analysis in combination with mass spectrometry is better for understanding the entire picture of cell function (reviewed in [132]).

5 AIMS OF THE THESIS

A number of compounds have been developed to reactivate p53 and some of them are already in clinical trials. The thesis aims to address p53-dependent and -independent functions of small molecules that target p53.

Specific aims:

1. To understand p53-independent anti-cancer mechanism of RITA along with other two intriguing compounds, aminoflavone and oncrasin-1 (**Paper I**)

2. To investigate the action of p53 inhibitor pifithrin-α on p53 as well as its function independent of p53 (**Paper II**)

3. To investigate the effect of RITA on mRNA translation (**Paper III**)

6 RESULTS AND DISCUSSION

The thesis is focusing on the studies of the p53-dependent and -independent mechanisms of p53 reactivating compounds and p53 inhibitor.

Paper I

Thermal proteome profiling identifies oxidative-dependent inhibition of major oncogenes transcription as new therapeutic mechanism for selective anticancer compounds

Sylvain Peuget, **Jiawei Zhu**, Gema Sanz Santos, Madhurendra Singh, Massimiliano Gaetani, Xinsong Chen, Yao Shi, Amir Ata Saei, Torkild Visnes, Mikael Lindström, Ali Rihani, Lidia Moyano-Galceran, Joseph Carlson, Elisabet Hjerpe, Ulrika Joneborg, Kaisa Lehti, Johan Hartman, Thomas Helleday, Roman Zubarev, and Galina Selivanova.

Analysis of the GI50 data of several thousand of compounds in NCI 60 cell lines allowed to identify sensitivity correlation profiles of Aminoflavone and Oncrasin-1 that are similar to RITA. Based on the analysis, we confirmed that these three compounds share a similar anti-cancer activity in a set of cancer cell lines. We found that the three compounds can efficiently kill MCF7, MCF7p53KO, U2OS, OVCAR3 and T47D cancer cell lines in a p53-independent but ROS-dependent manner, as co-treatment of antioxidants such as resveratrol and NDGA rescued their killing effect in either p53KO cells or p53 mutant cells. Our q-PCR and Western blot (WB) results both showed that pro-apoptotic genes such as Puma, Noxa are greatly activated while pro-survival genes, also known as oncogenes, such as MDM2, MDMX, Mcl-1, PPM1D, are inhibited by these three compounds. We confirmed that this phenomenon is also mediated by ROS induction and is independent of p53. WB results also showed that DNA damage and apoptotic signatures, such as γH2AX and PARP cleavage, are induced by these three compounds in a ROS-dependent manner and p53- independent manner. In addition, 8-oxoguanine is induced by three compounds, indicating oxidative DNA damage, as shown by immunofluorescence.

To investigate what is the common pathway that is affected by these compounds, first we performed Thermal proteome profiling (TPP) to identify what are the common targets of these three compounds. We identified 90 common targets between 2 compounds and 8 common targets among all three compounds. After clustering the pathways of these common targets by Gene Ontology analysis, we found RNA processing to be affected by all three compounds. To investigate the effect on RNA processing, we determined the rate of EU incorporation by immunofluorescence and the level of RNA polymerases by WB. Not surprisingly, all three compounds efficiently inhibited transcription machinery of cancer cells. EU incorporation is inhibited and RNA polymerase II and its Ser2-phosphorylated form are inhibited due to proteasome degradation.

It was previously found that RITA can cause DNA replication stalling. Therefore, to understand if the transcription inhibition was due to DNA replication stalling, we performed cell synchronization experiment, using double thymidine block to arrest the cells in G1/S phase and lovastatin treatment to arrest the cells in G1 phase. We found that transcription is inhibited by three compounds in G1 phase, thus indicating that inhibition of transcription is causing DNA replication stalling, but not vice versa. Consistently, the compounds known to inhibit RNA Pol II-mediated transcription, such as CDK12/13 inhibitor THZ531 and actinomycin D, also share microarray profiles similar to these three compounds, including oncogene repression. This we further confirmed by WB. We found that the global transcription inhibition by these compounds is through ROS induction as ROS inhibitor resveratrol partially rescued the inhibition of RNA polymerase II (Pol II) complex by three compounds, as detected by WB.

Not only oncogenes were repressed, but also genes that are involved in homologous recombination (HR), such as RNF168, RNF8 and Rad51, are also downregulated by the three compounds. Our HR reporter assay confirmed the impairment of HR upon these treatments. It is known that cancer cell survival relies on major DNA repair pathways, such HR, non-homologous end joining (NHEJ), single strand break repair (SSBR), and others. Breast and ovarian cancers with BRCA1/2 mutations, which confer a defect in HR, prefer SSBR regulated by ADP-ribose polymerase (PARP) to repair DNA. Several PARP inhibitors have been approved in clinic for the treatment of triple negative breast cancer patients with BRCA1/2 mutations. Therefore, since our three compounds inhibit HR, we hypothesize that their combination with PARP1 inhibitors will have better anti-cancer effect. Indeed, we observed strong synergistic effect in our cancer lines as well as in primary ovarian and breast cancer patient samples.

In conclusion, RITA, Aminoflavine and Oncrasin-1 increase intracellular ROS levels, leading to global transcription inhibition in a p53-independent manner. As the consequence of transcription inhibition, early expressed oncogenes are repressed, this leads to cell growth suppression. Further, genes that are involved in DNA repair, especially HR, are also inhibited, so that cancer cells become more vulnerable to treatment of PARP1 inhibitors (the model is presented in Figure 7). Our study also shows that TPP approach could be used to identify the mechanism of action of small molecules, which is important for drug development.

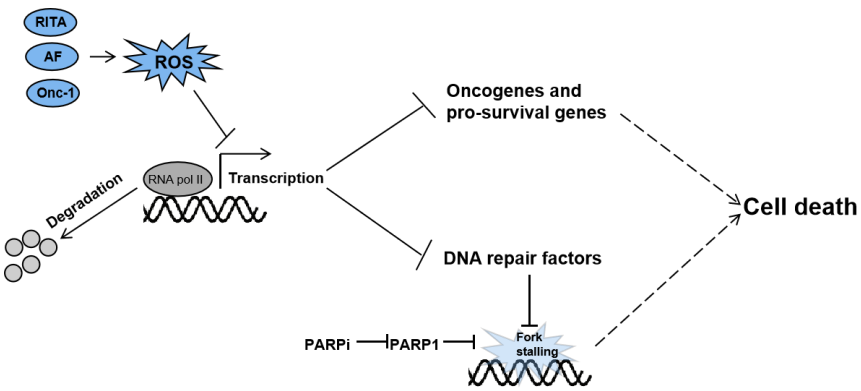

Figure 7. RITA, AF and Onc-1 inhibit transcription machinery, induce replication stress, and downregulate oncogenes through ROS accumulation, which leads to robust cancer cell death. Since RITA, AF and Onc-1 repress HR factors such as Rad51, RNF8, RNF168, addition of PARP inhibitors could lead to synergistic cell death effect in cancer cells with functional HR machinery.

Paper II

Pifithrin-α alters p53 post-translational modification pattern and differentially inhibits p53 target genes

Jiawei Zhu, Madhurendra Singh, Galina Selivanova and Sylvain Peuget.

To investigate PFT-α inhibition efficiency on p53, we treated MCF7 and A375 cells with Nutlin-3 to induce cell cycle arrest or apoptosis specifically through p53 activation. We found that PFT-α cannot prevent cancer cell growth suppression upon p53 activation. The same result was observed in immortalized fibroblasts. However, in primary fibroblasts, PFT-α by itself could enhance the growth which compensates for Nutlin-3-induced growth suppression. We also tested another p53 inhibitor PFT-μ, which can prevent the action of p53 in mitochondria, but no rescue of growth suppression was observed with PFT-μ in MCF7 and A375 cells as well.

It was originally described that PFT-α can inhibit p53 transcription activity by preventing p53 DNA binding ability. We selected a panel of p53 target genes and validated them by comparing MCF7 p53WT and MCF7 p53KO upon Nutlin-3 treatment. Our qPCR results showed that the addition of PFT-α, especially with 12h pre-treatment displays differential inhibition of p53 target genes. To be specific, *BBC3* (PUMA), *PIG3, TP53INP1*, *RRM2B, PPM1D* (WIP1), *SESN1* and *TIGAR* induction upon Nutlin-3 was moderately inhibited (decreased by 35% to 50%) by PFT-α, while the effect on the transcription of *ZMAT3* and *CDKN1A* (p21) was limited (induction decreased by only 23% and 25%, respectively). Moreover, no significant transcriptional inhibition was observed for *MDM2* and *DRAM*. We also confirmed by WB that MDM2 and p21 were not inhibited at protein level by PFT-α upon Nutlin-3-induced p53 stabilization.

To understand how PFT-α differentially inhibit p53 target genes, we performed WB to check the effects of PFT-α on total p53 level and phosphorylated Ser33 of p53 upon Nutlin-3 treatment. We found total p53 protein level is not changed upon PFT-α, but we observed that phosphorylation of p53 on Ser33 upon Nutin-3 treatment was significantly inhibited by PFT-α. Moreover, we treated MCF7 with doxorubicin to induce phosphorylation of p53 on residues Ser15 and Ser37 and PFT-α efficiently inhibited both Ser33 and Ser15 phosphorylation, but not Ser37 activated upon doxorubicin. We also observed robust inhibition of Ser33 and Ser15 phosphorylation of p53 in A375 and HCT116 cell lines. It indicates that PFT-α can affect specific p53 PTMs, which could change the conformation of p53 resulting in different expression of its targets. To investigate the mechanism of how PFT-α alters PTMs of p53, we

hypothesized that PFT-α could decrease ROS to weaken phosphorylation of p53 because ROS is one of the main regulators of PTMs, especially phosphorylation. We performed DCFD-A staining and checked intracellular ROS level upon PFT-α with flow cytometry. In both MCF7 and MCF7 p53KO cell lines, we found that PFT-α can decrease both basal ROS level and prevent ROS formation upon doxorubicin by activating AHR/NRF2 pathway independent of p53. PFT-α exerts antioxidant effect in mutant p53-expressing T47D and A375 p53KO cells with activation of NRF2 pathway but not in H1299 with low expression of AHR. We investigated whether PFT-α antioxidant activity leads to attenuation of p53 PTMs. In MCF7 cells treated with Nutlin-3, addition of another antioxidant, NAC, had no effects on Ser33 phosphorylation of p53, while PFT-α strongly inhibited it. Therefore, we could not link the antioxidant effect of PFT-α to its inhibition p53 PTMs.

In conclusion, the efficiency of PFT-α as specific p53 inhibitor appears to be highly questionable and context-dependent. In our models, it did not inhibit p53-dependent cell cycle arrest and apoptosis and had a target gene- and model- dependent effect on p53 transcription, probably due to indirect inhibition of p53 PTMs, such as inhibition of kinases including DYRK2, CHK1, CHK2, CK2, HIPK2, JNK, LRRK2, p38, PKCδ, and PLK3 that phosphorylate p53. In addition, PFT-α has notable p53-independent effects, such as strong induction of the AHR pathway which results in decrease of ROS levels. AHR is a potent transcription factor that can regulate a series of signaling pathways. Therefore, it is possible that PFT-α can affect p53 activity by activating AHR. Moreover, its differential properties in normal primary cells and cancer cells may have interesting clinical potential in combination with p53-reactivating compounds. However, it has been shown that antioxidants can promote tumor growth and metastasis if the tumor is already formed. This is because cancer cells are under high metabolism and ROS stress, antioxidants can decrease the stress in cancer cells. Therefore, using PFT-α to treat cancer patient might lead to opposite effects. The effect of PFT-α on cancer should be further evaluated in animal models.

Paper III

RITA requires eIF2α-dependent modulation of mRNA translation for its anti-cancer activity

Johannes Ristau, Vincent van Hoef, Sylvain Peuget, **Jiawei Zhu**, Bo-Jhih Guan, Shuo Liang, Maria Hatzoglou, Ivan Topisirovic, and Galina Selivanova & Ola Larsson.

To investigate the effect of RITA on mRNA translation, we treated MCF7 cells with RITA for 8h and performed polysome profiling to check the rate of engagement of mRNA and ribosomes. We found that RITA reduced actively translated mRNA (polysome fraction) with increase of 80S monosomes. Since we previously found that RITA efficiently kills cancer cells independent of p53, we speculated that RITA inhibits translation also regardless of p53. Indeed, polysome profiling analysis demonstrated that RITA inhibits mRNA translation in both MCF7 and MCF7 p53KO cells. Consistently, incorporation of S-methionine and S-cysteine were

reduced upon RITA treatment. It was reported that RITA induce apoptosis through ROS accumulation. We wanted to know if RITA inhibits translation because of ROS induction, since it was found that ROS inhibits protein synthesis. Although we found that NAC completely rescued ROS induction upon RITA treatment, as shown by CellROX Deep Red dye staining, PARP cleavage and translation inhibition persisted in the presence of NAC.

Next, we investigated if mTOR/4E-BP is mediating RITA-induced translation inhibition. 4E-BP1 knock down by shRNA could not rescue RITA-induced translation inhibition assessed by polysome tracing. In addition, mTOR inhibitor Torin1 could not affect mTOR-mediated phosphorylation of downstream targets S6K and 4E-BP1. Thus, RITA inhibits translation independent of ROS and mTOR/4E-BP axis.

Since phosphorylation of eIF2α decreases translation initiation, we checked if RITA inhibits translation through eIF2α phosphorylation. We found that RITA significantly induced eIF2α phosphorylation in MCF7, HCT116 and GP5d cells. Addition of integrated stress response inhibitor ISRIB partially rescued RITA-induced translation inhibition indicated by decrease of 80S monosomes peak with polysome tracing assay, as well as increased incorporation of S-methionine and S-cysteine. Because PERK can phosphorylate eIF2α, we wanted to investigate if RITA induces phosphorylation of eIF2α and translation inhibition through PERK activation. Indeed, PERK inhibitor GSK2606414 reduced eIF2α phosphorylation, translation inhibition, PARP cleavage and apoptosis upon RITA treatment. Moreover, we found that salubrinal, which inhibits eIF2α phosphatases and enhances eIF2α phosphorylation, enhanced apoptosis effect of RITA in both wild type and p53KO cells, while GSK2606414 had the opposite effect. Of note, we found that p53 activation and apoptosis effect upon RITA is also dependent on eIF2α phosphorylation by comparing HT1080 wild type and HT1080 KI with non-phosphorylatable eIF2α mutant.

We found that RITA strongly downregulates MDM2 and investigated the mechanism of how RITA inhibits MDM2 on its protein level. We hypothesized that the inhibition could be through proteasome, autophagy or microRNA-mediated translation inhibition. Therefore, we implemented proteasome inhibitor MG132, autophagy inhibitor chloroquine and DICER knock out (preventing microRNA-mediated inhibition of translation) to verify the mechanism. However, none of them rescued RITA-induced MDM2 inhibition (Figure 8). We addressed the question if the inhibition of MDM2 has impact on the effects of RITA. We manipulated MDM2 level by overexpressing or knocking out with CRISPR-Cas9 to see if these can affect RITA-induced apoptosis. We found that RITA can still efficiently downregulate survival genes and induce DNA damage signalling in both MDM2 overexpressing and knock out cell lines (Figure 9), suggesting that MDM2 downregulation does not play a key role in this model. Although these data were not included in the final paper, they served for the development of the hypothesis and further supported the mechanism that this paper described.

In conclusion, we found that the stress sensing eIF2α pathway is critical for RITA-induced mRNA translation inhibition and cell apoptosis. By using translatome analysis methods such

as polysome profiling, polysome tracing and S-methionine and S-cysteine incorporation, we could unravel the mechanism of action of small molecules, which is important for stratifying cancer patients.

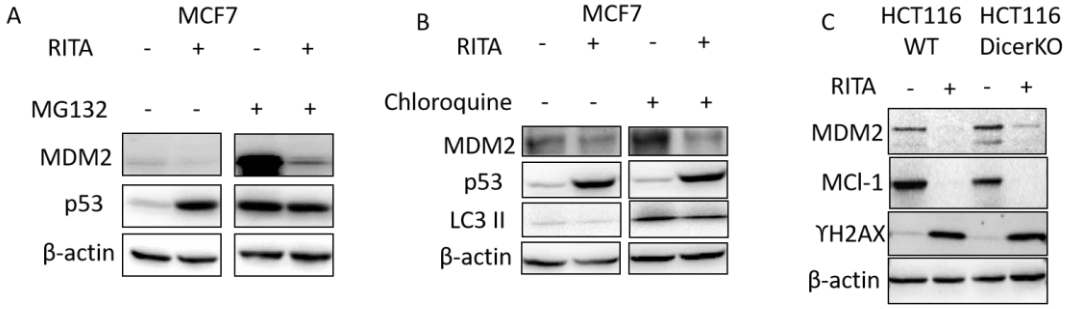

Figure 8. Investigation of the mechanism of MDM2 downregulation upon RITA. A) In MCF7 cells, MDM2 protein level is still greatly inhibited by RITA in the presence of proteasome inhibitor MG132 which restored basal protein level of p53. B) In MCF7 cells, autophagy is inhibited by using Chloroquine, as judged by LC3II upregulation. MDM2 downregulation was not rescued by inhibition of autophagy and p53 level was not affected. C) MDM2, Mcl-1 are inhibited, DNA damage persists upon RITA treatment in HCT116 DicerKO cells, in which microRNA-mediated mRNA translation inhibition is attenuated.

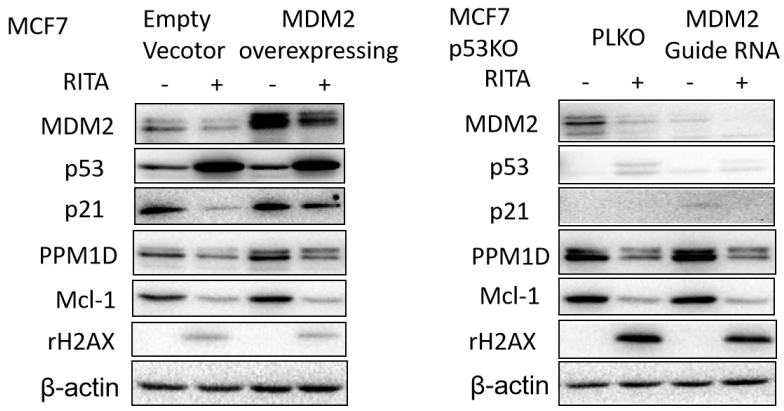

Figure 9. Investigation of the impact of MDM2 inhibition for RITA-induced cell killing effect. MDM2 is overexpressed in MCF7 cells, MDM2 KO is established in MCF7 p53KO cells to avoid p53-mediated cell death. Result demonstrates that RITA inhibits pro-survival genes such as p21, PPM1D and Mcl-1, and DNA damage persists upon RITA in either MDM2 overexpressing or MDM2 KO cell lines.

7 CONCLUDING REMARKS

All three papers are focused on addressing the molecular mechanisms of small molecules. Originally, each of them was designed to target a specific protein. Nevertheless, it is also necessary to identify any other targets (since they will most likely be) and depict the action of a compound targeting several proteins instead of a one drug-protein connection. The small compounds studied in this thesis are all designed to target p53, the well-known tumor suppressor, which has been studied for four decades. However, there are still no p53 targeting drugs that have officially entered clinic to treat patients. Challenges remain for the development of p53 targeting drugs and one of them is the off-target effects of drugs, which is a big concern for researchers and clinicians. Thus, identification of drug targets appears to be important for providing more comprehensive information for drug application.

In our first study, by performing high throughput approaches such as TPP assay, we managed to identify that transcription machinery is affected by RITA, Aminoflavone and Oncrasin-1 in a common p53-independent way, which has never been shown before in the study of these three drugs. TPP assay could provide hundreds of hits. Therefore, it is hard to identify what are the main targets of a compound. This is because TPP assay provides only physical compound-protein/protein complexes interaction information instead of biological response information. It is practically difficult to verify each single target in biological models. Therefore, we used Gene Ontology analysis to cluster the common pathways that are affected by all three compounds and found most of the common hits that are involved in RNA processing. Biological experiments not only further confirmed our findings but also provided more detailed information. Inspired by our finding of global transcription inhibition by the compounds, we found that these three compounds can repress genes such as RNF8, RNF168 and Rad51 which are key HR factors. It is generally accepted that combination treatments are less toxic and are often more effective than monotherapy. Therefore, we combined PARP inhibitors which inhibit SSBR with RITA, Aminoflavone and Oncrasin-1 *in vivo* and *ex vivo* and observed strong synergistic effect. Thus, our study shows that TPP assay followed by Gene Ontology analysis is a powerful tool to investigate the molecular mechanism of drug action. In addition, it is easier to find the main targets studying in parallel several drugs displaying similar biological effects.

While the first study is focused on the effect of transcription machinery, our next study addresses the action of mechanism of RITA at translational level. We performed translatome analysis using polysome profiling which provides information regarding the rate of mRNA translation by checking mRNA ribosome engagement. We found that RITA induces dramatic translation inhibition through phosphorylation of eIF2α in a p53-independent way. These two studies helped us to have a more comprehensive view on RITA action since we looked into both transcription and translation which are the basic mechanisms providing factors that regulate various cellular processes. In a future, it would be interesting to test whether the effect

of RITA on these two basic cellular processes are interdependent and whether the same or different targets are involved.

In the third study, we sought to understand how PFT-α inhibits p53 action and investigate any other targets except p53. We found that PFT-α can significantly inhibit phosphorylation of p53 on Ser33 and Ser15 which is induced by Nutlin-3 or doxorubicin treatment. Correspondingly, PFT-α also alters p53 transcription activity as indicated by differential inhibition of p53 target genes upon Nutlin-3 treatment. Nevertheless, we do not have straightforward evidence to prove that inhibition of p53 PTMs leads to the differential inhibition of p53 targets in our model. To provide evidence for this, we need to introduce mutation in p53 on either Ser33 or Ser15 or both and test p53's transcriptional activity. We found that PFT-α activates AHR/NRF2 pathway to exert antioxidant effect. However, its antioxidant effect is not the cause of inhibition of p53 phosphorylation. Surprisingly, PFT-α fails to rescue p53 activation-induced cell growth suppression in cancer cell lines and immortalized fibroblasts. Notably, it induces primary fibroblasts cell growth and rescue Nutlin-3-induced growth suppression. We think that this is related to its antioxidant effect, as NAC also induces cell growth in primary fibroblasts. Therefore, due to the differential effects of PFT-α on normal *versus* cancer cells, we think it might be a good strategy to combine PFT-α and p53 reactivating compounds to treat cancer to protect normal tissues from side effects of therapy as it has been suggested in previous studies which showed significant protective effect of PFT-α in normal mouse tissues upon chemo and irradiation therapy. We can foresee that the inhibition of p53 is a controversial idea, because PFT-α might be an effective p53 inhibitor in some tissues and inhibition of wild type p53 might lead to genome instability and carcinogenesis. In addition, it has been shown that antioxidants can promote cancer metastasis. Future studies are needed to investigate the above mentioned aspects and to establish whether the strategy of p53 inhibition could be applied in clinic.

We believe that our study provided an important message to researchers who work with p53 and use PFT-α to investigate p53-mediated cellular processes, that PFT-α is not a pan-p53 inhibitor as it is described. In the future it would be very interesting to find out which are the targets of PFT-α, using high-throughput approaches such as CRISPR-Cas9 screens or those described in this thesis.

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9 REFERENCES

1. Linzer DIH, Levine AJ. Characterization of a 54K Dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*. 1979;17(1):43-52. doi:10.1016/0092-8674(79)90293-9

2. Kress M, May E, Cassingena R, May P. Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. *J Virol*. 1979;31(2):472-483.

3. Chang C, Simmons DT, Martin MA, Mora PT. Identification and partial characterization of new antigens from simian virus 40-transformed mouse cells. *J Virol*. 1979;31(2):463-471.

4. Jenkins JR, Rudge K, Currie GA. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. *Nature*. 1984;312(5995):651-654. doi:10.1038/312651a0

5. Chen PL, Chen YM, Bookstein R, Lee WH. Genetic mechanisms of tumor suppression by the human p53 gene. *Science*. 1990;250(4987):1576-1580. doi:10.1126/science.2274789

6. Levine AJ, Oren M. The first 30 years of p53: growing ever more complex. *Nat Rev Cancer*. 2009;9(10):749-758. doi:10.1038/nrc2723

7. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature*. 1997;387(6630):296-299. doi:10.1038/387296a0

8. Schroeder M, Mass MJ. CpG Methylation Inactivates the Transcriptional Activity of the Promoter of the Humanp53Tumor Suppressor Gene. *Biochemical and Biophysical Research Communications*. 1997;235(2):403-406. doi:10.1006/bbrc.1997.6796

9. Mishra A, Brat DJ, Verma M. P53 Tumor Suppression Network in Cancer Epigenetics. *Cancer Epigenetics*. 2015:597-605. doi:10.1007/978-1-4939-1804-1_31

10. Soto-Reyes E, Recillas-Targa F. Epigenetic regulation of the human p53 gene promoter by the CTCF transcription factor in transformed cell lines. *Oncogene*. 2010;29(15):2217-2227. doi:10.1038/onc.2009.509

11. Meek DW, Anderson CW. Posttranslational Modification of p53: Cooperative Integrators of Function. *Cold Spring Harb Perspect Biol*. 2009;1(6):a000950. doi:10.1101/cshperspect.a000950

12. Murray-Zmijewski F, Slee EA, Lu X. A complex barcode underlies the heterogeneous response of p53 to stress. *Nature Reviews Molecular Cell Biology*. 2008;9(9):702-712. doi:10.1038/nrm2451

13. Feng L, Hollstein M, Xu Y. Ser46 phosphorylation regulates p53-dependent apoptosis and replicative senescence. *Cell Cycle*. 2006;5(23):2812-2819. doi:10.4161/cc.5.23.3526

14. West LE, Gozani O. Regulation of p53 function by lysine methylation. *Epigenomics*. 2011;3(3):361-369. doi:10.2217/EPI.11.21

15. Moll UM, Petrenko O. The MDM2-p53 Interaction. *Mol Cancer Res*. 2003;1(14):1001-1008.

16. Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. *Oncogene*. 1999;18(53):7644-7655. doi:10.1038/sj.onc.1203015

17. Carr MI, Jones SN. Regulation of the Mdm2-p53 signaling axis in the DNA damage response and tumorigenesis. *Transl Cancer Res*. 2016;5(6):707-724. doi:10.21037/tcr.2016.11.75

18. Shinozaki T, Nota A, Taya Y, Okamoto K. Functional role of Mdm2 phosphorylation by ATR in attenuation of p53 nuclear export. *Oncogene*. 2003;22(55):8870-8880. doi:10.1038/sj.onc.1207176

19. Callero MA, Loaiza-Pérez AI. The role of aryl hydrocarbon receptor and crosstalk with estrogen receptor in response of breast cancer cells to the novel antitumor agents benzothiazoles and aminoflavone. *Int J Breast Cancer*. 2011;2011:923250. doi:10.4061/2011/923250

20. Terzuoli E, Puppo M, Rapisarda A, et al. Aminoflavone, a ligand of the aryl hydrocarbon receptor, inhibits HIF-1alpha expression in an AhR-independent fashion. *Cancer Res*. 2010;70(17):6837-6848. doi:10.1158/0008-5472.CAN-10-1075

21. Callero MA, Rodriguez CE, Sólimo A, Joffé EB de K, Pérez AL. Abstract 4983: Aminoflavone acts as an immunomodulator of tumor growth in a breast cancer mouse model. *Cancer Res*. 2016;76(14 Supplement):4983-4983. doi:10.1158/1538-7445.AM2016-4983

22. Guo W, Wu S, Liu J, Fang B. Identification of a small molecule with synthetic lethality for K-Ras and protein kinase Ciota (PKCι). *Cancer Res*. 2008;68(18):7403-7408. doi:10.1158/0008-5472.CAN-08-1449

23. Unger T, Mietz JA, Scheffner M, Yee CL, Howley PM. Functional domains of wild-type and mutant p53 proteins involved in transcriptional regulation, transdominant inhibition, and transformation suppression. *Mol Cell Biol*. 1993;13(9):5186-5194.

24. Fuchs SY, Adler V, Buschmann T, Wu X, Ronai Z. Mdm2 association with p53 targets its ubiquitination. *Oncogene*. 1998;17(19):2543-2547. doi:10.1038/sj.onc.1202200

25. Poyurovsky MV, Katz C, Laptenko O, et al. The C terminus of p53 binds the N-terminal domain of MDM2. *Nat Struct Mol Biol*. 2010;17(8):982-989. doi:10.1038/nsmb.1872

26. Kawai H, Wiederschain D, Yuan Z-M. Critical contribution of the MDM2 acidic domain to p53 ubiquitination. *Mol Cell Biol*. 2003;23(14):4939-4947. doi:10.1128/mcb.23.14.4939-4947.2003

27. Geyer RK, Yu ZK, Maki CG. The MDM2 RING-finger domain is required to promote p53 nuclear export. *Nat Cell Biol*. 2000;2(9):569-573. doi:10.1038/35023507

28. Linke K, Mace PD, Smith CA, Vaux DL, Silke J, Day CL. Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their

ubiquitylation in trans. *Cell Death Differ*. 2008;15(5):841-848. doi:10.1038/sj.cdd.4402309

29. Wang Y, Suh Y-A, Fuller MY, et al. Restoring expression of wild-type p53 suppresses tumor growth but does not cause tumor regression in mice with a p53 missense mutation. *J Clin Invest*. 2011;121(3):893-904. doi:10.1172/JCI44504

30. Dittmer D, Pati S, Zambetti G, et al. Gain of function mutations in p53. *Nat Genet*. 1993;4(1):42-46. doi:10.1038/ng0593-42

31. Petitjean A, Mathe E, Kato S, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Human Mutation*. 2007;28(6):622-629. doi:10.1002/humu.20495

32. Chen JM, Rosal R, Smith S, Pincus MR, Brandt-Rauf PW. Common conformational effects of p53 mutations. *J Protein Chem*. 2001;20(2):101-105. doi:10.1023/a:1011065022283

33. Termén S, Tan E-J, Heldin C-H, Moustakas A. p53 regulates epithelial–mesenchymal transition induced by transforming growth factor β. *Journal of Cellular Physiology*. 2013;228(4):801-813. doi:10.1002/jcp.24229

34. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61(5):759-767. doi:10.1016/0092-8674(90)90186-i

35. Rivlin N, Brosh R, Oren M, Rotter V. Mutations in the p53 Tumor Suppressor Gene. *Genes Cancer*. 2011;2(4):466-474. doi:10.1177/1947601911408889

36. Wynford-Thomas D, Blaydes J. The influence of cell context on the selection pressure for p53 mutation in human cancer. *Carcinogenesis*. 1998;19(1):29-36. doi:10.1093/carcin/19.1.29

37. Garritano S, Inga A, Gemignani F, Landi S. More targets, more pathways and more clues for mutant p53. *Oncogenesis*. 2013;2(7):e54. doi:10.1038/oncsis.2013.15

38. Boysen M, Kityk R, Mayer MP. Hsp70- and Hsp90-Mediated Regulation of the Conformation of p53 DNA Binding Domain and p53 Cancer Variants. *Mol Cell*. 2019;74(4):831-843.e4. doi:10.1016/j.molcel.2019.03.032

39. Robles AI, Harris CC. Clinical Outcomes and Correlates of TP53 Mutations and Cancer. *Cold Spring Harbor Perspectives in Biology*. 2010;2(3). doi:10.1101/cshperspect.a001016

40. McBride KA, Ballinger ML, Killick E, et al. Li-Fraumeni syndrome: cancer risk assessment and clinical management. *Nat Rev Clin Oncol*. 2014;11(5):260-271. doi:10.1038/nrclinonc.2014.41

41. Gonzalez KD, Noltner KA, Buzin CH, et al. Beyond Li Fraumeni Syndrome: Clinical Characteristics of Families With p53 Germline Mutations. *JCO*. 2009;27(8):1250-1256. doi:10.1200/JCO.2008.16.6959

42. Walsh JH, Karnes WE, Cuttitta F, Walker A. Autocrine growth factors and solid tumor malignancy. *West J Med*. 1991;155(2):152-163.

43. Wong AJ, Ruppert JM, Bigner SH, et al. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A*. 1992;89(7):2965-2969.

44. Malumbres M, Barbacid M. RAS oncogenes: the first 30 years. *Nat Rev Cancer*. 2003;3(6):459-465. doi:10.1038/nrc1097

45. Bheda A, Creek K, Pirisi L. Loss of p53 induces epidermal growth factor receptor promoter activity in normal human keratinocytes. *Oncogene*. 2008;27(31):4315-4323. doi:10.1038/onc.2008.65

46. Yallowitz A, Li D, Lobko A, Nemajerova A, Marchenko N. Mutant p53 amplifies Epidermal Growth Factor Receptor family signaling to promote mammary tumorigenesis. *Mol Cancer Res*. 2015;13(4):743-754. doi:10.1158/1541-7786.MCR-14-0360

47. Weissmueller S, Manchado E, Saborowski M, et al. Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor beta signaling. *Cell*. 2014;157(2):382-394. doi:10.1016/j.cell.2014.01.066

48. Yang W, Wetterskog D, Matsumoto Y, Funa K. Kinetics of repression by modified p53 on the PDGF beta-receptor promoter. *Int J Cancer*. 2008;123(9):2020-2030. doi:10.1002/ijc.23735

49. Stecca B, Ruiz i Altaba A. A GLI1-p53 inhibitory loop controls neural stem cell and tumour cell numbers. *EMBO J*. 2009;28(6):663-676. doi:10.1038/emboj.2009.16

50. Yoon JW, Lamm M, Iannaccone S, et al. p53 modulates the activity of the GLI1 oncogene through interactions with the shared coactivator TAF9. *DNA Repair (Amst)*. 2015;34:9-17. doi:10.1016/j.dnarep.2015.06.006

51. Mazzà D, Infante P, Colicchia V, et al. PCAF ubiquitin ligase activity inhibits Hedgehog/Gli1 signaling in p53-dependent response to genotoxic stress. *Cell Death Differ*. 2013;20(12):1688-1697. doi:10.1038/cdd.2013.120

52. Sun Y, Klauzinska M, Lake RJ, et al. Trp53 regulates Notch 4 signaling through Mdm2. *J Cell Sci*. 2011;124(Pt 7):1067-1076. doi:10.1242/jcs.068965

53. Yogosawa S, Miyauchi Y, Honda R, Tanaka H, Yasuda H. Mammalian Numb is a target protein of Mdm2, ubiquitin ligase. *Biochem Biophys Res Commun*. 2003;302(4):869-872. doi:10.1016/s0006-291x(03)00282-1

54. Laws AM, Osborne BA. p53 regulates thymic Notch1 activation. *Eur J Immunol*. 2004;34(3):726-734. doi:10.1002/eji.200324772

55. Shao J, Fujiwara T, Kadowaki Y, et al. Overexpression of the wild-type p53 gene inhibits NF-kappaB activity and synergizes with aspirin to induce apoptosis in human colon cancer cells. *Oncogene*. 2000;19(6):726-736. doi:10.1038/sj.onc.1203383

56. Zindy F, Eischen CM, Randle DH, et al. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev*. 1998;12(15):2424-2433.

57. Lin AW, Lowe SW. Oncogenic ras activates the ARF-p53 pathway to suppress epithelial cell transformation. *Proc Natl Acad Sci U S A*. 2001;98(9):5025-5030. doi:10.1073/pnas.091100298

58. Ho JSL, Ma W, Mao DYL, Benchimol S. p53-Dependent Transcriptional Repression of c-myc Is Required for G1 Cell Cycle Arrest. *Mol Cell Biol*. 2005;25(17):7423-7431. doi:10.1128/MCB.25.17.7423-7431.2005

59. Buganim Y, Solomon H, Rais Y, et al. p53 Regulates the Ras Circuit to Inhibit the Expression of a Cancer-Related Gene Signature by Various Molecular Pathways. *Cancer Res*. 2010;70(6):2274-2284. doi:10.1158/0008-5472.CAN-09-2661

60. Riedel F, Götte K, Schwalb J, Schäfer C, Hörmann K. Vascular endothelial growth factor expression correlates with p53 mutation and angiogenesis in squamous cell carcinoma of the head and neck. *Acta Otolaryngol*. 2000;120(1):105-111. doi:10.1080/00016480060203334

61. Goodheart MJ, Ritchie JM, Rose SL, Fruehauf JP, Young BRD, Buller RE. The Relationship of Molecular Markers of p53 Function and Angiogenesis to Prognosis of Stage I Epithelial Ovarian Cancer. *Clin Cancer Res*. 2005;11(10):3733-3742. doi:10.1158/1078-0432.CCR-04-0056

62. Yu EY, Yu E, Meyer GE, Brawer MK. The relation of p53 protein nuclear accumulation and angiogenesis in human prostatic carcinoma. *Prostate Cancer Prostatic Dis*. 1997;1(1):39-44. doi:10.1038/sj.pcan.4500205

63. Hegde PU, Brenski AC, Caldarelli DD, et al. Tumor Angiogenesis and p53 Mutations: Prognosis in Head and Neck Cancer. *Arch Otolaryngol Head Neck Surg*. 1998;124(1):80-85. doi:10.1001/archotol.124.1.80

64. Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. *Nat Rev Cancer*. 2001;1(3):222-231. doi:10.1038/35106065

65. Chen J. The Cell-Cycle Arrest and Apoptotic Functions of p53 in Tumor Initiation and Progression. *Cold Spring Harb Perspect Med*. 2016;6(3). doi:10.1101/cshperspect.a026104

66. Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells*. 1998;3(11):697-707. doi:10.1046/j.1365-2443.1998.00223.x

67. Vaseva AV, Moll UM. The mitochondrial p53 pathway. *Biochim Biophys Acta*. 2009;1787(5):414. doi:10.1016/j.bbabio.2008.10.005

68. Fei P, El-Deiry WS. P53 and radiation responses. *Oncogene*. 2003;22(37):5774-5783. doi:10.1038/sj.onc.1206677

69. Bertheau P, Espié M, Turpin E, et al. *TP53* Status and Response to Chemotherapy in Breast Cancer. *Pathobiology*. 2008;75(2):132-139. doi:10.1159/000123851

70. Kowanetz M, Ferrara N. Vascular Endothelial Growth Factor Signaling Pathways: Therapeutic Perspective. *Clin Cancer Res*. 2006;12(17):5018-5022. doi:10.1158/1078-0432.CCR-06-1520

71. Sermeus A, Michiels C. Reciprocal influence of the p53 and the hypoxic pathways. *Cell Death Dis*. 2011;2(5):e164-e164. doi:10.1038/cddis.2011.48

72. Teodoro JG, Evans SK, Green MR. Inhibition of tumor angiogenesis by p53: a new role for the guardian of the genome. *J Mol Med*. 2007;85(11):1175-1186. doi:10.1007/s00109-007-0221-2

73. Yu JL, Rak JW, Coomber BL, Hicklin DJ, Kerbel RS. Effect of p53 Status on Tumor Response to Antiangiogenic Therapy. *Science*. 2002;295(5559):1526-1528. doi:10.1126/science.1068327

74. Yeh J-RJ, Mohan R, Crews CM. The antiangiogenic agent TNP-470 requires p53 and p21CIP/WAF for endothelial cell growth arrest. *PNAS*. 2000;97(23):12782-12787. doi:10.1073/pnas.97.23.12782

75. Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med*. 2006;12(8):895-904. doi:10.1038/nm1469

76. Lim S-O, Kim H, Jung G. p53 inhibits tumor cell invasion via the degradation of snail protein in hepatocellular carcinoma. *FEBS Letters*. 2010;584(11):2231-2236. doi:10.1016/j.febslet.2010.04.006

77. Moustakas A, Heldin C-H. Mechanisms of TGFβ-Induced Epithelial–Mesenchymal Transition. *J Clin Med*. 2016;5(7). doi:10.3390/jcm5070063

78. Araki S, Eitel JA, Batuello CN, et al. TGF-β1–induced expression of human Mdm2 correlates with late-stage metastatic breast cancer. *J Clin Invest*. 2010;120(1):290-302. doi:10.1172/JCI39194

79. Yang J-Y, Zong CS, Xia W, et al. MDM2 Promotes Cell Motility and Invasiveness by Regulating E-Cadherin Degradation. *Mol Cell Biol*. 2006;26(19):7269-7282. doi:10.1128/MCB.00172-06

80. Martínez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nat Rev Cancer*. 2011;11(3):161-176. doi:10.1038/nrc3025

81. Herbig U, Jobling WA, Chen BPC, Chen DJ, Sedivy JM. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Mol Cell*. 2004;14(4):501-513. doi:10.1016/s1097-2765(04)00256-4

82. Stampfer MR, Garbe J, Nijjar T, Wigington D, Swisshelm K, Yaswen P. Loss of p53 function accelerates acquisition of telomerase activity in indefinite lifespan human mammary epithelial cell lines. *Oncogene*. 2003;22(34):5238-5251. doi:10.1038/sj.onc.1206667

83. Zhang C, Liu J, Liang Y, et al. Tumour-associated mutant p53 drives the Warburg effect. *Nat Commun*. 2013;4(1):1-15. doi:10.1038/ncomms3935

84. Amelio I, Mancini M, Petrova V, et al. p53 mutants cooperate with HIF-1 in transcriptional regulation of extracellular matrix components to promote tumor progression. *PNAS*. 2018;115(46):E10869-E10878. doi:10.1073/pnas.1808314115

85. Berger A. Positron emission tomography. *BMJ*. 2003;326(7404):1449.

86. Kim J, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*. 2006;3(3):177-185. doi:10.1016/j.cmet.2006.02.002

87. Bensaad K, Tsuruta A, Selak MA, et al. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell*. 2006;126(1):107-120. doi:10.1016/j.cell.2006.05.036

88. Matoba S, Kang J-G, Patino WD, et al. p53 regulates mitochondrial respiration. *Science*. 2006;312(5780):1650-1653. doi:10.1126/science.1126863

89. Muñoz-Fontela C, Mandinova A, Aaronson SA, Lee SW. Emerging roles of p53 and other tumour-suppressor genes in immune regulation. *Nat Rev Immunol*. 2016;16(12):741-750. doi:10.1038/nri.2016.99

90. Cha YJ, Kim HR, Lee CY, Cho BC, Shim HS. Clinicopathological and prognostic significance of programmed cell death ligand-1 expression in lung adenocarcinoma and its relationship with p53 status. *Lung Cancer*. 2016;97:73-80. doi:10.1016/j.lungcan.2016.05.001

91. Cortez MA, Ivan C, Valdecanas D, et al. PDL1 Regulation by p53 via miR-34. *J Natl Cancer Inst*. 2016;108(1). doi:10.1093/jnci/djv303

92. Guo G, Yu M, Xiao W, Celis E, Cui Y. Local Activation of p53 in the Tumor Microenvironment Overcomes Immune Suppression and Enhances Antitumor Immunity. *Cancer Res*. 2017;77(9):2292-2305. doi:10.1158/0008-5472.CAN-16-2832

93. Eischen CM. Genome Stability Requires p53. *Cold Spring Harb Perspect Med*. 2016;6(6). doi:10.1101/cshperspect.a026096

94. Hanel W, Moll UM. Links between mutant p53 and genomic instability. *J Cell Biochem*. 2012;113(2):433-439. doi:10.1002/jcb.23400

95. Saadatzadeh MR, Elmi AN, Pandya PH, et al. The Role of MDM2 in Promoting Genome Stability versus Instability. *Int J Mol Sci*. 2017;18(10). doi:10.3390/ijms18102216

96. Bouska A, Lushnikova T, Plaza S, Eischen CM. Mdm2 Promotes Genetic Instability and Transformation Independent of p53. *Molecular and Cellular Biology*. 2008;28(15):4862-4874. doi:10.1128/MCB.01584-07

97. Cao Z, Xue J, Cheng Y, et al. MDM2 promotes genome instability by ubiquitinating the transcription factor HBP1. *Oncogene*. 2019;38(24):4835-4855. doi:10.1038/s41388-019-0761-2

98. Roy S, Tomaszowski K-H, Luzwick JW, et al. p53 orchestrates DNA replication restart homeostasis by suppressing mutagenic RAD52 and POLθ pathways. *eLife*. 7. doi:10.7554/eLife.31723

99. Klusmann I, Rodewald S, Müller L, et al. p53 Activity Results in DNA Replication Fork Processivity. *Cell Rep*. 2016;17(7):1845-1857. doi:10.1016/j.celrep.2016.10.036

100. Yeo CQX, Alexander I, Lin Z, et al. p53 Maintains Genomic Stability by Preventing Interference between Transcription and Replication. *Cell Reports*. 2016;15(1):132-146. doi:10.1016/j.celrep.2016.03.011

101. Karni-Schmidt O, Lokshin M, Prives C. The Roles of MDM2 and MDMX in Cancer. *Annual Review of Pathology: Mechanisms of Disease*. 2016;11(1):617-644. doi:10.1146/annurev-pathol-012414-040349

102. Khoo KH, Verma CS, Lane DP. Drugging the p53 pathway: understanding the route to clinical efficacy. *Nat Rev Drug Discov*. 2014;13(3):217-236. doi:10.1038/nrd4236

103. Kojima K, Konopleva M, Samudio IJ, et al. MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy. *Blood*. 2005;106(9):3150-3159. doi:10.1182/blood-2005-02-0553

104. Efeyan A, Ortega-Molina A, Velasco-Miguel S, Herranz D, Vassilev LT, Serrano M. Induction of p53-dependent senescence by the MDM2 antagonist nutlin-3a in mouse cells of fibroblast origin. *Cancer Res*. 2007;67(15):7350-7357. doi:10.1158/0008-5472.CAN-07-0200

105. Vaseva AV, Marchenko ND, Moll UM. The transcription-independent mitochondrial p53 program is a major contributor to nutlin-induced apoptosis in tumor cells. *Cell Cycle*. 2009;8(11):1711-1719.

106. Chang YS, Graves B, Guerlavais V, et al. Stapled α−helical peptide drug development: A potent dual inhibitor of MDM2 and MDMX for p53-dependent cancer therapy. *PNAS*. 2013;110(36):E3445-E3454. doi:10.1073/pnas.1303002110

107. Gu S, Cui D, Chen X, Xiong X, Zhao Y. PROTACs: An Emerging Targeting Technique for Protein Degradation in Drug Discovery. *BioEssays*. 2018;40(4):1700247. doi:10.1002/bies.201700247

108. Hines J, Lartigue S, Dong H, Qian Y, Crews CM. MDM2-recruiting PROTAC Offers Superior, Synergistic Anti-proliferative Activity via Simultaneous Degradation of BRD4 and Stabilization of p53. *Cancer Res*. January 2018:canres.2918.2018. doi:10.1158/0008-5472.CAN-18-2918

109. Herman AG, Hayano M, Poyurovsky MV, et al. Discovery of Mdm2-MDMX E3 Ligase Inhibitors Using a Cell-Based Ubiquitination Assay. *Cancer Discov*. 2011;1(4):312-325. doi:10.1158/2159-8290.CD-11-0104

110. Yang Y, Ludwig RL, Jensen JP, et al. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. *Cancer Cell*. 2005;7(6):547-559. doi:10.1016/j.ccr.2005.04.029

111. Bykov VJN, Issaeva N, Shilov A, et al. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med*. 2002;8(3):282-288. doi:10.1038/nm0302-282

112. Rökaeus N, Shen J, Eckhardt I, Bykov VJN, Wiman KG, Wilhelm MT. PRIMA-1^{MET}/APR-246 targets mutant forms of p53 family members p63 and p73. *Oncogene*. 2010;29(49):6442-6451. doi:10.1038/onc.2010.382

113. Issaeva N, Bozko P, Enge M, et al. Small molecule RITA binds to p53, blocks p53–HDM-2 interaction and activates p53 function in tumors. *Nat Med*. 2004;10(12):1321-1328. doi:10.1038/nm1146

114. Weilbacher A, Gutekunst M, Oren M, Aulitzky WE, van der Kuip H. RITA can induce cell death in p53-defective cells independently of p53 function via activation of JNK/SAPK and p38. *Cell Death & Disease*. 2014;5(7):e1318. doi:10.1038/cddis.2014.284

115. Komarov PG. A Chemical Inhibitor of p53 That Protects Mice from the Side Effects of Cancer Therapy. *Science*. 1999;285(5434):1733-1737. doi:10.1126/science.285.5434.1733

116. Gudkov AV, Komarova EA. Prospective therapeutic applications of p53 inhibitors. *Biochemical and Biophysical Research Communications*. 2005;331(3):726-736. doi:10.1016/j.bbrc.2005.03.153

117. Culmsee C, Zhu X, Yu QS, et al. A synthetic inhibitor of p53 protects neurons against death induced by ischemic and excitotoxic insults, and amyloid beta-peptide. *J Neurochem*. 2001;77(1):220-228. doi:10.1046/j.1471-4159.2001.t01-1-00220.x

118. Strom E, Sathe S, Komarov PG, et al. Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation. *Nat Chem Biol*. 2006;2(9):474-479. doi:10.1038/nchembio809

119. Bohlman S, Manfredi JJ. p53-Independent Effects of Mdm2. *Subcell Biochem*. 2014;85:235-246. doi:10.1007/978-94-017-9211-0_13

120. Thomasova D, Mulay SR, Bruns H, Anders H-J. p53-Independent Roles of MDM2 in NF-κB Signaling: Implications for Cancer Therapy, Wound Healing, and Autoimmune Diseases. *Neoplasia*. 2012;14(12):1097-1101.

121. Prives C, White E. Does control of mutant p53 by Mdm2 complicate cancer therapy? *Genes Dev*. 2008;22(10):1259-1264. doi:10.1101/gad.1680508

122. Muller PAJ, Vousden KH. p53 mutations in cancer. *Nature Cell Biology*. 2013;15(1):2-8. doi:10.1038/ncb2641

123. Terzian T, Suh Y-A, Iwakuma T, et al. The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. *Genes Dev*. 2008;22(10):1337-1344. doi:10.1101/gad.1662908

124. Peng Y, Chen L, Li C, Lu W, Chen J. Inhibition of MDM2 by hsp90 Contributes to Mutant p53 Stabilization. *J Biol Chem*. 2001;276(44):40583-40590. doi:10.1074/jbc.M102817200

125. Taubert H, Würl P, Bache M, et al. The p53 gene in soft tissue sarcomas: prognostic value of DNA sequencing versus immunohistochemistry. *Anticancer Res*. 1998;18(1A):183-187.

126. PIASKOWSKI S, ZAWLIK I, SZYBKA M, et al. Detection of P53 mutations in different cancer types is improved by cDNA sequencing. *Oncol Lett*. 2010;1(4):717-721. doi:10.3892/ol_00000125

127. Lomenick B, Olsen RW, Huang J. Identification of Direct Protein Targets of Small Molecules. *ACS Chem Biol*. 2011;6(1):34-46. doi:10.1021/cb100294v

128. Sharma S, Petsalaki E. Application of CRISPR-Cas9 Based Genome-Wide Screening Approaches to Study Cellular Signalling Mechanisms. *Int J Mol Sci*. 2018;19(4). doi:10.3390/ijms19040933

129. Schenone M, Dančík V, Wagner BK, Clemons PA. Target identification and mechanism of action in chemical biology and drug discovery. *Nat Chem Biol*. 2013;9(4):232-240. doi:10.1038/nchembio.1199

130. Jafari R, Almqvist H, Axelsson H, et al. The cellular thermal shift assay for evaluating drug target interactions in cells. *Nat Protoc*. 2014;9(9):2100-2122. doi:10.1038/nprot.2014.138

131. Mateus A, Määttä TA, Savitski MM. Thermal proteome profiling: unbiased assessment of protein state through heat-induced stability changes. *Proteome Sci*. 2017;15. doi:10.1186/s12953-017-0122-4

132. King HA, Gerber AP. Translatome profiling: methods for genome-scale analysis of mRNA translation. *Brief Funct Genomics*. 2016;15(1):22-31. doi:10.1093/bfgp/elu045