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INHIBITION OF CRUCIAL ONCOGENES BY PHARMACOLOGICALLY ACTIVATED p53

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Stockholm 2013

To my family

ABSTRACT

The tumor suppressor p53 is a transcriptional factor which is frequently inactivated in cancer, either by point mutations or by its negative regulators, such as Mdm2 and MdmX. Reactivation of p53 by small molecules is a promising strategy to treat cancer. The aim of this thesis is to elucidate the molecular mechanisms of the different biological responses induced by two p53-reactivating small molecules, RITA and nutlin.

We found that the induction of p53 pro-apoptotic target genes is not sufficient to induce a full-scale cell death; the inhibition of key survival genes is necessary to trigger robust apoptosis upon reactivation of p53. Our results reveal that two distinct transcriptional programs, activation of pro-apoptotic genes and repression of pro-survival genes are required to be orchestrated by p53 to produce a robust apoptotic outcome. In contrast to p53-mediated transactivation, transrepression by p53 is more strictly controlled by Mdm2 and requires a high ratio of p53/Mdm2 at the promoters of repressed genes.

Further investigation of the underlying mechanisms of the differential biological outcome upon p53 reactivation revealed that the inhibition of TrxR1 by RITA leads to the induction of ROS and activation of JNK. Activated JNK creates a positive feedback loop with p53 and converts p53 into an efficient transrepressor. We demonstrated that Wip1 is one of the crucial factors downstream of JNK, whose inhibition contributes to a robust and sustained transcriptional response by p53 and the subsequent cell death. Our data suggest that simultaneous activation of p53 and inhibition of TrxR1 lead to synthetic lethality in cancer cells. Our study points out that perturbing the redox system in tumors, which carry abnormally high level of ROS, might enable the pharmacologically reactivated p53 to selectively eliminate cancer cells.

Neuroblastoma is one of the most challenging childhood cancers. The ability of RITA to reactivate both wild type and mutant p53 prompted us to investigate the effect of RITA in a panel of seven neuroblastoma cell lines with different p53 status. We found that RITA induced apoptosis in all the neuroblastoma cell lines tested, irrespective of the status of p53. RITA-activated p53 induced a set of pro-apoptotic target genes. In addition, RITA-activated p53 repressed several key survival genes, including N-myc, Wip1, Aurora kinase, Mcl-1, Bcl-2, Mdm2 and MdmX. Moreover, RITA exhibited strong antitumor effect in xenograft models.

In summary, our data presented above demonstrate that concurrent activation of p53 in combination with inhibition of TrxR1 followed by the induction of ROS represent a promising strategy to treat cancer. Inhibition of pro-survival genes plays a fundamental role in a full-scale apoptosis induction in cancer cells upon pharmacological p53-reactivation.

LIST OF PUBLICATIONS

I. Grinkevich VV, Nikulenkov F*, **Shi Y***, Enge M, Bao W, Maljukova A, Gluch A, Kel A, Sangfelt O, Selivanova G.
Ablation of Key Oncogenic Pathways by RITA-Reactivated p53 Is Required for Efficient Apoptosis
Cancer Cell, 2009, May 5;15(5):441-53

II. **Yao Shi**, Fedor Nikulenkov, Joanna Zawacka-Pankau , Hai Li, Razif Gabdoulline, Jianqiang Xu, Sofi Eriksson, Elisabeth Hedström, Natalia Issaeva, Alexander Kel, Elias S.J. Arnér and Galina Selivanova.
ROS-dependent activation of JNK converts p53 into an efficient inhibitor of oncogenes leading to robust apoptosis
Cell Death Differ, under revision

III. Burmakin M, **Shi Y**, Hedström E, Kogner P, Selivanova G.
Dual Targeting of Wild-Type and Mutant p53 by Small Molecule RITA Results in the Inhibition of N-Myc and Key Survival Oncogenes and Kills Neuroblastoma Cells *In Vivo* and *In Vitro*
Clin Cancer Res, 2013, Sep 15;19(18): 5092–103

ASSOCIATED PUBLICATIONS

Insights into p53 transcriptional function via genome-wide chromatin occupancy and gene expression analysis
F Nikulenkov, C Spinnler, H Li, C Tonelli, **Y Shi**, M Turunen, T Kivioja, I Ignatiev, A Kel, J Taipale and G Selivanova
Cell Death Differ, 2012, Jul 13, *[Published ahead of print]*

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LIST OF ABBREVIATIONS

ARF	Alternative reading frame
ATM	Ataxia telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3-related
Bcl-2	B-cell lymphoma 2
COP1	Constitutively photomorphogenic 1
COX2	Cyclooxygenase 2
DBD	DNA binding domain
DDR	DNA damage response
E3	Enzyme 3
FBW7	F-box and WD repeat domain-containing 7
GPX	Glutathione peroxidase
GSH	Glutathione
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia sequence 1
Mdm2	Mouse double minute 2
MdmX	Mouse double minute X
mTOR	Mammalian target of rapamycin
NCI	National cancer institute
PI3K	Phosphatidyl inositol-3 kinase
Pirh2	p53-induced protein with a RING H2 domain
PPM1D	Protein phosphatase Mg^{2+}/Mn^{2+}-dependent 1D
Puma	p53 upregulated modulator of apoptosis
RITA	Reactivation of p53 and induction of tumor cell apoptosis
ROS	Reactive oxygen species
SH3	Sarcoma homolog domain 3
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
SOD2	Superoxide dismutase 2
TP53	Tumor protein p53
Trx	Thioredoxin
TrxR1	Thioredoxin reductase 1
Wip1	Wild type p53 induced phosphatase 1
UV	Ultraviolet

1   CANCER

Cancer is one of the leading causes of death in the human society. The incidence of cancer is not only dependent on the individual genetic background, but also heavily influenced by the environment and life style. Breast cancer, colorectal cancer and lung cancer are among the most frequent types of cancer that threaten humans (Jemal et al., 2011). Neuroblastoma is one of the most common pediatric tumors in children with 7.5 cases per 100 000 infants (Schwab et al., 2003) and it belongs to the most challenging childhood cancer.

The development of cancer is a multi-step process involving an accumulation of genetic and epigenetic changes that eventually lead to uncontrolled cell division and growth. Ten hallmarks are proposed to be required for normal cells to achieve a malignant state. These are: persistent proliferative signalling, escape from growth suppressors, resistance to cell death, limitless replicative potential, deregulated metabolism, genomic instability, tumor-promoting inflammation, avoidance from immune destruction, triggering angiogenesis and induction of invasion and metastasis (Hanahan and Weinberg, 2011).

1.1   Oncogenes and tumor suppressors

Oncogenes, which have the potential to cause cancer, are the result of a mutation or deregulation of the corresponding normal cellular genes (proto-oncogenes). The functions of oncogene-encoded oncoproteins are to regulate the development of several hallmarks of cancer; for instance, sustained proliferative signalling, resistance to cell death stimuli and malfunction of cellular energetics. Oncoproteins could be classified into several subgroups, such as transcriptional factors (c-Myc, N-Myc, c-Jun), growth factor receptors (EGFR, IGF-1R, Met), signal transducers (PI3K, Akt), inhibitors of apoptosis (Bcl-2, Mcl-1, Mdm2, Wip1) and others. Activation of oncogenes could be due to several mechanisms, including chromosomal translocation, point mutation and gene amplification. Mutations in microRNAs that regulate the expression of oncogenes could also cause their activation. All these mechanisms enable the abnormal growth of cells by changing the structure of the oncogene or deregulation of its protein expression (Croce, 2008).

Oncogene addiction, i.e., the dependency of cancer cells on oncogenic signalling, provides a new strategy to develop anti-cancer drugs. Currently, several small molecules have been developed and used in clinic to inhibit the kinase activity of the oncoproteins EGFR, Met and ERBB2.

Since a single oncogene is not sufficient to transform normal rodent cells, additional genetic changes are required to cooperate with the initial mutation; transformation in rodent cells usually requires collaboration between two or more mutant genes. In human cells, the situation is even more complicated. It has been proposed that a series of cellular and genetic changes are required to transform human cells: activation of Ras, maintenance of telomeres by hTERT, deregulation of protein phosphatase 2A (PP2A),

inactivation of cell cycle control by pRb and malfunction of the p53 pathway (Akagi, 2004).

pRb and p53 belong to another distinctive type of growth-controlling genes, which operates to constrain or suppress cell proliferation, namely tumor suppressor genes. Tumor suppressor genes are frequently inactivated in cancer by genetic mutation or epigenetic silencing via promoter methylation; inactivation of one copy of tumor suppressor gene might be followed by the loss of another copy, i.e., loss of the heterozygosity (LOH) at the tumor suppressor locus. The loss of the second allele of a tumor suppressor via LOH occurs more frequently than via mutations or promoter methylation. The tumor suppressor genes could also be inactivated by aberrant expression and activation of their negative regulators.

1.2   p53 as a guardian of the genome

In 1979, p53 was discovered as a non-viral protein which co-precipitated with the SV40 large T-antigen, with an apparent molecular weight 53 kDa (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979; Smith et al., 1979). As retroviruses have been shown to kidnap cellular genes to promote neoplastic transformation, p53 was logically considered to be a positive effector to transform the cells, leading to the conclusion that p53 was an oncogene. Indeed, p53 cDNAs cloned from both mouse and human genome were able to cooperate with several established oncogenes to transform the primary cells in culture. However, several years later, these p53 cDNAs were found to originate from the tumor cells. Subsequent studies revealed that the p53 cDNA cloned from normal cells conferred suppression, instead of promotion of transformation, establishing that wild type p53 is a tumor suppressor (Levine and Oren, 2009).

The tumor suppressor function of p53 has been further supported *in vivo*. Although p53-null mice were developmentally normal, they were clearly prone to spontaneous tumor formation. 74% of the homozygous p53-null mice developed a variety of neoplasm by the age of 6 months, while no tumor was found in wild type p53 mice by the age of 9 months (Donehower et al., 1992). This indicates that p53 is dispensable for embryonic development, but its absence could predispose the mice to cancer. Meanwhile, p53 has been found to be frequently mutated in diverse types of human cancer (Hollstein et al., 1991). Findings demonstrating that p53 induced by DNA damage can stop the proliferation of damaged cells or kill them lead to the idea of p53 as the guardian of the genome (Lane, 1992).

A variety of signals can cause the p53 induction; the most common sources are ultraviolet (UV) radiation, which we face almost all the time under the sun, and reactive oxygen species (ROS), which are the by-products of normal metabolism of the oxygen. p53 could also be induced by oncogene activation and chemotherapeutic drugs, which are widely used in clinic. Upon stabilization, p53 triggers diverse biological responses such as cell cycle arrest, senescence, apoptosis and DNA repair (Figure 1) (Mancini et al., 2009).
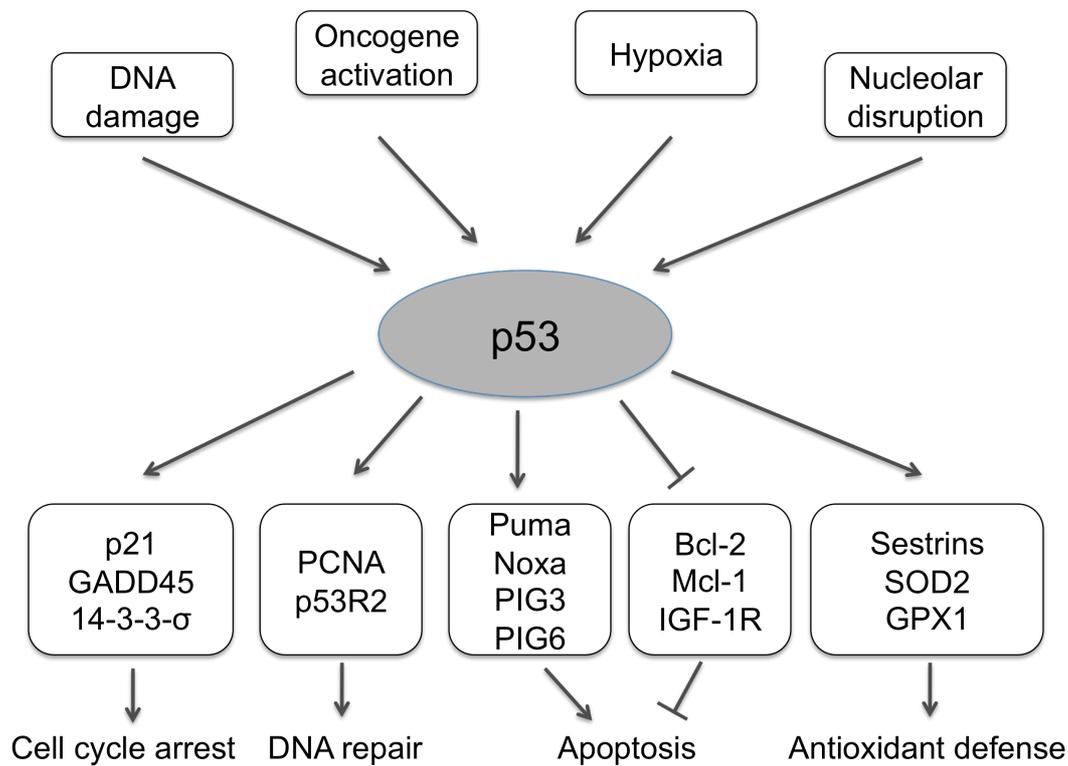
Figure 1. Upon diverse stress signals, p53 is activated and induces different biological responses via modulating the expression of a number of target genes. Puma, PIG3 and PIG6 also belong to the pro-oxidant genes.

1.3 p53 regulation

The p53 protein is comprised of 393 amino acid residues and could be divided into several functional domains (Figure 2): the N-terminal transactivation domain, which could be subdivided into two separate transactivation domains (TA1 and TA2), carries regions for Mdm2, MdmX and p300/CBP binding (Momand et al., 2000); proline-rich domain (PRD), which can bind SH3 domains on other proteins; DNA-binding domain (DBD); nuclear localisation signal domain (NLS); tetramerization domain (TET) and C-terminal regulatory domain (REG), which harbours acetylation and phosphorylation sites regulating the DNA binding specificity of p53.

p53 induction is mainly attributed to protein stabilization, but not to increased mRNA expression. Wild type p53 is a short-lived protein, with a half-life of about 20 minutes (Finlay et al., 1988). The underlying logic of the rapid turnover seems to ensure its immediate induction via protein stabilization upon stress to execute the suppressor function. Thus it is not surprising that the p53 protein level is very tightly controlled in cells.
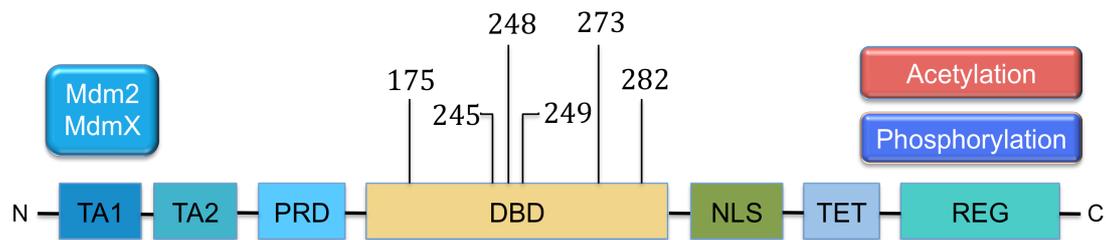
Figure 2. Schematic view of the six distinct domains of human p53 protein. N-terminal transactivation domain: TA1 and TA2, N-terminal transactivation domain 1, which contains regions for Mdm2, MdmX binding, and N-terminal transactivation domain 2; PRD: Proline rich domain, which binds SH3 domains and has a regulatory role in p53 tumor suppression function; DBD: DNA binding domain, where most of the point mutations in tumors occur and hotspots for mutations are indicated; NLS: nuclear localisation signal domain; TET: tetramerization domain; REG: C-terminal regulatory domain, which carries the key residues for acetylation and phosphorylation.

The major negative regulators of p53 are Mdm2 and MdmX. Mdm2 binds to the N-terminal transactivation domain of p53 and inhibits its transcription (Momand et al., 1992); Mdm2 also functions as an E3 ubiquitin ligase and targets p53 to proteasomal degradation (Kubbutat et al., 1997); moreover, Mdm2 mediated mono-ubiquitination of p53 could induce its nuclear export thus inhibiting its transcriptional activity (Li et al., 2003). Although MdmX could also bind p53 and block its transcriptional function, MdmX does not possess intrinsic E3 ligase activity (Stad et al., 2001) and is unable to target p53 for degradation. Interestingly, under certain conditions MdmX can cooperate with p53 in apoptosis induction (Mancini et al., 2009). In order to execute E3 ligase activity, Mdm2 is required to form oligomers with itself or MdmX through Ring-finger domains; however, hetero-oligomeriztion of Mdm2 and MdmX renders a more efficient E3 ligase towards p53 (Wade et al., 2013). Indeed, *in vivo* studies support the essential role of Mdm2 and MdmX in regulating p53 activity. Mice with either Mdm2 or MdmX deletion are embryonically lethal; interestingly, knockout of p53 completely rescues the embryonic lethality, suggesting the fundamental role of Mdm2 and MdmX in controlling p53 activity (Jones et al., 1995; Montes de Oca Luna et al., 1995; Parant et al., 2001).

In addition to Mdm2, other E3 ligases have also been identified to target p53 for degradation by the proteasome, such as COP1 (Dornan et al., 2004), Pirh2 (Leng et al., 2003), TRIM24 (Allton et al., 2009), CHIP (Esser et al., 2005), ARF-BP1 (Chen et al., 2005), Synoviolin (Yamasaki et al., 2007) and TOPORS (Rajendra et al., 2004). There are also several other E3 ligases which ubiquitinate p53 without causing its degradation (Jain and Barton, 2010; Love and Grossman, 2012). Moreover, the E3 ligases Mdm2, COP1 and Pirh2 are induced by p53, creating the auto-regulatory negative feedback

loops. The relative importance of other E3 ligases, except Mdm2, for the p53 function remains elusive.

p53 could be activated by various signals. The stabilization and activation of p53 by oncogene activation are partially mediated by ARF (Harris and Levine, 2005). The tumor suppressor ARF (p14^{ARF} in human and p19^{ARF} in mice) regulates p53 stability by counteracting Mdm2. ARF binds Mdm2 and sequesters it in the nucleolus; the association of ARF with Mdm2 also leads to the inhibition of Mdm2 binding to p53 and the prevention of p53 polyubiquitination, thus resulting in p53 stabilization (Michael and Oren, 2003).

Upon DNA damage, p53 is stabilized and activated by ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and Rad3-related). ATM and ATR play two major roles here: first, activated ATM and ATR lead to the phosphorylation and degradation of Mdm2 and MdmX; second, ATM and ATR could initiate a series of signalling cascades which could induce the phosphorylation of p53 at several sites at its N-terminal, resulting its disassociation from Mdm2 and MdmX; as a consequence, p53 is stabilized and activated (Meek, 2009).

Wip1 (Wild type p53 induced phosphatase 1 encoded by the *PPM1D* gene) is a type 2C phosphatase, which serves as a negative regulator of p53 by counteracting DNA damage response (DDR). The oncogenic Wip1 could directly dephosphorylate and inactivate the key DDR components, such as ATM, ATR, Chk1, Chk2, p53, γH2AX (Lu et al., 2008; Macurek et al., 2010; Moon et al., 2010), resulting in the attenuation of DDR; it could also remove the inhibitory phosphorylation marks in Mdm2 and MdmX (Lu et al., 2007; Zhang et al., 2009), leading to the inactivation of p53. Interestingly, Wip1 is a transcriptional target of p53. This creates a negative feedback loop that attenuates p53 activity, functioning to halt p53 response to allow cells to resume growth once DNA is repaired.

In addition to phosphorylation, p53 is subjected to acetylation, which could also activate its function. p53 has been shown to be acetylated by histone acetyl transferases (HATs) CBP (p300/CREB-binding protein) and PCAF (p300/CBP-associated factor) at various lysine sites at its C-terminal (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). Recently, another two acetyl transferases, hMOF and TIP60, have been shown to acetylate p53 at lysine 120 (Sykes et al., 2006; Tang et al., 2006). Acetylation of p53 stimulates its transcriptional activity, leading to diverse biological responses. The indispensible role of acetylation on p53 transactivation indicates that the acetylation of p53 should be tightly controlled in cells. Indeed, HDAC (histone deacetylase) such as SIRT1, could deacetylate p53 and impair its transcriptional function (Brooks and Gu, 2011).

Besides the above-mentioned modifications, p53 can undergo several other types of modifications that could either activate or inhibit p53 function, such as SUMOylation (Bischof et al., 2006; Gostissa et al., 1999; Kahyo et al., 2001; Schmidt and Muller, 2002), methylation (Huang et al., 2007; Scoumanne and Chen, 2008) and neddylation (Abida et al., 2007; Xirodimas et al., 2004).

1.4 p53 as a transcriptional factor

p53 executes its tumor suppressor function primarily through its capability to regulate the transcription of a broad range of genes involved in multiple biological responses (Figure 1). The canonical response element present in p53 target genes is composed of two decamer motifs 5′-RRRCWWGYYY-3′ (R represents a purine; W represents either A or T; Y represent a pyrimidine) separated by a spacer of 0-13 bp (el-Deiry et al., 1992; Funk et al., 1992). 162 genes have been validated as direct p53 target genes (Wang et al., 2009), and the list is increasing. Although p53-mediated transactivation is well-studied and easily linked to p53 consensus binding sites, p53-mediated transrepression is less well characterized due to the lack of uniform responsive element in its repressed genes (Nikulenkov et al., 2012; Riley et al., 2008). It remains unclear how p53 distinguishes between its repressed or activated genes (Riley et al., 2008).

1.41 p53-mediated transactivation

Ample evidences have shown that upon binding to its response element within a target gene, p53 could recruit general transcriptional factors and HATs through direct protein-protein interaction, leading to the acetylation of histones in vicinity and activation of transcription (Farmer et al., 1996; Gu and Roeder, 1997; Gu et al., 1997; Thut et al., 1995).

1.42 p53-mediated transrepression

p53-mediated transrepression involves both direct and indirect mechanisms. For instance, p53 directly binds the responsive elements in the apoptotic inhibitor *BIRC5* (Hoffman et al., 2002), cell cycle and proliferation regulator *MYC* (Ho et al., 2005; St Clair and Manfredi, 2006), and phosphatase *CDC25C*, leading to their repression (St Clair and Manfredi, 2006). At present, there are three well-established mechanisms governing direct transrepression by p53 as shown in Figure 3. First, steric hindrance, competing out transcriptional activators; second, sequestration of transcriptional activators; third, recruitment of transcriptional repressors (Riley et al., 2008; Rinn and Huarte, 2011).

p53-mediated repression via steric interference is achieved by direct binding the promoter-enhancer region of target genes, competing out other transactivators. For example, p53 responsive elements on the genes *BIRC5* (survivin), *AFP* (α-fetoprotein), *BCL2* (B-cell lymphoma-2) are overlapping or adjacent to the responsive elements of E2F1, HNF3 and POU4F1 (POU domain class 4 transcription factor-1), respectively (Budhram-Mahadeo et al., 1999; Lee et al., 1999; Nguyen et al., 2005; Raj et al., 2008). Other transcriptional factors that can repress genes via similar steric hindrance mechanisms are CEBP (CCAAT/enhancer binding protein), SP1 and NF-Y family proteins (Riley et al., 2008).

The p53-mediated sequestration of other transcriptional activators is achieved via direct protein-protein interaction. p53 is able to bind SP1 and impair its binding to the promoter DNA of *TERT* (telomerase reverse transcriptase), *IGF1R* (insulin-like growth factor 1 receptor), *VEGFA* (vascular endothelial growth factor A), leading to the repression of these genes (Bohlig and Rother, 2011).

Another strategy exploited by p53 to directly repress its target genes is the recruitment of repressors to chromatin by p53. For instance, p53-mediated recruitment of HDAC1 (histone deacetylase 1) is through co-repressor mSin3a, which binds both p53 and HDAC1 (Lin et al., 2005).

p53-mediated indirect transrepression might involve its target genes/miRNAs as the effectors. For example, cdk inhibitor p21, which is a direct p53 target, via inhibition of E2F has been implicated in the repression of *CDK1* (cdc2), *CDC25C*, *CHEK1*, *CCNA2* (cyclin A2), *CCNB1* (cyclin B1) and *FOXM1* (Rinn and Huarte, 2011). p53 could induce some miRNAs, which contribute to p53-mediated repression. The best-characterized miRNA involved in p53-mediated repression is miR-34, which is induced by p53 (He et al., 2007). For example, p53 target genes *CDK4*, *CCNE2* (cyclin E2) and *MET* are directly targeted and repressed by miR-34 (He et al., 2007).

*1.43   Biological role of p53-mediated transcription*

Upon various stress signals, p53 is stabilized and activated to induce different sets of genes involved in the regulation of cell cycle arrest, DNA repair, apoptosis, senescence, angiogenesis, metastasis, metabolism and ROS generation. The elucidation of molecular mechanisms underlying the choice of different biological responses by p53 remains a major challenge in the p53 field (Vousden and Prives, 2009).

The best-known p53 target gene involved in cell cycle control is *CDKN1A* encoding p21, which inhibits the cyclin-dependent kinases and induces cell cycle arrest both at G1 and G2 (Bunz et al., 1998; Harper et al., 1993). In addition to p21, other p53 targets, such as GADD45A (Hollander et al., 1999), 14-3-3σ (Hermeking et al., 1997), REPRIMO (Ohki et al., 2000) and CDC25C (Krause et al., 2001) have also been shown to control the cell cycle progression. Apoptosis triggered by p53 involves a number of its targets, such as puma (Jeffers et al., 2003; Nakano and Vousden, 2001), noxa (Oda et al., 2000), Fas (Tamura et al., 1995) and others (Vousden and Lu, 2002).

(1)
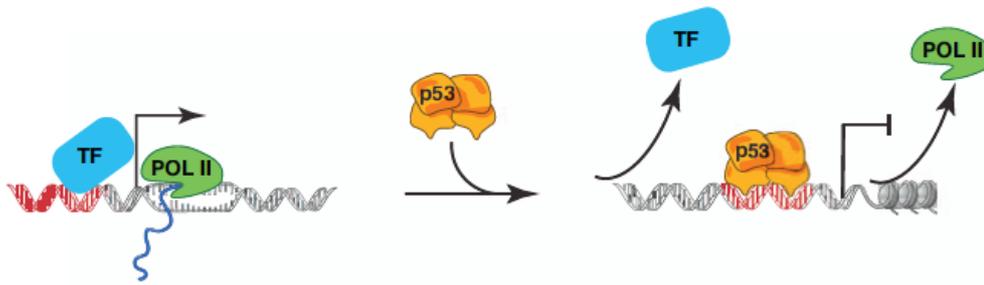
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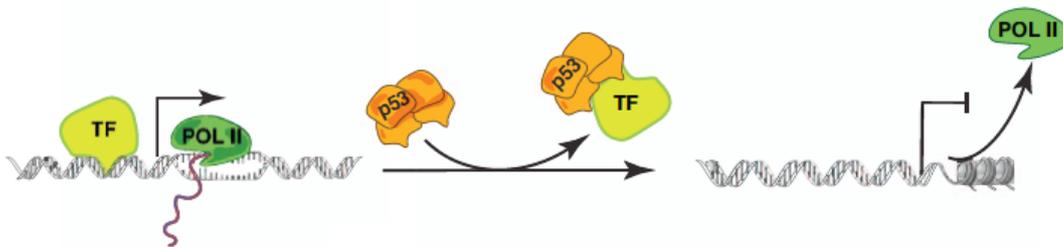
(3)
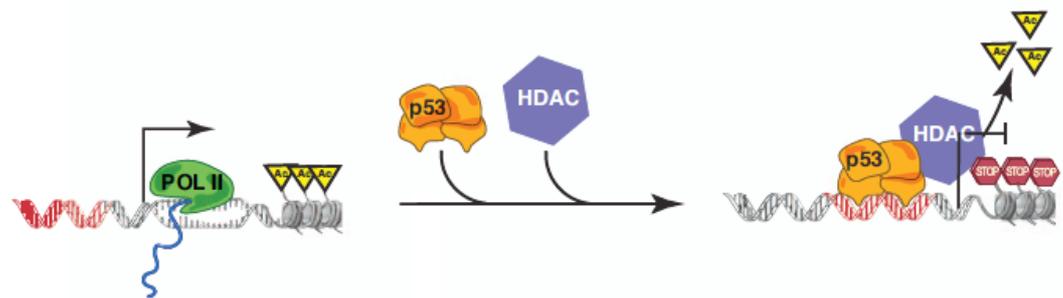
Figure 3. Mechanistic model of direct transcriptional repression by p53. (1) Steric hindrance: p53 binds the same sites in DNA as other transcriptional factors occupy. p53 binding competes out the other transcriptional factors thus inhibiting the transcription. (2) Sequestration of other transcriptional factors: p53 binds and sequesters other transcriptional factors that are required for the transactivation of the target genes. (3) Recruitment of transcriptional repressors: p53 binding to the gene promoter recruits transcriptional repressors, such as a histone deacetylase and silences the transcription. Reproduced from Rinn and Huarte 2011 with the permission from Elsevier.

It has been widely accepted that the induction of cell cycle arrest, apoptosis and senescence is the major tumor suppressor function of p53. However, this notion has been questioned by several recent studies.

Attardi and her colleagues showed that compared to the p53-null mice, homozygous knockin transactivation-deficient p53[25,26] (L25Q;W26S) mice do not display accelerated tumor formation in the KrasG12D-driven non-small cell lung cancer (NSCLC) model. Although the transactivation function of p53[25,26] is largely compromised, as evidenced from the reduced expression of p21, puma and noxa upon genotoxic stress, a low level of transcriptional activity was still observed. However, the transactivation-dead mutant p53[25,26,53,54] (L25Q;W26S;F53Q;F54S), which is unable to induce cell cycle arrest, apoptosis and senescence, still show partial tumor suppressor activity. This suggests that other mechanisms can also contribute to the tumor prevention by p53 (Brady et al., 2011).

Even more compelling evidence has been provided by the study of mutant p53[3KR/3KR] (K117R+K161R+K162R) mice. In spite of the abrogation of p53-mediated cell cycle arrest, apoptosis and senescence in p53[3KR/3KR] mice, and in contrast to p53-null mice, p53[3KR/3KR] mice do not suffer from the formation of early-onset spontaneous tumors. Therefore, regulation of energy metabolism and ROS production is proposed to be crucial for the tumor suppressor function of p53[3KR/3KR] (Li et al., 2012).

It is worth noticing that the expression of p21, puma, noxa is not completely absent, but only decreased in these p53 mutant mice; it is possible that the residual levels of these proteins might contribute to the observed tumor suppressor function of p53. To completely rule out the involvement of these three proteins in p53-mediated tumor suppression, Strasser and his colleagues generated the p21-/-puma-/-noxa-/- triple knockout mice and tested their predisposition to cancer. Although cells derived from the p21-/-puma-/-noxa-/- mice were resistant to p53-dependent apoptosis, cell cycle arrest and senescence induced by DNA damage, these mice were not predisposed to the spontaneous tumor development. The authors suggest that the ability of p53 to regulate DNA repair might be critical for tumor prevention by p53 (Valente et al., 2013).

1.5 The cross-talk between PI3K/Akt pathway and p53

The PI3K/Akt signalling pathway controls cell growth, proliferation, motility and metabolism. This pathway is frequently deregulated in cancer (Altomare and Testa, 2005). The phosphatidylinositol 3-kinases (PI3Ks) are a family of transducer kinases that play a central role in mediating growth factor signalling. PI3K is recruited to the ligand-activated receptor at the plasma membrane via its SH2 domain in the regulatory subunit, leading to the activation of PI3K; moreover, GTP-activated Ras could also bind and activate PI3K. Activated PI3K phosphorylates and converts Phosphatidylinositol 4,5-bisphosphate (PIP_2) to Phosphatidylinositol 3,4,5-triphosphate (PIP_3); then Akt/PKB (protein kinase B) could attach to PIP_3 via its pleckstrin homology (PH) domain, which has a very high affinity for PIP_3. Once

associated with plasma membrane, Akt/PKB becomes activated by PDK1 (Alessi et al., 1997). Activated Akt/PKB proceeds to phosphorylate mTOR (mammalian target of rapamycin) (Vander Haar et al., 2007), which further phosphorylates Akt, leading to its full activity (Sarbassov et al., 2005). Fully activated Akt/PKB acquires additional substrate specificity and phosphorylates a broad range of cytosolic and nuclear proteins, including GSK3β (glycogen synthase kinase β) (Nave et al., 1999), pro-apoptotic FOXO proteins (Guertin et al., 2006) and Mdm2 (Gottlieb et al., 2002; Zhou et al., 2001). Phosphorylated Mdm2 is released from its negative regulator ARF, becomes stabilized, and promotes the ubiquitination and degradation of p53 (Gottlieb et al., 2002; Zhou et al., 2001). The crosstalk between p53 and PI3K/Akt could also occur via the capability of p53 to upregulate the PI3K negative regulator PTEN (Stambolic et al., 2001) and repress PI3K catalytic subunit PIK3CA (Astanehe et al., 2008).

1.6 The interplay between ROS and p53

p53 is a redox-sensitive protein containing several cysteines, including three cysteines in the sequence-specific DNA binding domain crucial for its folding, thus it is subjected to regulation by ROS (reactive oxygen species). ROS are constantly generated by the biological reactions in the cells as products or side-products. ROS act as either toxic compounds or as secondary messengers by impacting various cellular processes, including cell proliferation, growth arrest and apoptosis. Ample evidence demonstrates that ROS levels are frequently elevated in cancer cells compared to normal cells (Kawanishi et al., 2006; Szatrowski and Nathan, 1991; Toyokuni et al., 1995). Cancer cells are under persistent oxidative stress due to various mechanisms, such as activation of oncogenes (Behrend et al., 2003; Vafa et al., 2002), malfunction of mitochondria (Brandon et al., 2006; Carew et al., 2003; Ishikawa et al., 2008) and inactivation of p53 function (Achanta et al., 2005; Liu et al., 2008). Therefore cancer cells might be well adapted to the increased ROS levels. It has been reported that a mild induction of ROS could even promote the proliferation and differentiation of cells (Boonstra and Post, 2004; Schafer and Buettner, 2001). However, excessive ROS insults might cause the oxidative damage to the macromolecules inside the cells (Perry et al., 2000). ROS could damage both the bases and sugar backbone of DNA and induce a variety of lesions, including DNA-protein adducts and cross-links of DNA-DNA (Berquist and Wilson, 2012). It has been shown that ROS play a role as both upstream regulators of p53 and downstream effectors of p53 (Hafsi and Hainaut, 2011; Maillet and Pervaiz, 2012). An integrated model about the crosstalk between ROS and p53 is shown in Figure 4.
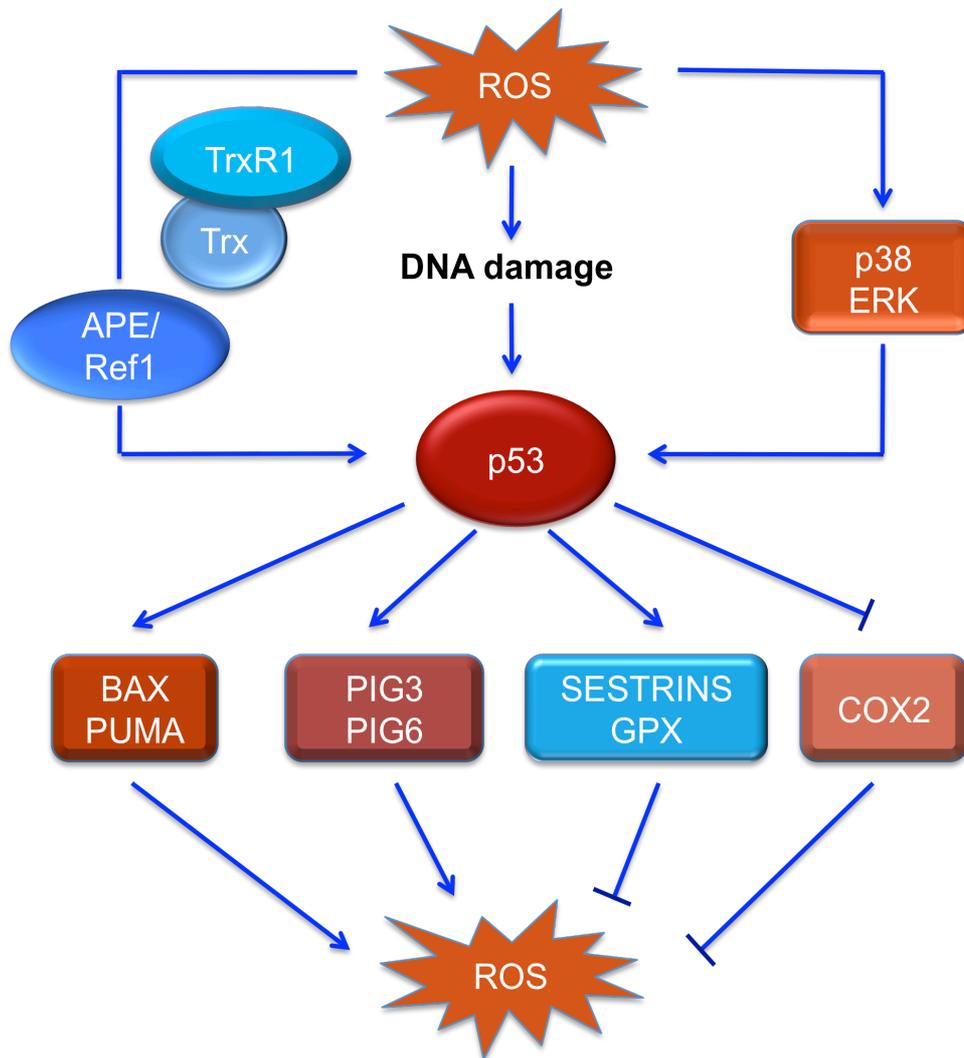
Figure 4. An integrated model of the interplay between ROS and p53. ROS could activate p53 via several mechanisms, such as induction of APE/Ref1, DNA damage response and activation of p38 or ERK; the basal activity of p53 could be regulated via APE/Ref1 or Trx-TrxR1; activated p53 controls the level of ROS by inducing a number of pro-oxidant genes, such as BAX, PUMA, PIG3, PIG6, and anti-oxidant genes SESTRIN1, SESTRIN2 and GPX or by inhibiting COX2, which is also an anti-oxidant gene.

1.61   *Redox regulation of p53*

The major redox control inside the cells is implemented by the glutathione system and the thioredoxin (Trx) system. GSH serves as a direct ROS scavenger or a substrate for glutathione peroxidase (GPX), which eliminates H_2O_2 (Townsend and Tew, 2003). It has been reported that p53 is a substrate for S-glutathionylation; moreover, glutathionylation of p53 impairs its DNA binding capability, leading to the reduced activity of p53 (Velu et al., 2007).

Trx is a family of evolutionary conserved proteins that could reduce the oxidized cysteine groups on proteins, with the support from TrxR (thioredoxin reductase), which could reduce Trx. Under physiological conditions, the basal stability and activity of p53 are regulated by TrxR1-Trx and APE/Ref-1 (Seemann and Hainaut, 2005). However, under stress conditions, the regulation of p53 by ROS is mainly attributed to DDR, which is induced by ROS (Hainaut and Mann, 2001). Besides the kinases involved in DDR, other kinases downstream of ROS could also modify and stabilize p53, such as mitogen-activated protein kinases (MAPK) p38 and ERK (Liu et al., 2008) (Figure 4).

1.62   p53 regulation of redox state of a cell

p53 regulates the redox signalling via its capability to regulate a set of genes involved in redox control. For example, p53 could bind to the promoter of GPX and induce its expression upon treatment with etoposide (Tan et al., 1999). Besides GPX, p53 target genes sestrin1 and sestrin2 were also reported to be involved in anti-oxidant defence (Budanov et al., 2004; Budanov et al., 2002; Kopnin et al., 2007). On the other hand, p53 could also transactivate PIGs (p53-induced genes), which have an impact on cellular redox status (Polyak et al., 1997); PIG3 and PIG6 have been shown to promote ROS production (Ostrakhovitch and Cherian, 2005; Rivera and Maxwell, 2005). Moreover, the p53 pro-apoptotic target genes Bax and Puma could also induce ROS in the mitochondria. In addition to transactivation, p53 could transrepress COX2 and SOD2, which have also been linked to ROS production (Jiang et al., 2004; Pani et al., 2000). Thus, via its target genes p53 can either prevent or induce ROS production, as illustrated in Figure 4.

1.7   Inactivation of p53 in cancer

As discussed above, p53 plays a fundamental role in the prevention of cancer formation and it is inactivated in almost all the tumors. There are two major ways to abrogate p53 function, namely mutations and deregulation of its negative regulators.

1.71   Mutations in p53

More than 50% of human cancers carry p53 mutations, 75% of which are missense mutations. Interestingly, 97% of these mutations occur in the exons encoding the DNA binding domain, suggesting the crucial role of its transcriptional function in cancer prevention. There are six hotspot mutations in this domain: R175, G245, R248, R249, R273 and R282 (Olivier et al., 2002; Petitjean et al., 2007). Individuals carrying the germline TP53 mutations display Li-Fraumeni syndrome or Li-Fraumeni-like syndrome, which are characterized by a high incidence of tumor development (Malkin, 1993). In addition to inactivating p53 transcription function, mutations in p53 have two other major consequences: dominant negative effect over the remaining wild type p53 allele (Petitjean et al., 2007) and gain-of-function effect (Goh et al., 2011; Lozano, 2007; Oren and Rotter, 2010).

The p53 transcriptional activity is dependent on the formation of tetramers, which are dimers of dimers. Dominant-negative effect of mutant p53 is due to impaired DNA binding and transcriptional activity of p53 tetramers formed via hetero-oligomerization between the mutant and wild type p53. The p53 hetero-oligomers might shift their binding specificity thereby activating some oncogenes, which can contribute to mutant p53 gain-of-function (Strano et al., 2007). Moreover, mutant p53 might bind other proteins, including NF-Y (Di Agostino et al., 2006; Liu et al., 2011) and p53 family proteins p63 and p73 (Gaiddon et al., 2001; Irwin, 2004; Strano et al., 2002), interfering with their activity and leading to the gain-of-function effect. Indeed, *in vivo* studies support the idea of gain-of-function of mutant p53. Compared to p53-null mice, mice expressing one hotspot mutant p53 allele show a broader tumor spectrum, increased metastasis and genomic instability (Lang et al., 2004; Liu et al., 2010; Olive et al., 2004).

Mutations in p53 could be roughly divided into two categories based on their influence on the thermodynamic stability of p53, namely DNA contact mutations which impair the DNA binding of p53, and structure mutations which change the folding of p53 (Muller and Vousden, 2013). However, even mutations in the DNA contact residues have also been reported to unfold p53 to some extent (Bullock et al., 1997), leading to the increased flexibility of p53 and the altered binding to its partners and DNA.

1.72   Inhibition by negative regulators

In cancers retaining the wild type p53 gene, the p53 pathway is also abrogated. The malfunction of the p53 pathway occurs primarily via the elevated expression of the p53 negative regulators, such as Mdm2, MdmX and Wip1. Upregulation of Mdm2 could be due to gene amplification, increased stability of its mRNA, elevated translation (Patterson et al., 1997; Riley and Lozano, 2012). In addition, inactivation of Mdm2 inhibitor ARF also releases Mdm2 and blocks p53 function (Esteller et al., 2001; Sherr and Weber, 2000). There are also other mechanisms which deregulate Mdm2 in cancer, including alterations in kinases regulating Mdm2, such as activation of Akt and inactivation of checkpoint kinases. Increased expression of MdmX is mainly attributed to gene amplification (Markey, 2011). Wip1 amplification and overexpression are frequently observed in breast cancers, neuroblastomas and adenocarcinomas (Bulavin et al., 2002; Li et al., 2002; Saito-Ohara et al., 2003); moreover, Wip1 has been reported to stabilize Mdm2 and MdmX, leading to the reduced activity of p53 (Lu et al., 2007; Zhang et al., 2009).

2   p53 AS A THERAPEUTIC TARGET

Since the p53 function is inhibited in the majority of tumors, suggesting that its inactivation is required for tumor growth, and that the p53 protein, albeit inactive, is expressed in cancers, pharmacological reactivation of p53 seems to be a promising strategy to combat cancer. Several elegant *in vivo* studies have validated that restoration of p53 confers increased survival of mice with established tumors in Eμ-myc lymphoma model (Martins et al., 2006) and leads to the regression of established tumors in autochthonous lymphoma and sarcoma model (Ventura et al., 2007) and liver carcinoma model (Xue et al., 2007). Interestingly, restoration of p53 fails to perturb the early-stage tumors, but rather induces the regression in the high-grade tumors (Feldser et al., 2010; Junttila et al., 2010). The capability of p53 to eliminate the late-stage tumors was correlated with the high levels of oncogenic signaling, such as MAPK signaling, and activation of p19^{ARF} (Feldser et al., 2010; Junttila et al., 2010). The selective eradication of advanced tumors by p53 reinstatement might impede the therapeutic effect of p53 reactivation, as the early lesions within the heterogeneous tumors might eventually develop into malignant tumors again. However, patients coming into clinic are usually diagnosed with malignant tumors which account for most of the tumor mass. Therefore, pharmaceutical reactivation of p53 is expected to increase the survival and extend the lifetime of these patients.

2.1   Pharmacological reactivation of wild type p53

In light of the crucial role of the negative regulators Mdm2 and MdmX for p53 inactivation in cancer, disruption of the interaction between p53 and Mdm2/MdmX is the most promising approach to treat cancer with intact wild type p53.

Several traditional chemotherapeutic drugs, such as 5-FU (fluorouracil) and Cisplatin can inhibit the interaction between p53 and Mdm2/MdmX followed by p53 activation via triggering DNA damage response. However, they induce severe side effects in normal tissues. On the contrary, targeted therapy is expected to have less side effects as it specifically targets pathological proteins or protein-protein interactions and thus blocks the growth and proliferation of cancer cells, leaving the normal cells less perturbed.

Unlike inhibiting the enzymatic activity of tyrosine kinases, targeting protein-protein interactions presents a big challenge, as the binding surfaces between two proteins are usually large and contain numerous intermolecular contacts. Fundamental work by Clackson and Wells illustrated that in the large binding surface between two proteins, there exist some key residues that account for most of the binding free energy (Clackson and Wells, 1995). Moreover, the dimension of these key residues resembles the size of a small organic molecule; thus targeting the key residues in the binding cleft by small molecules provides a powerful strategy to disrupt the interaction between two proteins.

Examples of small molecules that could disrupt the interaction between p53 and Mdm2 are nutlins (Vassilev et al., 2004), RITA (Issaeva et al., 2004), MI219 (Shangary et al., 2008), benzodiazepenes (Grasberger et al., 2005) and others.

Wild type p53 could also be activated by other mechanisms. For example, tenovins reactivate p53 via the inhibition of SIRT1 and SIRT2 (Lain et al., 2008); actinomycin D stabilizes and activates p53 by releasing ribosomal proteins RPL5 and RPL11, which could interact with MDM2 and block MDM2 function (Lohrum et al., 2003); leptomycin B prevents p53 nuclear export by inhibiting the export protein CRM1 (Mutka et al., 2009).

2.2   Pharmacological reactivation of mutant p53

95% of p53 mutations occur in the core domain and 75% of the mutations are missense mutations, resulting in the expression of a full-length, but misfolded p53 protein with impaired transcriptional activity. Moreover, mutant p53 tends to aggregate inside the cells (Ano Bom et al., 2012; Wang and Fersht, 2012; Wilcken et al., 2012). Thus, small molecules that could increase thermo-stability of mutant p53 and restore the misfolded mutant p53 protein to wild type p53 conformation, have the potential to rescue the wild type p53 transcriptional function.

Examples of small molecules that could which restore the mutant p53 to its wild- type conformation are PRIMA-1^{MET} (APR-246) (Bykov et al., 2002), CP31398 (Foster et al., 1999) and Phikan083 (Boeckler et al., 2008).

2.3   Small molecules studied in the thesis

As my thesis focuses on the study of the wild type p53 reactivators nutlin and RITA, I will introduce them in a more detailed manner.

2.31   Nutlins

Nutlins are a family of small molecules identified in a biochemical screen aimed to identify molecules that disrupt the interaction between p53 and Mdm2 (Vassilev et al., 2004). Crystal structure of the nutlin-bound Mdm2 protein demonstrated that nutlin directly binds the p53-binding pocket in the Mdm2 protein, verifying the target specificity. Further study showed that nutlin3a is the most potent inhibitor of the p53 and Mdm2 interaction in this class of molecules, while its enantiomer nutlin3b is 150 times less active (Vassilev et al., 2004). Importantly, nutlin3a induced p53-dependent growth suppression both *in vitro* and *in vivo*. Interestingly, nultin3a preferentially induced apoptosis in SJSA-1 and RKO cells, while cell cycle arrest is more prominent in other cancer cell lines (Enge et al., 2009; Tovar et al., 2006). Importantly, the nutlin derivative RG7112 has recently been tested in liposarcoma patients in the phase I clinical trial and showed very encouraging results (Ray-Coquard et al., 2012).

2.32 RITA

RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis, known also as NSC 652287) has been identified by our laboratory in a cell-based screen of NCI library compounds (Issaeva et al., 2004). RITA directly binds p53, induces a conformation change of p53 and disrupts the interaction between p53 and Mdm2, leading to the stabilization and activation of p53. RITA triggers p53-dependent growth inhibition both *in vitro* and *in vivo*. Notably, RITA induced p53-dependent apoptosis in a variety of cancer cell lines of different origin (Issaeva et al., 2004). In addition to reactivation of wild type p53, RITA can also reactivate mutant p53 and induce apoptosis in a mutant p53-dependent manner (Zhao et al., 2010).

RITA was originally reported to induce the cross-links of DNA-DNA or DNA-protein, resulting in the DNA damage response (Nieves-Neira et al., 1999). However, a recent study demonstrated that the induction of DNA damage response by RITA is p53-dependent (Yang et al., 2009), ruling out the possibility that RITA is a general DNA intercalator.

Interestingly, RITA preferentially induces apoptosis in a panel of cancer cell lines containing wild type p53, while nutlin3a triggers cell cycle arrest (Enge et al., 2009). A mechanistic study revealed that upon RITA treatment, Mdm2 is released from p53 and targets hnRNP K, which is a p53 transcriptional cofactor for induction of p21, to the proteasomal degradation. Moreover, Mdm2 itself binds p21 and brings it to the proteasome for degradation. In contrast, upon nutlin3a treatment, Mdm2 is bound by nutlin3a and is unable to degrade hnRNP K and p21 (Enge et al., 2009). However, nutlin3a-bound, but not RITA-released Mdm2 is able to degrade HIPK2, a kinase that could phosphorylate Ser46 of p53 and promote pro-apoptotic function of p53 (Rinaldo et al., 2009). The exact mechanism of how Mdm2 achieves its different target specificity upon treatment with RITA and nutlin remains elusive.

3 AIMS OF THE THESIS

p53 is a potential therapeutic target. For the clinical application of p53-based therapies we must decipher the molecular mechanisms of the p53-mediated pro-apoptotic function upon its pharmacological activation.

Specific aims:

To investigate the molecular mechanism of apoptosis induced by RITA-activated p53 in cancer cells (**Paper I**)

To study the role of ROS on the p53-mediated apoptosis response (**Paper II**)

To investigate whether and how RITA triggers the apoptosis and inhibition of oncogenes in neuroblastoma cells *in vitro* and *in vivo* (**Paper III**)

4 RESULTS AND DISCUSSION

This thesis is comprised of three papers focusing on deciphering the molecular mechanisms of the anti-tumor effects of pharmacologically activated p53 and the potential therapeutic implications.

Paper I

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

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* equal contribution

Genome-wide gene expression analysis revealed that the expression of a set of crucial oncogenes including *IGF1R, PIK3CA, PIK3CB, MYC, EIF4E, BCL2, MAP4, MCL1* and *BIRC5* was significantly inhibited upon RITA treatment in HCT116 and MCF7 cells carrying wild type p53, but not in the isogenic p53-null HCT116 cells. The p53-dependent downregulation of this set of oncogenes was validated by quantitative real-time PCR (qPCR) in the p53-positive and –negative cells and further supported by the experiments performed with p53 pifithrin-α, which inhibits p53 transcriptional activity. The p53-dependent downregulation of IGF-1R, c-Myc, survivin, Mcl-1 was also observed on the protein level both *in vitro* and *in vivo*. Importantly, the decrease of these factors was not observed in non-tumorigenic cells.

RITA-activated p53 inhibited the activity of several key components of the PI3K/Akt/mTOR pathway, such as Akt, mTOR and eIF4E. The inhibition of eIF4E led to the reduced translation of IGF-1R and c-Myc. Moreover, activation of GSK3β due to the inhibition of Akt also contributed to the c-Myc depletion. Notably, downregulation of c-Myc and cyclin E was mediated by FBW7/hCdc4, an E3 ubiquitin ligase which was induced by RITA-activated p53. Thus, we conclude that RITA-activated p53 triggers the downregulation of c-Myc via both transcriptional and posttranscriptional mechanisms.

Intriguingly, the protein levels of p53 and the pro-apoptotic p53 target genes Puma and Noxa were induced to a similar extent by 0.1 and 1 μM RITA. However, these two doses of RITA triggered different biological responses, namely transient growth arrest and apoptosis, respectively. A meticulous investigation of the underlying mechanism demonstrated that the set of oncogenes mentioned above was differentially regulated by 0.1 and 1 μM RITA: only 1 μM RITA was able to trigger the dramatic downregulation of c-Myc, Mcl-1 and survivin on the protein level, while the decrease of these oncogenes was either absent or less prominent upon 0.1 μM RITA treatment. qPCR confirmed the dose-dependent repression of these genes. At this stage, we concluded that the induction of pro-apoptotic genes by p53 is not sufficient to trigger robust apoptosis; the inhibition of pro-survival factors by p53 is necessary for the induction of a full-scale apoptotic response.

As the total level of p53 induced by these two doses of RITA was similar, we examined whether there were any differences in the subcellular localization of p53. Strikingly, compared to 0.1 µM RITA, 1 µM RITA led to a much higher level of chromatin-bound p53. This could be the reason for a more robust p53 response upon higher dose of RITA.

As Mdm2 has been reported to bind p53 at the promoters of p53 target genes, we also investigated the level of chromatin-bound Mdm2 upon 0.1 and 1 µM RITA treatment. We found that in contrast to 0.1 µM RITA, 1 µM RITA was more efficient to deplete Mdm2 on the chromatin. This indicates that the ratio of p53/Mdm2 on the chromatin is much higher upon 1 µM RITA treatment. Moreover, ChIP experiments showed that both doses of RITA could increase the ratio of p53/Mdm2 at the promoter of p53-activated gene *CDKN1A*. However, only 1 µM RITA was capable to increase the ratio of p53/Mdm2 on the promoter of p53-repressed gene *MCL1*. This indicates that p53 mediated transcriptional repression is more tightly controlled by Mdm2 than transcriptional activation.

To address the contribution of oncogene inhibition to p53-mediated apoptosis, we combined the depletion of key oncogenes by siRNA with 0.1 µM RITA treatment. We found that siRNA-mediated depletion of c-Myc or Mcl-1 significantly enhanced the apoptosis induced by 0.1 µM RITA, supporting the crucial role of oncogene inhibition in p53-mediated apoptotic response. Moreover, inhibition of Mcl-1 or c-Myc converted growth arrest induced by nutlin into cell death.

Our results suggest that the ability of p53 to preferentially kill cancer cells, but not normal cells, might be attributed to the transcriptional repression of major oncogenes, thus targeting 'oncogene addiction', i.e., dependence of cancer cells on oncogenic signaling. It would be interesting to address in future studies whether reconstitution of p53 in established cancers can disable the survival program in cancer cells.

In this study, we have shown for the first time that the ability of p53 to activate and repress gene expression is differentially regulated. This leads us to investigate in our next study, which factors could regulate p53-mediated transcriptional repression and make p53 such a potent repressor of transcription, as discussed below (Paper 2).

In conclusion, our data indicate that the activation of pro-apoptotic genes and the inhibition of oncogenes are differentially regulated by p53, via a threshold mechanism involving p53 and Mdm2, as illustrated in Figure 5. We suggest a model in which two distinct p53-dependent transcriptional programs are essential for the induction of a full-scale apoptosis. The simple induction of the pro-apoptotic proteins might not be enough to overcome the pro-survival buffer, leading to the incomplete apoptotic response. Downregulation of pro-survival factors could be orchestrated with the upregulation of pro-apoptotic proteins to shift the survival/death balance to trigger a robust apoptotic response. In addition to controlling the stability of p53, Mdm2 also contributes to the regulation of these two transcriptional programs via direct

association with p53 on the promoters of p53 target genes. Compared to transcriptional activation, p53-mediated transcriptional repression might be more tightly controlled by Mdm2.

Our study also implies the potential application of combinational treatment with p53 activators and oncogene inhibitors in the clinic setting.
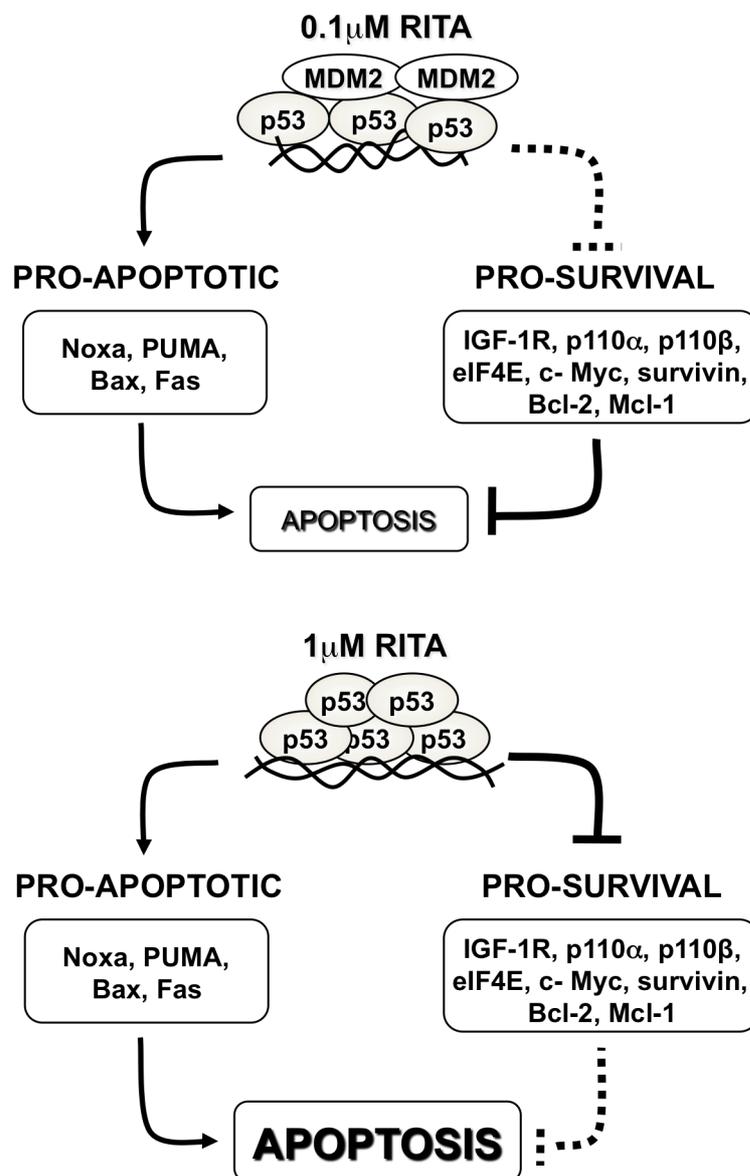

Figure 5. Two distinct branches of p53-dependent transcriptional program are required for a robust apoptosis response. Upper panel; a low dose of RITA is able to release Mdm2 from the promoter of p53 pro-apoptotic genes and activate these genes; however, this low dose of RITA is not sufficient to release Mdm2 from the pro-survival genes, leaving the pro-survival genes unperturbed and leading to the incomplete apoptosis. Lower panel: 1 μM RITA is capable to release Mdm2 from both p53 pro-apoptotic genes and p53-repressed pro-survival genes, resulting in the concurrent activation of p53 pro-apoptotic genes and inhibition of pro-survival genes. Engagement of these two branches induces a full-scale apoptosis.

Paper II

ROS-dependent activation of JNK converts p53 into an efficient inhibitor of oncogenes leading to robust apoptosis

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Systematic clustering analysis of the microarray data obtained in breast carcinoma MCF7 cells treated with 10 μM nutlin, 0.1 and 1 μM RITA at 10 different time points revealed that only 1 μM RITA induced a sustained response (either induction or repression) of several clusters of genes. In contrast, the response of the same clusters of genes was transient upon 0.1 μM RITA and 10 μM nutlin treatment. In accordance, transcriptional activation or repression of p53 target genes was also sustained upon the treatment with 1, but not 0.1 μM RITA.

We speculated that the transient versus sustained response might be due to the DNA damage response (DDR). Indeed, the DNA damage signaling was induced by 1, but not by 0.1 μM RITA in tumor cells. In addition, it was not induced by 1 μM RITA in non-tumorigenic cells. Intriguingly, no strand breaks were observed upon 1 μM RITA treatment. Moreover, the DNA damage signaling induced by RITA was independent of the canonical ATM/ATR-mediated DDR pathway. Further investigation demonstrated that it was the induction of ROS that triggered the DNA damage signaling. Induction of ROS was due to the efficient inhibition of the reducing activity of TrxR1 by RITA, but not its oxidase activity.

Further, we found that ROS was also the determinant of the apoptotic response upon p53 reactivation. Notably, a low dose of the TrxR1 inhibitor auranofin was synthetically lethal with p53 reactivation by the low dose of RITA or nutlin, while single treatment with these compounds was not sufficient to induce robust apoptosis. Furthermore, the ROS scavenger NAC could revert the synthetic lethality upon these combinational treatments. Notably, ROS inhibitors could rescue the depletion of the survival genes upon RITA treatment. These data suggest that the induction of ROS by RITA is a cause of the profound downregulation of several key survival genes, such as Wip1 (encoded by *PPM1D*), Mcl-1 and MdmX (*Mdm4*).

As extensively discussed in paper I, p53-dependent inhibition of oncogenes plays a crucial role in a full-scale induction of apoptosis upon RITA treatment. Therefore we investigated which factors mediate the ROS signaling to p53 and the inhibition of oncogenes. Taking into account that JNK has been reported to be activated by the inhibition of TrxR1 and the fact that JNK could modulate p53 activity, we thought that JNK could be a link between the ROS signaling and p53. Therefore we examined the role of JNK in mediating the ROS signaling to p53. We found that JNK was phosphorylated and activated in a p53- and dose-dependent manner upon RITA treatment. Moreover, activation of JNK by RITA was also ROS-dependent,

suggesting that JNK is downstream of ROS signaling. Notably, p53-dependent activation of DNA damage signaling and inhibition of the crucial oncogenes Wip1 (encoded by *PPM1D*), Mcl-1 and MdmX (*Mdm4*) were JNK-dependent. In addition to *PPM1D*, *MCL1* and *MDM4*, the repression of *PIK3CA*, *PIK3CB* and *EIF4E* was also JNK-dependent. Strikingly, the Wip1 level was differentially regulated by 0.1 μM RITA and 1 μM RITA: while 0.1 μM RITA could induce the expression of Wip1, 1 μM RITA led to the profound downregulation of Wip1; moreover, the repression of Wip1 by 1 μM RITA was rescued by the JNK inhibitor, as well as by siRNA mediated depletion of JNK. This indicates that activation of JNK could convert p53 from a transactivator to a transrepressor of Wip1.

Since Wip1 is a critical negative regulator of the DNA damage response, we went on to evaluate the role of Wip1 in p53-mediated DNA damage signaling and apoptotic response. The depletion of Wip1 significantly enhanced the DNA damage signaling and promoted the apoptosis induced by RITA. Microarray analysis showed that the Wip1 depletion facilitated the p53-mediated transcriptional activation, but not transcription repression, as further validated by qPCR.
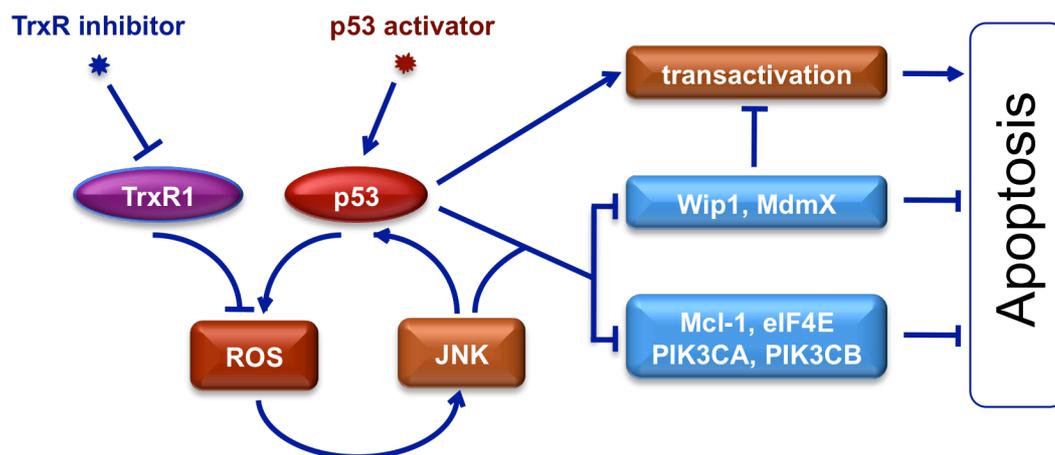

Figure 6. Concordant activation of p53 and inhibition of TrxR1 lead to the synthetic lethality in cancer cells. p53 is stabilized and activated upon its release from Mdm2; at the same time, TrxR1 inhibition results in the generation of ROS followed by the activation of JNK, which promotes the further activation of p53; in turn, activated p53 induces its pro-oxidant target genes, such as PIG3, PIG6 and Puma, leading to the further accumulation of ROS and activation of JNK. Activated JNK converts p53 into an efficient transcriptional repressor of Mcl-1, eIF4E, PIK3CA and PIK3CB, as well as p53 own inhibitors Wip1 and MdmX, thus further activating p53. Establishment of the positive feedback loop of ROS-JNK-p53 and disruption of the negative feedback loop of p53-Wip1 promotes the sustained p53 transcriptional response, leading to the robust apoptosis.

Based on our results, we propose a model (Figure 6) in which inhibition of TrxR1 followed by accumulation of ROS works in concert with p53 released from its negative regulator Mdm2 to activate JNK, which further promotes the p53 activation.

Activated JNK converts p53 into an efficient repressor of several crucial pro-survival factors including Mcl-1, eIF4E, PIK3CA and PIK3CB, as well as Wip1 and MdmX, which are p53's own negative regulators. This creates a positive feedback loop of ROS-JNK-p53 and disrupts a negative feedback loop of p53-Wip1, resulting in a robust apoptotic response.

Taken together, our results demonstrate that simultaneous activation of p53 and inhibition of TrxR1 followed by generation of ROS result in synthetic lethality in cancer cells.

Previous study has shown that RITA could induce DNA-DNA as well as DNA-protein cross-links; however it does not intercalate into DNA, nor induce DNA strand breaks (Nieves-Neira et al., 1999). A recent study has demonstrated that DDR induced by RITA is p53-dependent, confirming that RITA is not a general DNA intercalator (Yang et al., 2009). Based on these data and our previously published results that the induction of ROS by RITA is p53-dependent and only occurs in tumor cells, but not in normal cells, we propose the following idea. We speculate that the induction of ROS might be the cause of the observed DNA-DNA and DNA-protein cross-links and p53-dependent DDR upon RITA treatment.

Due to oncogene activation, aberrant energy metabolism, deregulation of mitochondria and several other mechanisms, cancer cells are under persistent intrinsic oxidative stress and frequently exhibit increased ROS levels. However, excessive ROS insults could result in oxidative damage to cells, leading to apoptosis. The adaptation of cancer cells to high endogenous ROS levels is achieved mainly via the development of mechanisms to efficiently scavenge excessive ROS and evade the apoptosis. Thus, it is believed that compared to normal cells, cancer cells are more dependent on the anti-oxidant systems to survive. Thus, inhibition of glutathione system or thioredoxin system is a feasible strategy to selectively kill the cancer cells. As auranofin is a FDA-proved drug and nutlin is currently under clinical trials, our data on the synthetic lethality of nutlin and auranofin combination might encourage the combinational treatment of these drugs in clinic and thus can help patients to combat cancer.

Paper III

Dual targeting of wild type and mutant p53 by small molecule RITA results in the inhibition of N-Myc and key survival oncogenes and kills neuroblastoma cells in vivo and in vitro

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To test whether RITA inhibits the growth of neuroblastoma cells, we performed the cell proliferation assay using WST-1 in a set of seven neuroblastoma cell lines with different status of p53 and N-Myc. We found that RITA efficiently suppressed the growth of all these cell lines. This indicates that RITA could reactivate both wild type and mutant p53 in neuroblastoma and inhibit the growth of N-myc amplified neuroblastoma cells. Moreover, RITA induced apoptosis in all these seven neuroblastoma cell lines. Importantly, the apoptosis induced by RITA was p53-dependent, as depletion of p53 by RNAi or pifithrin-α blocked the apoptosis induced by RITA.

RITA-activated p53 led to the induction of the p53 pro-apoptotic target genes Puma, Noxa and Bax on the protein level, irrespective of p53 status, suggesting that RITA restored both wild type and mutant p53 transactivation function. Moreover, induction of Puma (*BBC3*) and Bax also occurred on the mRNA level, as analyzed by the quantitative real-time PCR. Moreover, RITA-activated p53 triggered the profound downregulation of several crucial oncogenic factors, including Bcl-2, Mcl-1, Wip1, MdmX and Mdm2 on the protein level. In accordance, we observed the prominent downregulation of Bcl-2, Mcl-1, Wip1 and Aurora kinase on the mRNA level, which was p53-dependent, as pifithrin-α prevented the downregulation of these oncogenes by RITA.

However, inhibition of N-Myc was not observed on the mRNA level, but only on the protein level upon RITA treatment. Moreover, the depletion of N-Myc was dependent on the E3 ligase FBW7, which is a p53 target gene induced by RITA-activated p53. Interestingly, in the SKN-DZ cells carrying the wild type p53, inhibition of N-Myc was partially p53-dependent, while the inhibition of N-Myc in the SKN-BE(2) cells which harbor mutant p53, was p53 independent, suggesting that N-Myc downregulation in this cell line is controlled via other mechanisms.

To investigate whether RITA could suppress the tumor growth *in vivo*, we injected SKN-DZ cells, which carry wild type p53 and N-Myc amplification subcutaneously into the SCID mice. Intra-peritoneal injection of RITA led to the substantial suppression of tumor growth without the loss of body weight.

We conclude that RITA could reactivate both wild type and mutant p53, leading to the induction of p53 proapoptotic target genes, as well as the downregulation of key oncogenes including N-Myc, MdmX, Wip1, Mcl-1, Bcl-2, Mdm2 and Aurora kinase, resulting in strong anti-tumor effect both *in vitro* and *in vivo*.

It has been shown that nutlin reactivates wild type p53 but not mutant p53 in neuroblastoma. Notably, continuous treatment with nutlin leads to the generation of *de novo* p53 mutations, resulting in the drug resistance and relapse. Our data suggest that RITA could overcome this problem by reactivating both wild type and mutant p53.

Given the critical role of Bcl-2 and Mcl-1 in buffering the pro-apoptotic stimuli, the simultaneous inhibition of these two oncogenes by RITA-activated p53 might be essential to produce a robust apoptotic response. Moreover, the deregulation of N-Myc and Wip1 has been reported to be linked to a poor prognosis and resistance to therapy. Therefore, down-regulation of N-Myc and Wip1 by RITA might be important for the therapeutic effect. Aurora A, which is another poor prognostic factor of reduced survival in neuroblastoma, was also suppressed by RITA. The capability of p53 to down-regulate multiple pro-survival genes might allow this tumor suppressor to kill cancer cells irrespective of the particular combinations of mutations in a given cell, as well as of genetic heterogeneity of tumors.

5 CONCLUDING REMARKS

It was a long way for me to approach the cancer biology field with specific focus on targeted therapy. Up till now, I still clearly remember that when I was only nine years old, my grandfather was diagnosed with late stage lung cancer; since then, he had been lying in bed for approximately two years without any efficient therapy before he passed away. It was such a painful and helpless period that I will always remember.

Ten years later, I did not hesitate to take the pharmaceutical engineering as my major for the bachelor study. After four years of my undergraduate studies, it was time for me to select the subject for further study. At that time, I believed the most important thing to do for cancer therapy was to find the best drugs; therefore in 2003, I joined professor Yuezhong Li's lab working with myxobacterium *Sorangium cellulosum*, which produces the epothilone, a paclitaxel-like microtubule stabilization compound. Comparing to paclitaxel, epothilone has better water solubility, simpler chemical structure and is much more efficient in clinical trials. My research focused on increasing the yield of epothilone by fermentation from *Sorangium cellulosum*. However, soon I realized that epothione also belongs to a type of drug called "chemotherapy drug", which is commonly used in clinics but associated with severe side effects. Targeted therapy should be much more efficient in eliminating cancer cells, while leaving the normal cell less perturbed. At that time, the best characterized targeted therapy was the inhibitors of tyrosine kinases, such as imatinib, which inhibits the BCR-Abl.

Two years later in 2005, with the help of China Scholarship Council, I got a chance to study in the master program of Molecular Biotechnology and Bioinformatics in Uppsala University. I really expected to learn a lot from this program. Indeed, it opened several new fields to me, such as RNA biology, protein crystallography, protein-protein interactions and bioinformatics. During my master thesis, I joined Per Jemth's group, who is investigating the structure-function relationships of proteins using biophysical methods, as I think the final identity that executes the biological function in a cell is the protein and it is so important to understand the function of protein based on its structure, or even on its amino acid sequence.

In 2007, after I finished my master program study, I decided to perform my PhD education in a lab with specific focus on the study of some targeted therapy drugs or some lead compounds which have great potential in the future clinic application. There are several excellent groups working with promising lead compounds at Karolinska Institutet, and one of them is Galina Selivanova's group. Her group is working on the small molecule RITA, which has already been shown to bind p53, disrupt the interaction between p53 and Mdm2, and inhibit tumor growth without obvious toxicity both *in vitro* and *in vivo*. To my delight, professor Galina provided me the opportunity to perform my PhD study in her group. It is the best choice I have ever made in my career, because she introduced me to such a fantastic and challenging research filed!

p53 is a master tumor suppressor whose function is abrogated in almost all types of human cancers either by point mutations or via deregulation of its negative regulators, such as Mdm2. From the therapeutic point of view, p53 is a very good target as its inactivation is tumor specific and restoration of its function should prevent the tumor growth while leaving normal cells less perturbed. Recent *in vivo* studies have proven that reinstatement of p53 function is very efficient in eliminating cancer cells in established tumors; notably, the capability of restored p53 to suppress tumor growth is more prominent in advanced tumors, but not in early lesions, suggesting that certain signaling pathways exist in malignant tumors contributing to p53 tumor suppression function. In this sense, my finding that concurrent restoration of p53 function and ablation of TrxR1 activity followed by the generation of ROS could be a good strategy to selectively kill cancer cells and it is worth to test this idea *in vivo*.

Several p53-reactivating molecules, including PRIMA-1^{MET}(APR-246), Nutlin analog (RG7112) and MI-219 have entered the clinical trials and shown promising therapeutic benefits, while the side effects of these compounds are much less pronounced than the ones observed with the traditional chemotherapeutic drugs. I have no doubt that p53-reactivating compounds will be a common prescription used in the clinics in the near future and I beleive that they will rescue the life of a tremendous amount of patients.

6 ACKNOWLEDGEMENTS

7 REFERENCES

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Ablation of Key Oncogenic Pathways by RITA-Reactivated p53 Is Required for Efficient Apoptosis

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DOI 10.1016/j.ccr.2009.03.021

SUMMARY

Targeting "oncogene addiction" is a promising strategy for anticancer therapy. We report a potent inhibition of crucial oncogenes by p53 upon reactivation by small-molecule RITA in vitro and in vivo. RITA-activated p53 unleashes the transcriptional repression of antiapoptotic proteins Mcl-1, Bcl-2, MAP4, and survivin; blocks the Akt pathway on several levels; and downregulates c-Myc, cyclin E, and β-catenin. p53 ablates c-Myc expression via several mechanisms at the transcriptional and posttranscriptional level. We show that the threshold for p53-mediated transrepression of survival genes is higher than for transactivation of proapoptotic targets. Inhibition of oncogenes by p53 reduces the cell's ability to buffer proapoptotic signals and elicits robust apoptosis. Our study highlights the role of transcriptional repression for p53-mediated tumor suppression.

INTRODUCTION

The notion that initial oncogenic lesions remain essential for tumor maintenance is supported by a number of studies, including in vivo experiments in mice switching off Myc (Felsher and Bishop, 1999; Pelengaris et al., 2002), BCR-ABL (Huettner et al., 2000), or H-ras (Chin et al., 1999). "Oncogene addiction," i.e., the dependency of tumor cells on oncogenic activity that initially contributed to tumor phenotype, first coined by Weinstein (2002), potentially reveals an "Achilles' heel" of cancer cells. Targeting this "Achilles' heel" is currently a major strategy for the development of novel anticancer drugs.

Strategies aimed toward restoring the function of the tumor suppressor p53 have been much less popular so far. Recent studies in mice with "switchable" p53 demonstrated that restoration of p53 function leads to the suppression of already established tumors, such as lymphomas, soft tissue sarcomas, and

hepatocellular carcinomas (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). The important conclusion from these studies is that developed tumors remain vulnerable to p53 restoration. Taken together with the identification of *TP53* as the most commonly mutated gene in a recent systematic study of genetic alterations in breast and colon cancer (Sjoblom et al., 2006), these findings firmly support the notion that restoring p53 function might be an attractive strategy for treating cancer. Reactivation of p53 appears to be feasible, because p53 protein is usually expressed in tumors, although it is functionally inert.

Different strategies of p53 rescue for the selective elimination of tumors could be envisioned, depending on the type of p53 inactivation. Refolding mutant p53 in tumors carrying *TP53* point mutations appears to be a promising approach (Bykov et al., 2002). In tumors carrying wild-type p53, p53's function is often inhibited by MDM2, which binds p53, inhibits its transcriptional function, and promotes proteasomal degradation of p53 (Haupt

SIGNIFICANCE

p53 reinstatement leads to impressive regression of established tumors in mice, supporting the idea that restoring p53 is a good strategy in cancer treatment. Our study adds another dimension to the p53 story, demonstrating that p53 reactivation triggers ablation of crucial oncogenes. The multitude of oncogenes inhibited by p53 and the multiple levels on which they are targeted create external robustness of the p53 response. This capability might allow p53 to cope with the daunting challenge of anticancer therapy: multiple genetic abnormalities in individual cancers. Our finding that a combination of a low dose of p53-reactivating drug with oncogene inhibitors produced a synergistic effect provides a rationale for drug combinations to minimize side effects and newly developed resistance in patients.

et al., 1997; Kubbutat et al., 1997). Several classes of small molecules inhibiting the p53/MDM2 interaction or targeting the enzymatic activity of MDM2 have been reported (Lain et al., 2008; Vassilev et al., 2004; Yang et al., 2005). We have identified a small molecule RITA, which induces p53 accumulation and activation and suppresses the growth of tumor cells and human tumor xenografts in mice in a p53-dependent manner without obvious toxic effects (Issaeva et al., 2004). In addition to serving as lead compounds for the development of anticancer drugs, p53-reactivating molecules, such as RITA, can be useful tools for the study of p53 functional activity.

It has been well established that p53 is a transcriptional factor that regulates the expression of genes involved in control of the cell cycle and cell death upon activation by genotoxic or oncogenic stress (Vogelstein et al., 2000). p53 can activate the transcription of the proapoptotic genes *PUMA*, *PMAIP*, *Bax*, *Fas*, and others (Vogelstein et al., 2000), along with repression of the transcription of the survival genes *Bcl-2*, *MAP4*, *BIRC5* (survivin), *Mcl-1*, *IGF-1R*, *MYC*, *EIF4E*, and *PIK3CA* (Miyashita et al., 1994; Murphy et al., 1996; Hoffman et al., 2002; Pietrzak and Puzianowska-Kuznicka, 2008; Werner et al., 1996; Ho et al., 2005; Zhu et al., 2005; Astanehe et al., 2008). According to the current view, transrepression by p53 might occur via different mechanisms, including steric interference, squelching of the transcriptional activators, and p53-mediated recruitment of histone deacetylases (Riley et al., 2008). However, the relative contribution of transactivation and transrepression functions in the p53-induced biological response has not been established yet.

The question of how p53 chooses between its different targets received great attention, due to its paramount relevance to cancer therapy (Oren, 2003). The response of cells to p53 can vary greatly depending on a cellular context, the key component being the presence of survival signals, which render cells resistant to apoptosis. The overexpression of factors blocking apoptosis downstream of p53, such as Mcl-1 or Bcl-2, might lead to escape from p53-induced cell death. It is believed that when survival signals prevail, p53 activation will more likely result in growth arrest (Lowe et al., 2004; Oren, 2003). Thus, it remains to be elucidated whether p53 activation can counteract survival signaling, which is persistently expressed in cancer cells.

Using the p53-reactivating molecule RITA, we addressed the questions of whether and how p53 can overcome antiapoptotic and survival signals. We demonstrate that p53 activated by RITA represses the set of prosurvival oncogenes that play a critical role in p53-induced apoptosis.

RESULTS

Transcriptional Repression of Oncogenes upon p53 Reactivation by RITA

To explore the effects of restoring p53 function in tumor cells, we analyzed the changes in gene expression in isogenic p53-positive and p53 null HCT116 colon carcinoma cells after treatment with 1 μM RITA by using genome-wide DNA microarrays (Affymetrix; for details, see Enge et al. [2009]). Upon RITA treatment, a significant number of genes were downregulated in a p53-dependent manner, including the oncogenes *IGF-1R*, *PIK3CA*, *PIK3CB*, *MYC*, *EIF4E*, *BCL-2*, *MAP-4*, and *MCL-1* (Figure 1A). To test whether a similar effect occurs in a tumor cell line of

a different origin, we performed a DNA microarray experiment in breast carcinoma MCF7 cells addressing the kinetics of transcriptional repression upon RITA treatment (Figure 1B). We observed a very good correlation with the HCT116 microarray data. p53 reactivation resulted in strong transcriptional repression of the same set of oncogenes, with the exception of *EIF4E* and *MAP-4*, whose levels were not affected.

To verify our microarray data, we examined the mRNA levels of these genes by quantitative real-time PCR (qPCR). We observed a marked downregulation of the mRNA levels of *IGF-1R*, *PIK3CA*, *PIK3CB*, *MYC*, *EIF4E*, *BCL-2*, *MAP-4*, and *MCL-1* in both HCT116 and in MCF7 cells (Figures 1C and 1D, respectively). According to qPCR, transcriptional repression of oncogenes was much stronger in MCF7 cells, compared with HCT116 cells.

Transrepression of oncogenes was dependent on p53, because we did not detect any changes in the expression of these genes after RITA treatment in the p53 null cell lines HCT116 *TP53*$^{-/-}$ (Figure 1C), Saos-2, and H1299 (Figure 1E). In order to address p53 dependence in MCF7 cells, we blocked p53 function by using the small-molecule p53 inhibitor pifithrin-α (Komarov et al., 1999) or p53shRNA. Pifithrin-α was a superior p53 inhibitor compared to p53 depletion by shRNA, completely blocking p53 induction by RITA, whereas p53shRNA had only a partial effect (Figure 2A and Figure S1A available online, respectively); therefore, we used pifithrin-α in our subsequent experiments. Repression of the oncogenes by p53 (Figure 1D) in MCF7 cells, as well as transactivation of p53 targets (data not shown), was efficiently prevented by pifithrin-α, supporting the notion that downregulation of oncogenes is p53 dependent. In general, we observed a very good correlation of microarray data with qPCR in both cell lines, with the exception of *EIF4E*, whose repression in MCF7 cells was detected by qPCR, but not by microarray. In addition, qPCR showed a clear p53-dependent reduction of expression of another p53 target gene, *BIRC5* (survivin) in both cell lines (Figures 1C and 1D), which was not detected in microarray experiments. These differences probably reflect a poor hybridization with the probes in the array.

Consistent with the decrease of mRNA levels, protein levels of IGF-1R, c-Myc, survivin, and Mcl-1 were downregulated by RITA in wild-type p53-expressing HCT116, MCF7, A549, and U2OS cells, but not in the p53 null cell lines HCT116 *TP53*$^{-/-}$, Saos-2, and H1299 and in cells pretreated with pifithrin-α (Figures 2A and 2B).

Importantly, the transcriptional program resulting in oncogene inhibition by p53 was not restricted to the in vitro phenomenon. We applied RITA to HCT116 and HCT116 *TP53*$^{-/-}$ xenografts in *SCID* mice. Upon 18 hr of RITA treatment, we observed a decline of c-Myc, Mcl-1, survivin, and IGF-1R in p53-positive, but not p53-negative tumors (Figure 2C).

Taken together, our results demonstrate that reactivation of p53 by RITA markedly ablated the expression of a set of important oncogenes in tumor cells in vitro and in vivo. Because most of these factors are crucial for the viability of both tumor and normal cells, it appears important to assess the effect of RITA on this set of genes in nontransformed cells.

RITA Does Not Affect the Expression of Survival Genes in Nontransformed Cells

We examined the effect of RITA on survival genes in several nontransformed cell lines: human diploid fibroblasts (HDFs); and two
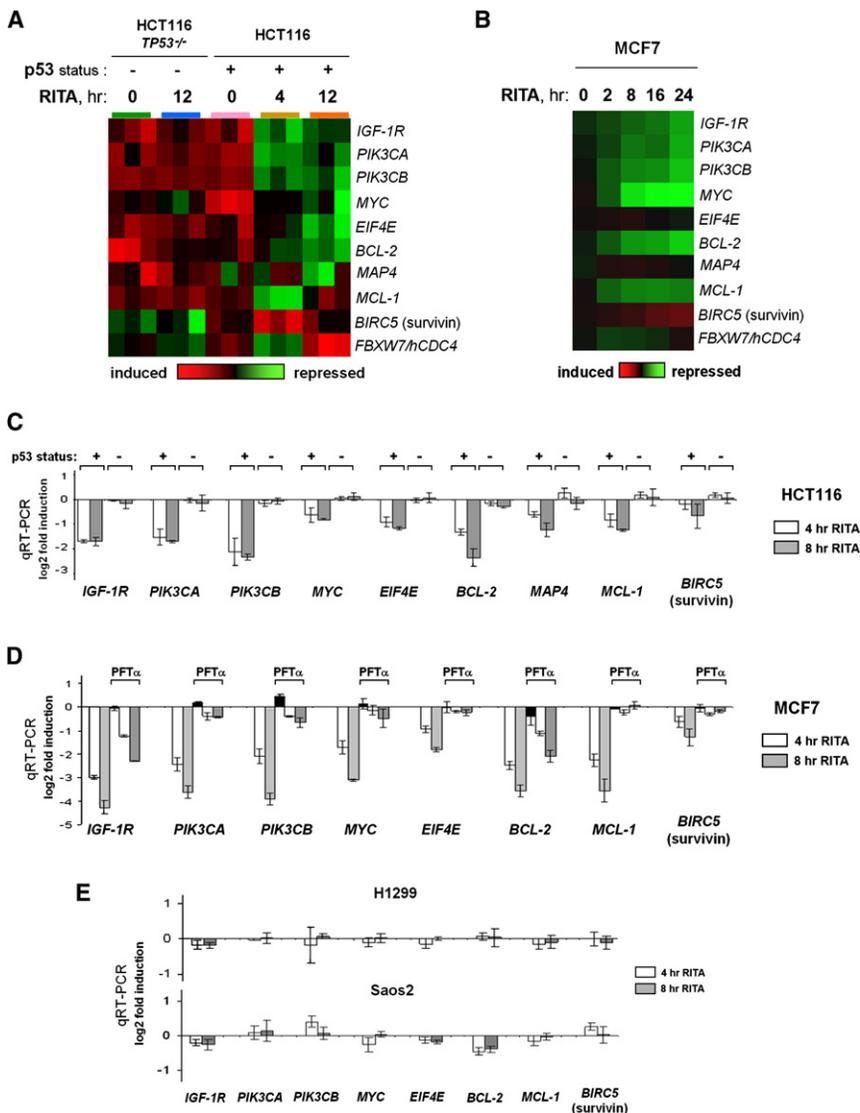
Figure 1. p53-Induced Transcriptional Repression of a Set of Oncogenes

(A) Microarray analysis of gene expression in wtp53-expressing HCT116 and p53 null HCT116 *TP53*$^{-/-}$ after 4 hr and 12 hr of treatment with 1 μM RITA. Shown is the heatmap of genes differentially expressed at 1% FDR, F-test. Vertical rows indicate separate arrays, and horizontal rows indicate genes. Values are normalized by row. Green indicates low expression; red indicates high expression.

(B) Microarray analysis of MCF7 cells treated with 1 μM RITA for 2–24 hr presented as in (A). Values are normalized to untreated control.

(C) mRNA levels of oncogenes were detected by qPCR in HCT116 and HCT116 *TP53*$^{-/-}$ cells 4 and 8 hr after treatment with 1 μM RITA (mean ± SEM, n = 3).

(D) mRNA levels of oncogenes were detected by qPCR in untreated MCF7 cells or upon pretreatment with 10 μM of the p53 inhibitor pifithrin-α 4 and 8 hr after RITA treatment (mean ± SEM, n = 3).

(E) mRNA levels of oncogenes in p53 null Saos-2 and H1299 cells, as detected by qPCR 4 and 8 hr after RITA treatment (mean ± SEM, n = 3).

stream effects of RITA-induced inhibition of oncogenes in tumor cells and the contribution of oncogene inhibition to the p53-mediated biological response.

Inhibition of Key Downstream Players of the Akt Pathway

Pathway analysis of microarray data obtained in HCT116 cells identified the PI(3)K/Akt pathway as one of the most affected by RITA (Enge et al., 2009). Indeed, we found that several genes involved in Akt signaling were repressed, as illustrated in Figure 3A. These include *IGF-1R*, *EIF4E*, as well as *PIK3CA* and *PIK3CB*, which encode catalytic subunits of PI(3) kinase, p110α and p110β, respectively (for the details of the Akt pathway, see Figure 3A).

Next, we examined whether inhibition of IGF-1R and PI(3)K affects the abundance and phosphorylation status of downstream factors. Upon treatment with RITA, we observed a p53-dependent decline of the active, phosphorylated form of Akt kinase, as well as phosphorylated mTOR downstream of Akt (Figures 3B and 3C). Furthermore, Akt kinase activity was significantly reduced in RITA-treated cells, as manifested by a decreased ability of Akt to phosphorylate its substrate GSK3αβ in vitro (Figure 3G).

Along with inhibition of mTOR phosphorylation, mRNA of *EIF4E*, one of the important downstream mediators of mTOR, was significantly downregulated (Figures 1A, 1C, and 1D). Because eIF4E is implicated in the regulation of translation of several important oncoproteins, including c-Myc (Averous and Proud, 2006), we set out to investigate whether inhibition of

mammary epithelial lines, MCF10A and 184A1. The levels of IGF1R, c-Myc, Mcl-1, and survivin were not affected by RITA in these cell lines (Figure 2D). This was matched by the lack of induction of p53 and its target gene *PUMA*, in line with the absence of p53 activation in nontransformed fibroblasts and lymphocytes, reported by us previously (Issaeva et al., 2004). The viability of nontransformed cell lines was not affected by RITA either (Figure 2E; Figure S1B). However, the chemotherapeutic agent 5-fluorouracil (5-FU), known to cause DNA damage, induced p53 and PUMA in these cells and reduced the expression of c-Myc and survivin (Figure 2D), along with the induction of cell death (Figure 2E; Figure S1B). We therefore conclude that targeting p53 by RITA does not result in p53 activation and/or block of survival gene expression in nontransformed cells, in contrast to DNA-damaging drugs. Tumor-selective inhibition of proproliferative and antiapoptotic genes might provide a powerful weapon against cancer cells without evoking toxic effects in normal tissues. Thus, we set out to explore in more detail the down-

Figure 2. p53-Dependent Downregulation of the Oncoproteins c-Myc, IGF-1R, Mcl-1, and Survivin In Vitro and In Vivo

(A) Immunoblotting of IGF-1R, c-Myc, Mcl-1, and survivin in cell extracts from the wtp53 cell lines U2OS, MCF7, A549, and HCT116 treated with RITA or with RITA in combination with pifithrin-α.

(B) Immunoblotting of oncoproteins in cell extracts from p53 null Saos-2 and H1299 cells, treated or nontreated with RITA.

(C) Protein levels of IGF-1R, c-Myc, survivin, and Mcl-1 upon 18 hr of RITA treatment (1 mg/kg) of HCT116 and HCT116 $TP53^{-/-}$ tumor xenografts in $SCID$ mice, as detected by immunoblotting.

(D) Protein levels of oncoproteins in nontransformed human diploid fibroblasts (HDFs) and the mammary epithelial cell lines MCF10A and 184A1 upon 12 hr treatment with 1 μM RITA or 100 μM 5-FU was detected by western blot.

(E) Phase-contrast microscopy of nontransformed MCF10A and 184A1 cells treated with 1 μM RITA or 100 μM 5-FU for 48 hr. Scale bars represent 100 μm.

GSK3β was reduced by RITA in HCT116 cells, but not in p53 null cells (Figure 3H).

Rescue of GSK3β activity due to inhibition of Akt is expected to result in proteasomal degradation of GSK3β substrates. Indeed, as shown in Figure 3I, activation of p53 by RITA led to a profound downregulation of the GSK3β substrates c-Myc, β-catenin (Doble and Woodgett, 2003), Mcl-1 (Maurer et al., 2006), and cyclin E (Figures 4G and 4H) in a p53-dependent manner. In line with these findings, GSK3β-dependent phosphorylation of c-Myc was increased (Figure 4D), supporting the notion that GSK3β activity is induced by RITA.

p53 Induces GSK3β-Dependent Degradation of c-Myc

The data presented above suggest that, in addition to transcriptional repression of *MYC* (Figures 1A–1D), c-Myc might also be targeted at a protein level due to phosphorylation by GSK3β.

To address the impact of a posttranscriptional mechanism on c-Myc inhibition, we tested whether c-Myc expressed from a p53-independent promoter will be affected. RITA treatment resulted in strong reduction of overexpressed ectopic c-Myc, indicating regulation on a posttranscriptional level (Figure 4A).

Next, we examined the involvement of proteasomal degradation in the depletion of c-Myc. The proteasomal inhibitor MG132 partially prevented downregulation of c-Myc by RITA (Figure 4B; Figure S2A). Consistent with these data, we observed a decrease in c-Myc half-life upon p53 activation by RITA (Figure 4C). However, the stability of Mcl-1, another putative target of GSK3β, was not decreased (Figure S2B). Thus, p53 appears to unleash the proteasomal degradation of c-Myc, but not of Mcl-1.

In order to validate whether GSK3β is required for c-Myc downregulation, we blocked GSK3β activity by the specific

EIF4E plays a role in the downregulation of oncoproteins upon RITA treatment.

We employed a translational reporter construct, encoding a luciferase whose mRNA translation is CAP dependent and regulated by eIF4E. Indeed, CAP-dependent translation was inhibited by RITA in p53-positive cells, but not in p53 null cells (Figure 3D). However, we did not observe a general inhibition of translation, as growth suppressor proteins were induced upon RITA treatment (Figure 3E). Notably, ectopic expression of eIF4E alleviated the block of CAP-dependent translation (Figure 3D), supporting the notion that the effect is eIF4E dependent.

Next, we assessed whether eIF4E can rescue the decline of oncoproteins by RITA (Figure 3F). Ectopic expression of eIF4E conferred only partial protection of the c-Myc level at a late time point (24 hr), indicating a minor contribution of translational block to c-Myc depletion. Downregulation of Mcl-1 was not restored at all. Unexpectedly, we observed a potent rescue of IGF-1R level upon eIF4E overexpression, indicating that in addition to repression of *IGF-1R* transcription, p53 induces downregulation of IGF-1R protein via an eIF4E-dependent mechanism.

Subsequently, we studied the status of another downstream target of Akt, GSK-3β (Figure 3A). In accordance with inhibition of Akt activity (Figure 3G), phosphorylation of endogenous

Figure 3. p53-Dependent Inhibition of the Akt Pathway upon RITA Treatment

(A) Scheme depicting the major players in the PI(3)K/Akt pathway and oncogenes that are transcriptionally repressed upon RITA treatment (in bold).

(B) Levels of IGF-IR and phosphorylated Akt HCT116 and HCT116 $TP53^{-/-}$ cells were analyzed by western blot.

(C) Phosphorylation of mTOR upon RITA treatment was assessed by western blot.

(D) Effect of RITA on CAP-dependent translation was evaluated by using the luciferase translation reporter in the presence or absence of ectopic expression of eIF4E (mean ± SEM, n = 4).

(E) Levels of GADD45α, Fas, and p53 proteins were detected by western blot.

(F) IGF-IR, c-Myc, and Mcl-1 protein levels upon 1 μM RITA in the presence or absence of ectopic expression of eIF4E in HCT116 cells, as detected by immunoblotting.

(G) Akt kinase activity was determined by an in vitro kinase assay with Akt kinase immunoprecipitated from HCT116 and HCT116 $TP53^{-/-}$ cells and purified GST-GSK3αβ as a substrate. Phosphorylation of GST-GSK3αβ was analyzed by western blot with phospho-specific antibodies.

(H) Phosphorylation of cellular GSK3β upon treatment with 1 μM RITA of HCT116 and HCT116 $TP53^{-/-}$ cells was assessed by immunoblotting with phospho-specific antibodies.

(I) p53-dependent downregulation of the GSK3β substrates β-catenin, c-Myc, and Mcl-1 upon treatment with 1 μM RITA was analyzed by western blot.

inhibitor B1686 BIO. This resulted in a partial rescue of c-Myc levels, evident at 8 hr after RITA treatment (Figure 4E). However, after 24 hr, c-Myc levels were reduced to the same level as in the absence of the GSK3β inhibitor, presumably due to the transcriptional repression of *MYC*.

In contrast to c-Myc, the level of Mcl-1 was not rescued by B1686 BIO (Figure 4E), indicating that downregulation of Mcl-1 by RITA is not GSK3β dependent. Taken together with our results presented above, that stability or translation of Mcl-1 were not affected by RITA, this allowed us to conclude that the observed decline of Mcl-1 protein occurs only on an mRNA level. On the other hand, c-Myc is targeted for degradation, at least partially due to GSK3β-induced phosphorylation.

Impact of the p53 Target Fbxw7/hCdc4 on c-Myc and Cyclin E Downregulation

GSK3β-phosphorylated c-Myc is a substrate for the F box protein Fbxw7/hCdc4, the substrate specificity factor of SCF$^{Fbxw7/hCdc4}$ E3 ubiquitin ligase (Yada et al., 2004). Microarray analysis (Figure 1A) and qPCR (Figure 4F) showed that the mRNA levels of two *FBXW7/hCDC4* isoforms (β and γ) were significantly

upregulated by RITA in a p53-dependent manner. Induction of the β isoform is consistent with published data demonstrating that the *FBXW7/hCDC4* gene is a direct p53 target (Kimura et al., 2003), whereas the γ isoform has not yet been demonstrated to be regulated by p53.

In order to examine the impact of Fbxw7/hCdc4 on c-Myc degradation, we compared the levels of c-Myc upon RITA treatment of HCT116 and HCT116 $hCDC4^{-/-}$ cells in which the *FBXW7/hCDC4* gene has been deleted. In the absence of Fbxw7/hCdc4, the kinetics and extent of c-Myc depletion were significantly impeded, confirming the involvement of Fbxw7/hCdc4 (Figure 4G). Nevertheless, the level of c-Myc was not completely rescued in these cells upon p53 reactivation by RITA, supporting our data that more than one mechanism contributes to c-Myc downregulation. Importantly, the level of another critical oncoprotein, cyclin E, a well established substrate for the SCF$^{Fbxw7/hCdc4}$ E3 ubiquitin ligase (Strohmaier et al., 2001), was downregulated in a p53-dependent manner (Figure 4H). Contrary to c-Myc, cyclin E was completely rescued by Fbxw7/hCdc4 deficiency (Figure 4G), implicating Fbxw7/hCdc4 as the major factor contributing to cyclin E decline. However, deletion of *FBXW7/hCDC4* was not sufficient to protect cells from growth inhibition by RITA, as shown by using a short-term cell proliferation assay and a long-term colony formation assay (Figure 4I, left and right panels, respectively).
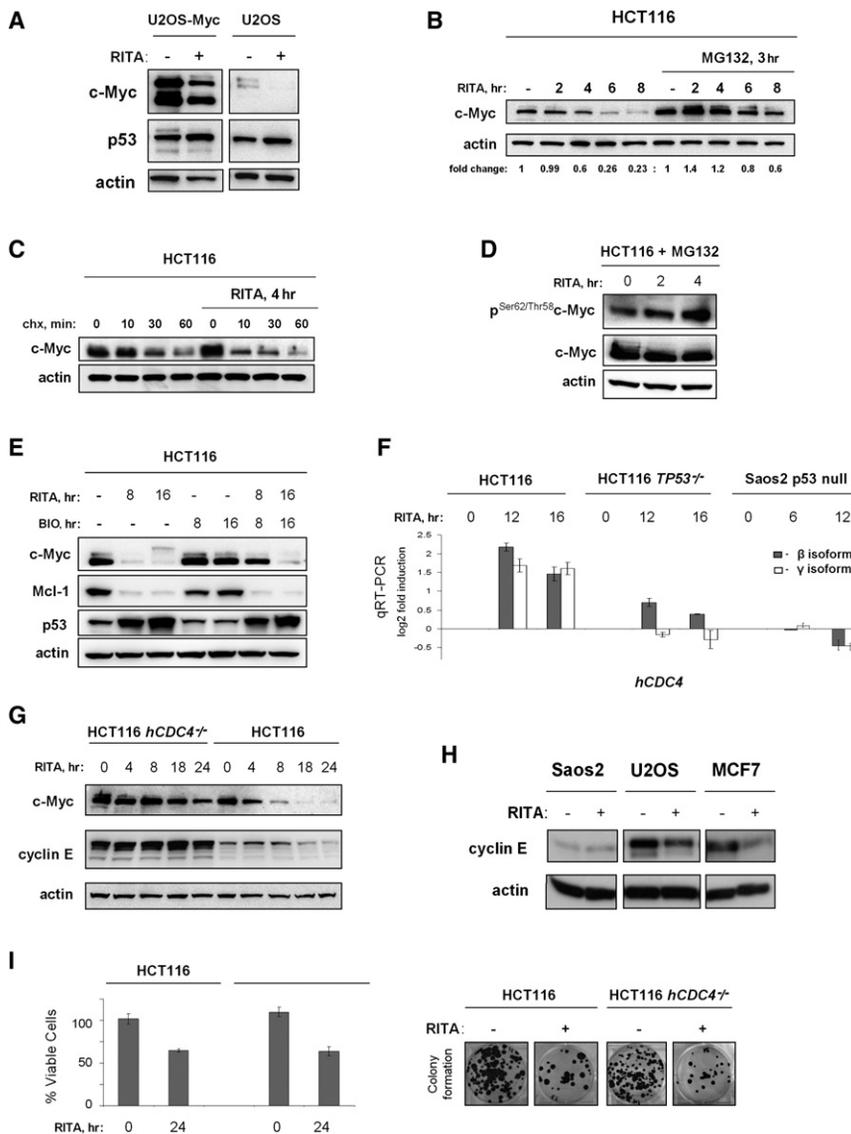
Figure 4. Reactivation of p53 by RITA Induces Proteasomal Degradation of c-Myc via Activation of GSK3β and Fbxw7/hCdc4

(A) The level of c-Myc expressed under a Tet-regulatable promoter in U2OS-Myc cells treated or nontreated with RITA was detected by western blot.

(B) Immunoblotting of c-Myc upon proteasomal inhibition with MG132 combined with RITA treatment.

(C) Half-life of c-Myc after RITA treatment, as assessed by immunoblotting of c-Myc upon treatment with cycloheximide for the indicated periods.

(D) Western blot of phosphorylated c-Myc 2 and 4 hr after treatment with 1 μM RITA. HCT116 cells were pretreated with MG132 to prevent downregulation of c-Myc.

(E) c-Myc and Mcl-1 levels upon RITA treatment combined with inhibition of GSK3β with B1686 BIO, as detected by immunoblotting.

(F) mRNA levels of β and γ isoforms of *FBXW7/hCDC4* in HCT116 cells were detected by qPCR (mean ± SEM, n = 3).

(G) c-Myc and cyclin E levels in HCT116 *CDC4$^{-/-}$* cells after RITA treatment as assessed by western blot.

(H) Cyclin E levels in wtp53 expressing U2OS and MCF7 and in p53 null Saos-2 cells were detected by western blot.

(I) Growth suppression by RITA was assessed by a cell proliferation assay (left panel) (mean ± SEM, n = 3) and a long-term colony formation assay in HCT116 and HCT116 *CDC4$^{-/-}$* cells (right panel).

at 1, but not at 0.1 μM, in both HCT116 and MCF7 cells, whereas p53 target genes encoding p21 and Noxa were readily induced at a low dose (Figures 5C and 5D).

Notably, in the absence of oncogene inhibition at 0.1 μM RITA, tumor cells died much less efficiently compared to 1 μM (Figure 7A), indicating that inhibition of oncogenes contributes to apoptosis induction by p53. To rule out the possibility that downregulation of survival factors was a consequence of apoptosis and/or caspase activation, we examined their level upon blocking apoptosis by the pan-caspase inhibitor Z-VAD-fmk. Caspase inhibition did not prevent the downregulation of Mcl-1, survivin, and c-Myc by RITA (Figure 5E), supporting the notion that their decline is due to p53-mediated transcriptional repression.

To address the differences underlying the regulation of proapoptotic and prosurvival genes by p53, we examined the subcellular distribution of p53 upon treatment with 0.1 and 1 μM RITA. We repeatedly noted a striking disproportion in the subnuclear distribution of p53 upon these two doses of RITA. Abundance of p53 in the chromatin-bound fraction was greatly enhanced by 1, but not by 0.1 μM, RITA (Figure 6A). Thus, a higher level of p53 on chromatin triggered by 1 μM RITA correlated with the induction of transrepression by p53. As a reference transcriptional factor implicated in both transcriptional activation and transcriptional

Thus, we conclude that induction of Fbxw7/hCdc4 by p53 triggers proteasome-dependent degradation of c-Myc and cyclin E.

### Dose-Dependent Repression of Oncogenes by RITA

Our results suggest that pharmacologically reactivated p53 acts as a potent repressor of a number of oncogenic and survival factors, as well as functions as a powerful trigger of proapoptotic proteins (Figure 5A) (Enge et al., 2009). Furthermore, we found that the transactivation of proapoptotic genes requires a lower dose of RITA than transrepression of prosurvival genes. As evident from Figure 5A, the response to 0.1 and 1 μM RITA was quite similar in terms of induction of p53 and its targets PUMA and Noxa. In contrast, oncogenes were regulated differently: whereas 1 μM RITA was sufficient to trigger a sharp downregulation of c-Myc, Mcl-1, and survivin, upon treatment with 0.1 μM RITA the decline of these oncogenes was either absent or less pronounced (Figure 5B).

qPCR confirmed that the transcriptional repression of *MCL-1*, *MYC*, *BIRC5*, *EIF4E*, *PIK3CA*, and *PIK3CB* was fully unleashed

Figure 5. Dose-Dependent Effect of RITA on the Transcriptional Activation of Proapoptotic Genes and the Repression of Oncogenes

(A) Levels of proapoptotic factors Puma and Noxa upon 0.1 and 1 μM RITA in HCT116 and MCF7 cells were detected by immunoblotting.

(B) Immunoblot of p53, c-Myc, Mcl-1, and survivin in HCT116 and MCF7 cells after treatment with 1 and 0.1 μM RITA.

(C and D) Upper panels: mRNA levels of *MCL-1*, *MYC*, *BIRC5*, *EIF4E*, *PIK3CA*, and *PIK3CB* after 1 and 0.1 μM RITA as detected by qPCR. Lower panels: mRNA levels of *CDKN1A* and *PMAIP* after 0.1 μM RITA. Experiments in (C) and (D) were performed in HCT116 and MCF7 cells treated with RITA for 12 and 8 hr, respectively (mean ± SEM, n = 3).

(E) Levels of c-Myc, Mcl-1, and cleaved PARP upon treatment with RITA combined with the caspase inhibitor zVAD (80 μM).

Furthermore, we compared the relative abundance of p53 and MDM2 on p53-activated versus p53-repressed promoters by using ChIP. We found that in untreated cells, the p53/MDM2 ratio on the p53-activated *CDKN1A* promoter was significantly higher than on p53-repressed *MCL-1* promoter (Figure 6C). Treatment with 0.1 μM RITA increased the p53/MDM2 ratio on *CDKN1A*, but not on the *MCL-1* promoter (Figure 6D), whereas 1 μM RITA increased the p53/MDM2 ratio on both promoters (Figure 6D). Taken together, our results are consistent with the idea that MDM2 is more easily dislocated by RITA from p53-activated than from p53-repressed promoters. It is therefore possible that transactivation of p53 might be less tightly controlled by MDM2 than transrepression. If this is the case, the prediction is that the basal levels of expression of survival genes that p53 can repress should be similar in the absence and presence of p53, whereas the expression of at least some p53-transactivated genes should be higher in p53-positive cells. Indeed, the analysis of microarray data of the gene expression profiles of untreated HCT116 and HCT116 *TP53*$^{-/-}$ cells revealed a significant difference between the basal levels of expression of these two groups of genes. A number of genes known to be positively regulated by p53, including *CDKN1A*, *FAS*, *DDB2*, and others had a higher level of expression in p53-positive than in p53 null cells. On the contrary, the mRNA levels of the p53-repressed genes *IGF1R*, *MYC*, *EIF4E*, *BCL2*, *MAP4*, *MCL1*, and *BIRC5* did not differ between the lines (Figure 6E).

Taken together, our data suggest that p53-mediated transrepression is more tightly controlled than transactivation; MDM2 associated with chromatin might play an important part in this process. The dose-dependent effect of RITA on the expression of oncogenes appears to be due to a less efficient release of MDM2 from the promoters of p53-repressed genes.

repression (Adhikary and Eilers, 2005), we tested subcellular distribution of c-Myc. c-Myc was also present in the chromatin fraction in untreated control cells (Figure 6A), whereas its level was reduced in treated cells, in line with results demonstrated above.

Recent chromatin immunoprecipitation (ChIP) studies demonstrated that p53 is already bound to most of its target genes in cancer cells before the genotoxic stress (Kaeser and Iggo, 2002; Shaked et al., 2008). However, in spite of being present at the promoters, p53 is not fully active as a transcriptional factor in the absence of stress, suggesting the involvement of a p53 inhibitor that blocks p53 function directly on promoters. A possible candidate for this role is MDM2, which can associate with chromatin in a p53-dependent manner (Minsky and Oren, 2004; White et al., 2006). We therefore tested whether the presence of MDM2 on chromatin is affected by RITA. We readily detected MDM2 in the chromatin fraction in nontreated MCF7 and HCT116 cells (Figure 6B). The amount of MDM2 in this fraction decreased upon RITA treatment, mirroring the increase of chromatin-bound p53 (Figure 6B). However, although both concentrations of RITA reduced the amount of p53/MDM2 complexes and induced p53 accumulation in the soluble fraction to the same extent (Figures 5A and 5B; Figures S3A and S3B), a lower dose of RITA was less efficient in releasing MDM2 from chromatin-bound p53 (Figure 6B).

Figure 6. Dose-Dependent Differences in the Subcellular Distribution of p53 Correlate with Distinct Transcriptional Programs Induced by p53

(A) Cytoplasmic fraction, soluble nuclear fraction (extracted with 300 mM NaCl), and chromatin-bound fraction (nuclear pellet after extraction) were obtained from MCF7 cells treated with 0.1 and 1 μM RITA and analyzed by immunoblotting. We used actin as a marker and loading control for the cytoplasmic fraction, PARP for both the soluble nuclear and chromatin-bound fractions, and Histone H3 for the chromatin-bound fraction. A cell-equivalent amount of each fraction was used for the comparisons.

(B) Abundance of p53 and MDM2 on chromatin upon treatment with 0.1 and 1 μM RITA was detected as in (A).

(C) The ratio between p53 and MDM2 bound to *MCL-1* and *CDKN1A* promoters in untreated HCT116 cells was detected by chromatin immunoprecipitation (ChIP).

(D) Changes in the p53/MDM2 ratio on *MCL-1* and *CDKN1A* promoters upon treatment with 0.1 and 1 μM RITA were detected by ChIP.

(E) Basal levels of mRNAs of p53-transactivated and p53-repressed genes were estimated using microarray analysis of HCT116 and HCT116 $TP53^{-/-}$ cells (mean ± SEM, n = 3).

HCT116 lines, whereas IGF1R and Mcl-1 were not affected at all (Figure 7E). These three lines are known to be only partially susceptible to nutlin3a-induced apoptosis (Enge et al., 2009; Tovar et al., 2006). However, in nutlin3a-sensitive SJSA cells, Mcl-1 is downregulated (Wade et al., 2008). To evaluate whether the depletion of Mcl-1 or c-Myc will affect the response to nutlin3a, we combined nutlin3a with siRNA to c-Myc or Mcl-1. Indeed, depletion of c-Myc or Mcl-1 synergized with nutlin3a in cell killing (Figure 7E), confirming that downregulation of c-Myc and Mcl-1 plays an important role in the apoptosis induced upon pharmacological reactivation of p53.

Next, we tested whether inhibition of the PI(3)K-Akt pathway contributes to p53-mediated cell death. Blocking the PI(3)K pathway by the pharmacological inhibitor LY294002 induced a low number of apoptotic cells, similarly to 0.1 μM RITA (Figures 7F and 7G; Figure S4C). Notably, a combination of both treatments induced apoptosis much more efficiently, in a synergistic manner, indicating that the lack of inhibition of the PI(3)K pathway by 0.1 μM RITA impedes efficient apoptosis induction.

Taken together, our data imply that ablation of oncogenes and survival factors plays an important role in the induction of apoptosis by pharmacologically reactivated p53.

DISCUSSION

Given the pivotal role of apoptosis in successful anticancer therapy, it is of crucial importance to understand the

Contribution of Oncogene Ablation to the Induction of Apoptosis by p53

To address whether the inhibition of oncogenes is essential for the apoptosis induction by p53, we used genetic and pharmacological approaches. We selected three representative oncogenes—prosurvival and proproliferation factor Akt, proproliferative c-Myc, and antiapoptotic Mcl-1—and "restored" their depletion at 0.1 μM RITA by applying a chemical inhibitor or corresponding siRNA.

Downregulation of Mcl-1 by siRNA, although it exerted only a weak proapoptotic effect per se, synergized with 0.1 μM RITA in apoptosis induction (Figures 7B–7D; Figures S4A and S4B). The effect of c-Myc ablation was also synergistic, albeit less pronounced (Figures 7B–7D; Figures S4A and S4B). Furthermore, we examined whether the downregulation of survival genes plays a role in apoptosis induction by another known p53 activator, nutlin3a (Vassilev et al., 2004). The effect of nutlin3a on survival genes was not prominent in MCF7, U2OS, and HCT116 lines (Figure 7E). Nutlin3a caused a decline of c-Myc and survivin in MCF7 cells, but not in U2OS and

Figure 7. Inhibition of Oncogenes Plays a Significant Role in Apoptosis Induction by Pharmacologically Activated p53

(A) Detection of apoptotic cells by FACS of annexin-stained HCT116 and MCF7 cells after 24 hr of treatment with 0.1 and 1 μM RITA and by phase-contrast microscopy of MCF7 cells.

(B and C) Cell death induction was assessed by trypan blue staining of cells treated with a low dose of RITA upon knockdown of c-Myc and Mcl-1 by siRNA in (B) HCT116 and (C) MCF7 cells (mean ± SEM, n = 3).

(D) Phase-contrast microscopy of MCF7 cells treated with 0.1 μM RITA upon c-Myc or Mcl-1 knockdown. Scale bars represent 100 μm.

(E) Upper panel: levels of IGF1R, c-Myc, Mcl-1, survivin, and p53 in MCF7, U2OS, and HCT116 cells treated with 10 μM nutlin3a were assessed by western blot. Lower panel: cell death induced by nutlin3a in the presence or absence of c-Myc or Mcl-1 depletion by siRNA was detected by trypan blue staining (mean ± SD, n = 3).

(F and G) Cell death of HCT116 cells treated with the indicated combinations of RITA and the PI3-kinase inhibitor LY294002 was assessed by (F) trypan blue staining (mean ± SD, n = 3) or (G) phase-contrast microscopy.

The scale bars in (A), (G), and (D) represent 100 μm. The asterisk in (B), (C), (E), and (F) denotes an expected additive effect.

iments showing induction of proapoptotic factors in the absence of transcriptional repression of survival genes at a submicromolar concentration of RITA. We show that induction of the transcriptional repression program correlated with a higher p53/MDM2 ratio on chromatin as a result of increased p53 and reduced MDM2 abundance on chromatin. Previous studies demonstrated that p53-dependent association of MDM2 on chromatin blocks transcriptional activation by p53 (Minsky and Oren, 2004; White et al., 2006). It has only begun to be examined how p53 and MDM2 interrelate on chromatin. Interesting mechanism of blocking p53 transcriptional activation on the promoters has been discovered (Minsky and Oren, 2004), which is mediated by MDM2-dependent ubiquitination of histones; there are likely to be other mechanisms. Our results suggest that p53-mediated transrepression is controlled more tightly than transactivation and involves MDM2 associated with the promoters of p53-repressed genes. The mechanism(s) by which MDM2 blocks transrepression by p53 awaits further investigation. It is possible that association of MDM2 with promoters of p53-repressed genes might favor recruitment of histone acetylases, such as p300, instead of histone deacetylases. In spite of intensive research, the mechanisms behind p53-mediated transcriptional repression remain largely unknown (Laptenko and Prives, 2006; Riley et al., 2008). Dose-dependent induction of p53-mediated transactivation versus transrepression by RITA might provide a new tool

mechanisms behind tumor cell susceptibility and resistance to cell death and, in particular, to p53-mediated apoptosis. Here, we applied the p53-reactivating compound RITA (Issaeva et al., 2004) to further decipher the consequences of restoration of p53 function in tumor cells. We previously demonstrated that transactivation of proapoptotic genes is required for cell death induced by RITA-reactivated p53 (Enge et al., 2009). In the present study, we show that upregulation of proapoptotic targets is not sufficient for a full-scale induction of cell death by RITA. We found that p53 triggers a dramatic and rapid downregulation of a number of critical oncogenes, thus overcoming survival signaling. Functional studies demonstrated that this facet of p53 activity is critical for a robust induction of apoptosis by pharmacologically reactivated p53.

Importantly, our results indicate that induction of proapoptotic genes and inhibition of antiapoptotic/survival genes represent two branches of the p53 response, which are differentially regulated. Evidence for this comes from the dose-dependent exper-

Figure 8. Model: Two Branches of the p53 Transcriptional Program Are Required for Efficient Apoptosis Induction

Upper panel: a low dose of RITA can displace MDM2 from p53 proapoptotic targets, but is insufficient to displace MDM2 from p53-repressed survival genes. Transcriptional activation of the proapoptotic p53 targets PUMA, Noxa, Fas, and Bax is counteracted by prosurvival signaling, blocking apoptosis at the submicromolar dose of RITA. Lower panel: 1 μM RITA efficiently dislocates MDM2 both from p53-activated target genes and from p53-repressed targets. This triggers the transcriptional repression of prosurvival and proproliferative oncogenes by p53. Simultaneous activation of proapoptotic genes and repression of oncogenes results in robust apoptosis.

by which to address the molecular mechanisms of transrepression.

A number of p53-repressed genes that play a role in cell survival have been identified in previous studies (Laptenko and Prives, 2006; Oren, 2003). However, it is still unclear whether reactivation of p53 can overcome survival signaling in cancer cells. Our data suggest that only upon simultaneous engagement of both branches, i.e., activation of proapoptotic genes and inhibition of survival genes, can an efficient apoptotic response can be elicited. This is consistent with recently published in vivo data, suggesting that other p53 functions, such as transcriptional repression, may be the key to an efficient apoptotic response. It has been shown that the VP16-p53 chimeric protein displayed profound apoptotic defects in a variety of settings, despite being fully competent in the transcriptional upregulation of proapoptotic genes (Johnson et al., 2008).

Based on our results, we propose a model in which two distinct p53-dependent transcriptional programs are required to trigger a full-scale apoptotic response (Figure 8). Our data suggest that induction of just one branch, i.e., enhanced expres-

sion of proapoptotic proteins, might be insufficient to shift the survival/death balance and to produce a robust apoptotic outcome. Concurrent downregulation of prosurvival factors might work in concert with the upregulation of proapoptotic factors to cross a threshold for firing the apoptotic program, because only when proapoptotic factors outweigh the prosurvival buffer can the program run to completion. In addition to the degradation of p53, MDM2 controls both branches of the p53-mediated response directly on promoters of p53 target genes. The threshold for displacing MDM2 from p53-repressed genes is higher than that for p53-activated genes. This creates an additional level of regulation of the p53 choice between the life and death of a cell.

Our results show that the initial transcriptional repression of individual genes by p53 unleashes a cascade of events leading to inhibition of oncogenic factors at several different levels, including transcriptional, translational, and posttranslational changes. Reactivated p53 represses transcription of the antiapoptotic target genes *BCL-2*, *MCL-1*, and *BIRC5* (survivin) and a set of target genes encoding upstream and downstream components of the Akt survival pathway, *IGF-1R*, *PIK3CA*, and *EIF4E*. Consequently, the block of PI(3)K signaling and inhibition of Akt phosphorylation/activity induce pleiotropic effects and result in profound changes in the survival program. As a result of mTOR and eIF4E inhibition, translation of c-Myc and IGF-1R mRNAs was also decreased. Moreover, active GSK3β promoted the proteasomal degradation of its downstream targets c-Myc, cyclin E, and β-catenin, which was facilitated by p53-mediated induction of the E3 ubiquitin ligase Fbxw7/hCdc.

We believe that the pleiotropic effect of p53 on c-Myc, i.e., repression of c-Myc transcription, block of its translation, and induction of proteasomal degradation, creates an external robustness of the p53-mediated ablation of c-Myc. This ensures that downregulation of c-Myc by p53 is achieved irrespective of the particular combination of mutations in a given cell. Dysfunction of one mechanism of c-Myc downregulation by p53, such as, for example, loss of *FBXW7/hCDC4*, constitutive activation of Akt, *MYC* gene translocation, or mutation, will be compensated for by other branches in the hierarchy. Since tumors are often dependent on deregulated c-Myc expression (Felsher and Bishop, 1999), its elimination might be an essential component for anticancer therapies targeting p53.

We have analyzed the effect of p53 on a number of oncogenic factors, but we possibly obtained only a glimpse of the whole picture of p53-induced effects. Systems biology studies aimed at characterizing the whole proteome of cancer cells upon p53 activation will help to better characterize the p53 network in the future.

Rescue of p53 tumor suppressor function by blocking the inhibitory role of MDM2 is a promising strategy by which to combat cancer that is pursued both in academia and industry (Lain et al., 2008; Yang et al., 2005). However, the question remains as to whether p53 reactivation by small molecules will be harmful for normal cells. A number of studies pointed toward the ability of p53 to kill cancer cells without detrimental effects in normal cells in vitro, although the mechanism of this phenomenon has not been defined (Selivanova, 2004). We have demonstrated that p53 induction by RITA in the absence of oncogene expression in nontransformed cells is transient and does not

induce growth suppression (Issaeva et al., 2004). Our present study extends these observations and indicates that the ability of p53 to target oncogene addiction might provide selective killing of cancer cells by molecules reactivating p53. We speculate that tumor cells might be particularly sensitive to p53 reactivation due to p53's ability to target oncogene addiction and disable survival programs that tumor cells are critically dependent on. Consequently, normal cells that are not dependent on oncogenes for their survival will remain largely unaffected. Additional studies including animal models will be required to address this issue.

Side effects and development of drug resistance remain a formidable barrier for the successful treatment of cancer. One way to solve these problems is to apply drug combinations, because multitargeted therapies will decrease the chance of mutations conferring resistance. At the same time, drug combinations that produce synergistic effects will allow a lower dose to be used and thus will decrease nonspecific toxicity of drugs. Combining targeted drugs in a more effective manner is a challenge; therefore, it becomes increasingly important to decipher the interactions between signaling pathways in cancer cells. Our data might help to identify pathways and/or factors whose targeting can provide a synergy with p53-reactivating compounds. Importantly, we show that combination of a low dose of p53-reactivating compound with inhibition of the PI(3)K/Akt pathway, c-Myc, or Mcl-1 produced a synergistic effect. Further work aimed at detailed characterization of molecular events upon p53 activation might help to guide rational development of more efficient and less toxic drug combinations.

EXPERIMENTAL PROCEDURES

Cell Lines, Plasmids, shRNA, and siRNA

Colon carcinoma HCT116, HCT116 $TP53^{-/-}$, and HCT116 $CDC4^{-/-}$ cells were gifts from B.Vogelstein and K.W. Kinzler. Osteosarcoma U2OS cells stably transfected with a Tet-regulatable c-Myc construct were obtained from J. Bartek. Translational reporter pcDNA/REN/HCV/FF was obtained from J. Pelletier. The eIF4E expression vector pcDNA3-3HA-meIF4Ewt was a gift from N. Sonenberg. Lentiviral p53 shRNA constructs were obtained from A. Jochemsen and from P. Chumakov. *MYC* siRNA was kindly provided by L.-G. Larsson, *MCL-1* siRNA was purchased from Santa Cruz, and GFP siRNA was purchased from Oligoengine. Plasmid DNA and siRNA transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cell viability assays were performed as we described (Enge et al., 2009).

Reagents

RITA was obtained from the National Cancer Institute (NCI) and was used at a concentration of 1 μM, unless otherwise stated. The proteasomal inhibitor MG132 was used at a concentration of 20 μM, the inhibitor of GSK3β kinase B1686 BIO was used at 5 μM, and the PI3-kinase inhibitor LY294002 was used at 20 μM (all from Sigma). Z-VAD-fmk (R&D Systems) was used at 20 μM concentration, and the p53 inhibitor PFTα, a gift from A. Gudkov, was used at 10 μM concentration.

Genome-Wide Analysis of Gene Expression Profiles

Analysis of gene expression profiles in HCT116 cells was performed as described (Enge et al., 2009). Microarray analysis in MCF7 cells treated with 1 μM RITA for 2–24 hr was performed by using hgu133a2 chips (Affymetrix). Raw data (.cel files) were analyzed by using the ExPlain software package (Wingender et al., 2007). Normalization and the quality control of the data were done with MAS 5.0 ("Quantiles," normalization method; "PM only," PM correction method). The data from arrays representing 2-4, 6-8-10, and 12-14-16 hr (indicated in Figure 1B as 2, 8, and 16 hr, respectively) were pooled

together, and the average fold change was calculated by using the t test method implemented in R package.

In Vitro Assays

For quantitative real-time reverse transcriptase-PCR analysis, mRNA from cells was isolated by using the RNeasy Kit (Quiagen). mRNA quantification was performed by using a fluorescence-based real-time RT-PCR technology (Power SYBR Green PCR Master Mix [ABI]). Primer sequences are described in Table S1. The preparation of cell extracts and western blot were performed according to standard procedures. Antibodies for immunoblotting were as follows: Phospho-Akt (anti-Ser473, 587F11), Akt, mTOR, Phospho-GSK3αβ (27C10), and Phospho-c-Myc (Ser62/Thr58) were from Cell Signaling; p53 (DO1), IGF-IR (C-20), Mcl-1 (S-19), c-Myc (N-262), PARP (H-250), GADD45α (C4), β-catenin, Bcl-2 (C-2), cyclin E (HE-12), survivin (FL-142), Fas (N-18), and MDM2(SMP14) were from Santa Cruz; β-actin (Sigma) and Phospho-mTOR (S2448) were from R&D Systems; p21 was from Beckton Dickinson; Noxa and PUMA were from Calbiochem; and Histone H3 was from Abcam. Secondary HRP-conjugated antibodies and Super Signal West Dura Extended Duration Substrate were from Pierce. To detect human c-Myc in xenografts, we used c-Myc (A-14) antibody (Santa-Cruz). Akt kinase activity was assessed by using the Nonradioactive Akt Kinase Assay Kit (Cell Signaling) according to the manufacturer's instructions. To measure CAP-dependent translation, cells were transiently transfected with the luciferase translational reporter construct pcDNA/REN/HCV/FF, and 24–48 hr after transfection the signal from Firefly luciferase was detected by using the Dual-Glo Luciferase Assay System (Promega). Small-scale biochemical fractionation to purify cytoplasmic, nuclear, and chromatin fractions was performed as described (Wysocka et al., 2001). Chromatin immunoprecipitation (ChIP) was performed as described (Enge et al., 2009), and the ChIP primers are presented in Table S1.

Animal Experiments

The Northern Stockholm Animal Ethical Committee approved all animal studies, and animal care was in accordance with Karolinska Institutet guidelines. Male SCID mice, 4–6 weeks old, were implanted subcutaneously with 1×10^6 HCT116 or HCT116 $TP53^{-/-}$ cells in 90% Matrigel (Becton Dickinson). Palpable tumors were established 7 days after cell injection; at this point, we injected 1 mg/kg RITA in tumors in a total volume of 100 μl phosphate-buffered saline.

Calculation of Expected Additive Effect

The expected additive effect was calculated using the following formula: $D = A + (B - A) + (C - A)$, where D is the expected additive effect, A is the percentage of apoptosis in untreated cells, and B and C are the percentages of apoptosis in cells upon first or second treatments, respectively.

ACCESSION NUMBERS

Microarray data described herein have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession numbers GSE11578 and GSE13291.

SUPPLEMENTAL DATA

Supplemental Data include four figures and one table and can be found with this article online at http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00110-X.

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Supplemental Data

Ablation of Key Oncogenic Pathways

by RITA-Reactivated p53 Is Required

for Efficient Apoptosis

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A

U2OS   MCF7

                shp53   shp53

RITA: -   +   -   +   -   +   -   +

p53

actin

B

MCF10A   184A1

contol   3,9%   10,3%

RITA   8,9%   14,4%

5FU   25,6%   41,9%
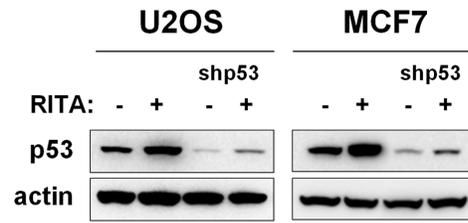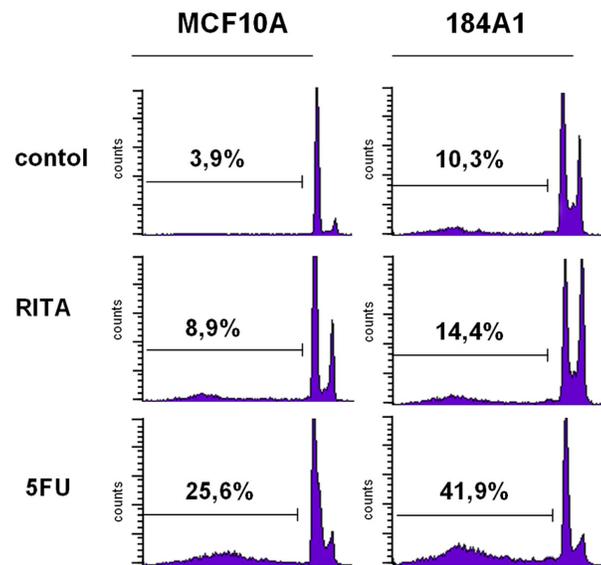
Supplemental Figure 2 Grinkevich et al.

A

| | cont | MG132 | RITA, 6 hr | RITA, 6 hr + MG132 |

HCT116

HCT116 *TP53-/-*

B

HCT116   HCT116 + RITA

chx, min: 0 10 20 30 40 60   0 10 20 30 40 60

Mcl-1

actin
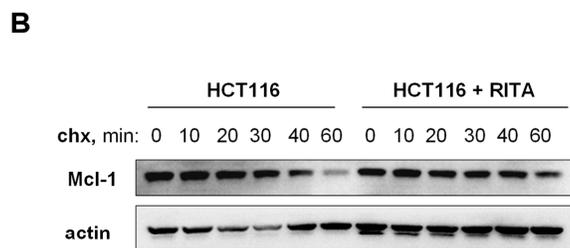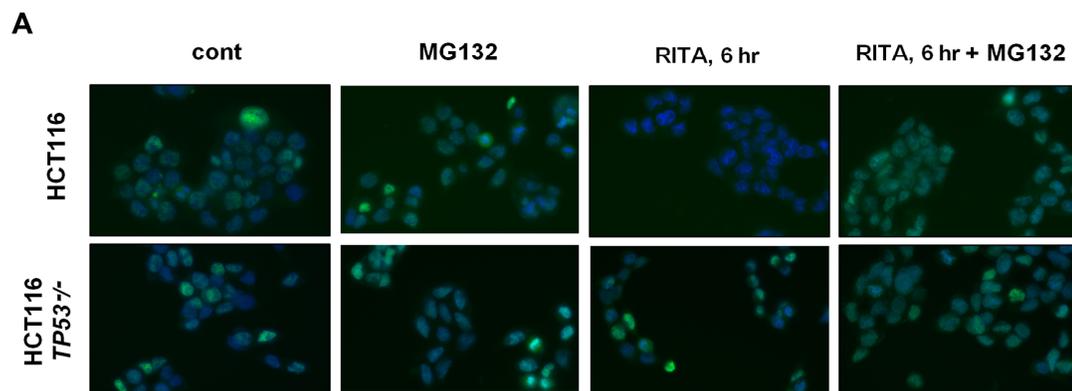
A
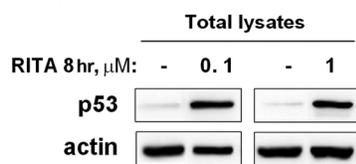
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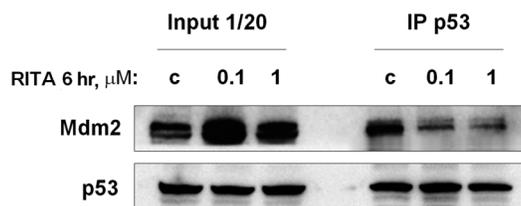
Supplemental Figure 4

A

B
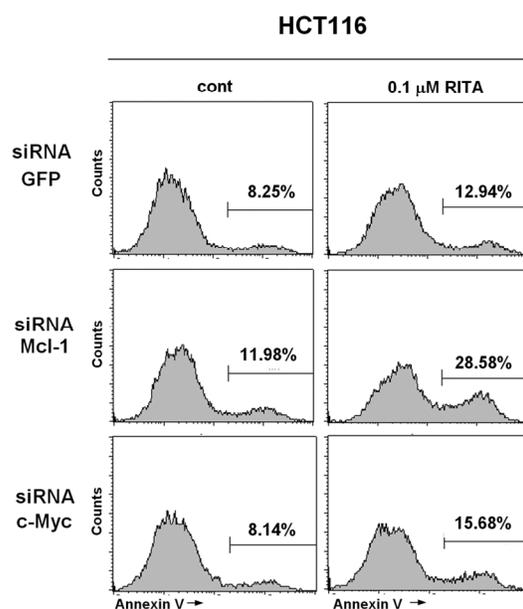
C
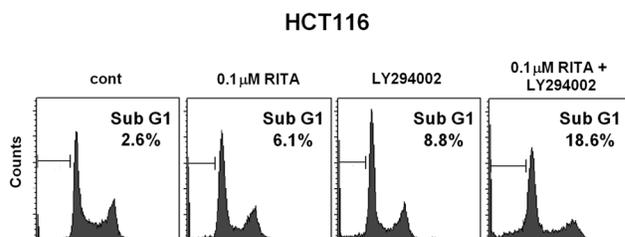
Figure S1.

(A) Depletion of p53 with two different lentivirus constructs encoding p53shRNA in U2OS and MCF7 cells did not prevent p53 accumulation after RITA treatment, as assessed by Western blot.

(B) FACS of Propidium Iodide (PI)-stained cells shows that 5-fluorouracil dramatically increased apoptosis (subG1 fraction) in non-transformed cell lines MCF10A and 184A1, in contrast to RITA.

Figure S2.

(A) Immunofluorescence analysis demonstrated that decrease of c-Myc after 6h of RITA treatment was prevented by proteasome inhibitor MG132. Scale bars 50 μm. Green, signal for anti-c-Myc; blue, nuclei stained with Hoechst. For immunostaining we used c-Myc (N-262) antibodies (Santa Cruz) and secondary FITC-conjugated goat-anti-rabbit antibodies from Jackson ImmunoResearch.

(B) Half-life of Mcl-1 did not change after 1μM RITA treatment in HCT116 cells as assessed by immunoblotting of Mcl-1 upon treatment with cycloheximide for indicated periods.

Figure S3.

(A) p53 is upregulated to the same extent upon 0.1 and 1 μM RITA treatment in MCF7 cells as assessed by Western Blot.

(B) Both 0.1 and 1 μM RITA induce disruption of interaction between p53 and Mdm2. Immunoprecipitation (IP) was performed using p53 (FL-393) conjugated agarose beads (Santa Cruz). IP samples were normalised to p53 levels.

Figure S4.

(A) Efficiency of c-Myc and Mcl-1 knock-down by siRNA in HCT116 and MCF7 cell lines was assessed by Western blot.

(B) FACS of Annexin-stained cells shows increased apoptosis upon combination of 0.1 μM RITA with c-Myc or Mcl-1 knockdown.

(C) FACS of PI stained cells shows increase of subG1 fraction upon treatment of HCT116 cells with sub-micromolar dose of RITA in combination with PI(3)-kinase inhibitor LY294002.

Supplemental Table 1

Primers (5'-3')		Sequence
QRT-PCR		
PIK3CA	F	TATGATGCAGCCATTGACC
	R	ATGAAACAGTTGTCCATCGT
PIK3CB	F	TTGGATCTTCGGATGTTGC
	R	AGGGCATCTTTGTTGAAGG
EIF4E	F	CATATAGGGAGGGTATACAAGGA
	R	CTATGAGAATACTCAGAAGGTGTC
MCL-1	F	TGGCTAAACACTTGAAGACC
	R	GTCCTCTACATGGAAGAACTC
BCL-2	F	GCCTTCTTTGAGTTCGGT
	R	AGTTCCACAAAGGCATCC
MAP4	F	AGGTGGTAATGTTCAGATTCAG
	R	TCAATCTTGACATCTCCTCCA
MYC	F	CAGCTGCTTAGACGCTGGATT
	R	GTAGAAATACGGCTGCACCGA
IGF-1R	F	CCATTCTCATGCCTTGGTCT
	R	TGCAAGTTCTGGTTGTCGAG
BIRC5	F	AAGAACTGGCCCTTCTTGGA
	R	CAACCGGACGAATGCTTTT
hCDC4β	F	GTTGCCGGTTCTGCTCCCTAATCT
	R	CCTGTAGGTGGCTGGACAGATGT
hCDC4γ	F	CCATGGCTTGGTTCCTGTTGATCTT
	R	CCTGTAGGTGGCTGGACAGATGT
CDKN1A (p21)	F	CAGAGGCAGTAACCATGC
	R	TGTAGGACCTTCGGTGAC
PMAIP1 (NOXA)	F	AAGTGCAAGTAGCTGGAAG
	R	TGTCTCCAAATCTCCTGAGT
BAX	F	GCTGTTGGGCTGGATCCAAG
	R	TCAGCCCATCTTCTTCCAGA
GAPDH	F	TCATTTCCTGGTATGACAACG
	R	ATGTGGGCCATGAGGT
Chromatin Immunoprecipitation		
MCL-1	F	CTTGACGGAAGGCTCAG
	R	AGAAACAGGTTCCTAATTACGG
CDKN1A (p21)	F	CCCTCCATCCCTATGCT
	R	CAGCCTCTTCTATGCCAG

ROS-dependent activation of JNK converts p53 into an efficient inhibitor of oncogenes leading to robust apoptosis

Running tile: ROS-JNK-p53 Axis Confers Robust Apoptosis

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Keywords: TrxR, ROS, JNK, p53, Wip1, inhibition of oncogenes

Abstract

Rescue of the p53 tumor suppressor is an attractive cancer therapy approach. However, pharmacologically activated p53 can induce diverse responses ranging from cell death to growth arrest and DNA repair, which limits the efficient application of p53-reactivating drugs in clinic. Elucidation of the molecular mechanisms defining the biological outcome upon p53 activation remains a grand challenge in the p53 field. Here, we report that concurrent pharmacological activation of p53 and the inhibition of TrxR followed by generation of reactive oxygen species (ROS), result in the synthetic lethality in cancer cells. ROS promote the activation of JNK, which establishes a positive feedback loop with p53. This converts the p53-induced growth arrest/senescence to apoptosis. We identified several survival oncogenes inhibited by p53 in JNK-dependent manner, including Mcl-1, PI3K, eIF4E, as well as p53 inhibitors Wip1 and MdmX. Further, we show that Wip1 is one of the crucial executors downstream of JNK whose ablation confers the enhanced and sustained p53 transcriptional response contributing to cell death. Our study provides novel insights for manipulating p53 response in a controlled way. Further, our results may enable new pharmacological strategy to exploit abnormally high ROS level, often linked to higher aggressiveness in cancer, to selectively kill cancer cells upon pharmacological reactivation of p53.

Introduction

The p53 tumor suppressor is a promising target for cancer therapy; several compounds targeting p53 are currently being tested in clinical setting [1]. *In vivo* studies support the idea of pharmacological restoration of p53 to combat cancer [2, 3, 4]. Activation of p53 can lead to growth arrest, senescence or cell death, but elucidation of the molecular mechanisms driving the life/death decision by p53 remains one of the grand challenges in p53 biology [5]. Since the p53-mediated senescence or growth arrest can prevent cancer cell killing by chemotherapy thus leading to poor clinical outcome [6], it is imperative to understand the mechanism of p53-mediated cell fate decisions for the efficient clinical application of drugs activating p53.

We have previously shown that in spite of different transcriptional programs induced by p53 in breast cancer cells upon administration of different p53-activating compounds, p53 binds the same set of genes, irrespective of the type of treatment [7]. This finding supports the view that the heterogeneous response and selective regulation of p53 target genes is likely to be influenced by other signal transduction pathways. A wealth of studies have looked into the p53 interactions with its partners and the type of p53 posttranslational modifications, but it still remains elusive, when, how and which factors direct p53 to a certain transcriptional program [5]. A number of p53-modifying enzymes have been identified, including checkpoint kinases ATM/ATR, Chk2 [5], as well as mitogen-activated protein kinases (MAPK) p38 and c-Jun N-terminal kinase JNK [8] induced by oxidative stress.

Cancer cells frequently have increased burden of oxidative stress [9] and therefore are likely to be more sensitive to the damage promoted by further ROS insults. Recent studies have revealed the dependency of cancers on redox-regulating mechanisms, such as the glutaredoxin and the thioredoxin systems, to be the cancer-specific vulnerability thereby offering a target for treatment of malignancies [9, 10]. The NADPH-dependent selenoprotein thioredoxin reductase (TrxR), often overexpressed in cancer, is one of the promising anticancer drug targets, which is inhibited by several anti-cancer drugs in clinical use [11, 12].

In the present study, we identified ROS-activated JNK as a crucial p53 co-regulator, revealing a strategy to switch the p53 transcriptional response from growth arrest to apoptosis upon its pharmacological activation.

Results

Transient versus sustained changes in gene expression upon p53-mediated growth arrest and apoptosis

To address the mechanisms of the differential biological outcome upon p53 activation, we used as molecular probes p53-reactivating molecules RITA and nutlin, which inhibit p53/MDM2 interaction [13]. As a model we applied a pair of cell lines, breast carcinoma MCF7 and colon carcinoma HCT116, in which activation of p53 by 10 μM nutlin and 0.1 μM RITA leads to growth inhibition, whereas 1 μM RITA induces apoptosis [14, 15]. Since high doses of nutlin induce

p53-independent cell death (Supplementary Figure S1C), we used 10 μM nutlin in our experiments.

We compared the kinetics of gene expression changes in MCF7 cells upon treatment with 10 μM nutlin, 0.1 or 1 μM RITA at ten time points using microarray analysis. Systematic clustering analysis showed that the genes involved in cell cycle regulation, metabolic and biosynthetic processes were continuously repressed upon 1 μM RITA; in contrast, these genes were only transiently repressed after nutlin and 0.1 μM RITA treatment (clusters 0001 and 0002 in Figure 1a and Table S1)..

Another gene cluster, comprising the stress response genes, was continuously induced by 1 μM RITA, but only transiently upregulated by nutlin and 0.1 μM RITA (cluster 0004 in Figure 1a).

Next we analyzed whether known p53 target genes are regulated in a similar differential manner. We found that 1 μM RITA led to the sustained induction of *ENC1, GADD45A, PMAIP1, LIF* and *SESN1,* and inhibition of pro-survival genes *IGF1R, MCL1, MYC, BCL2, PIK3CA* and *PIK3CB*; however, changes in the expression of these genes were only transient upon 0.1 μM RITA (Figure 1b).

In conclusion, the induction of apoptosis was associated with the sustained p53-mediated transcriptional response. This prompted us to investigate the factors underlying this phenomenon.

ATM-independent induction of p53-dependent DNA damage response upon 1 μM **RITA**

In line with the previous findings [16], we observed an increased phosphorylation of H2AX on Ser139 (γH2AX), a hallmark of DNA damage response (DDR), and phosphorylation of p53 on Ser15 (p-S15-p53) upon 1 μM RITA in a time-dependent manner in MCF7, HCT116 (Figure 2a) and U2OS cells (Supplementary Figure S1A). In contrast, low dose of RITA only barely affected the DDR (Figures 2a, 4a, 5b and Supplementary Figure S1A), as did nutlin [17], which correlates with the inefficient apoptosis induction, as evidenced by cleaved PARP levels (Figure 4a and [14, 15]).

Importantly, the induction of γH2AX by RITA was observed only in the presence of p53, i.e. in p53-positive HCT116 cells, but not in p53-null HCT116 p53-/-, osteosarcoma Saos2 and lung adenocarcinoma H1299 cells (Figure 2c, left and right panel). The p53-dependence of γH2AX induction was further supported by the ablation of γH2AX by the p53 inhibitor pifithrin-α [18] and upon p53 depletion with siRNA (Figure 2c, middle panel and Supplementary Figure S1B, respectively). We ruled out the possibility that DDR was induced upon DNA fragmentation during apoptosis, since the pre-treatment with a pan-caspase inhibitor Z-VAD-fmk did not prevent γH2AX, while it did prevent PARP cleavage (Supplementary Figure S1D).

Alkaline comet assay revealed that a "comet tail", indicating DNA strand breaks, was barely detectable upon 1 μM RITA, while positive control doxorubicin produced a clear pattern (Figure 2d). We did not detect DNA strand breaks using pulse-field electrophoresis assay either (Supplementary Figure S1E). Thus, in line with the previously published data [16, 19], RITA produces a low number of strand breaks, if any. Importantly, RITA did not

induce the DDR and apoptosis in the non-tumorigenic cells MCF10A and 184A1 (Figure 2b and Supplementary Figure S2F).

To find out the mechanism of DDR induction, we tested the involvement of checkpoint kinases. Depletion of ATM by siRNA did not prevent γH2AX and p53 accumulation, neither did the pretreatment with the ATM inhibitor KU55933 or with the major DDR kinase inhibitor caffeine (Supplementary Figures S1F-H), ruling out the involvement of these kinases. However, the kinetics and the extent of p53 accumulation were partially affected by caffeine (Supplementary Figure S1H), suggesting that DDR contributes to the faster and robust induction of p53, perhaps via amplification of the signaling to p53.

In conclusion, the induction of DDR was p53-, but not ATM/ATR-dependent and correlated with the induction of apoptosis.

Generation of ROS leads to DDR and confers synthetic lethality upon p53 reactivation

Since ROS can cause DNA damage response [20], we reasoned that the induction of γH2AX could be due to the p53-dependent induction of ROS resulting from the inhibition of TrxR1 by 1 μM RITA, reported previously by us [21]. More detailed analysis of the effect of RITA on TrxR1 in *in vitro* enzymatic assay revealed that while RITA inhibited the reducing activity of TrxR1 on two different substrates, it barely affected its NADPH oxidase function (Figure 3a), which endows the enzyme with pro-oxidant activity [22, 23]. Thus, both the inhibited reductase and the sustained oxidase activities of TrxR1 upon RITA

should contribute to ROS accumulation. Indeed, 1 μM RITA induced intracellular ROS in MCF7 and HCT116 cells, whereas a low dose of RITA or nutlin did not trigger ROS (Figure 3b, Supplementary Figures S2A, S3A-B). In line with our previously published results[21], ROS were not induced in non-transformed MCF10A cells (Supplementary Figures S2E and S3C), correlating with the absence of DDR. Thus, DDR was associated with the induction of ROS.

Next we addressed the question whether generation of ROS is the cause of DDR and whether it contributes to p53-mediated cell death. We found that ROS scavenger *N*-Acetyl-L-cysteine (NAC), as well as a low dose (1 μM) of antioxidant resveratrol [24] inhibited ROS induced by RITA (Figure 3c, Supplementary Figures S2B, S3A-B). Notably, both antioxidants prevented the induction of γH2AX (Figure 3d and Supplementary Figure S2C), supporting our idea that the induction of DDR is triggered by ROS.

However, NAC or resveratrol did not prevent the accumulation of p53 by RITA, indicating that p53 induction is not due to ROS.

NAC and resveratrol partially reversed the apoptosis triggered by RITA, as evidenced by the decreased PARP cleavage and the rescue of cell viability (Figure 3d and Supplementary Figures S2C, S4A). These data corroborated the involvement of ROS in p53-mediated apoptosis. Accordingly, FACS analysis confirmed that resveratrol, as well as another potent antioxidant nordihydroguaiaretic acid (NDGA) [25] significantly inhibited apoptosis upon RITA (Supplementary Figure S2D).

To reinforce the role of ROS as a possible denominator of the apoptotic response upon p53 reactivation, we examined whether blocking

TrxR by auranofin, a well-known inhibitor of TrxR [26] and inducer of ROS (Figure 3b and Supplementary Figure S2A), could convert the growth arrest/senescence induced by nutlin into apoptosis. As evident from Figure 3e, a low dose of auranofin was synthetic lethal with p53 activation by nutlin. This is manifested by the robust induction of apoptosis upon auranofin/nutlin combination, while both agents barely induced cell death, when taken alone; importantly, NAC reverted the synthetic lethality induced by the combination of nutlin and auranofin (Figures 3e-f, and Supplementary Figure S4C). We also observed a synergistic effect upon the combination of low dose of RITA and auranofin; similarly, NAC reverted the synthetic lethality upon low dose of RITA and auranofin as well (Figures 3f-g and Supplementary Figure S4C).

To elucidate whether ROS contribute to the apoptosis induction upon other types of pharmacological activation of p53, we used cisplatin (CDDP), which has been shown to activate p53 and inhibit the TrxR enzyme activity [27, 28]. We found that pretreatment with NAC, resveratrol or NDGA prevented the elimination of tumor cells by CDDP (Supplementary Figure S4B).

Taken together, our data suggested that disabling the oxidative defense mechanisms in cancer cells, for instance, via TrxR inhibition, is synthetic lethal when combined with the pharmacological restoration of p53.

Activation of JNK triggered by ROS mediates the synthetic lethality upon p53 activation and inhibition of TrxR

Next, we investigated which factor mediates the synthetic lethal effect of p53 activation combined with TrxR inhibition. The activation of MAP kinase JNK

upon inhibition of TrxR [29] and its ability to modulate p53 make JNK an attractive candidate mediator of ROS signaling to p53. We found that RITA induced JNK phosphorylation (p-JNK) in a p53- and dose-dependent manner (Figures 4a-b). Notably, the induction of p-JNK was conferred by the increased ROS levels, since NAC and resveratrol inhibited JNK activation (Figure 4c and Supplementary Figure S5A). Importantly, JNK serves as a critical mediator of the p53-induced apoptosis, as evidenced by the rescue of PARP cleavage, growth suppression and subG1 fraction by JNK inhibitor SP600125 and siRNA-mediated depletion of JNK (Figures 4d, 4h and Supplementary Figures S5B-C).

Furthermore, we found that the synthetic lethal effect of the low dose of RITA/auranofin combination is mediated by p-JNK. Auranofin combined with the low dose of RITA led to the robust induction of p-JNK. Notably, the induction of apoptosis upon this combination treatment, manifested as PARP cleavage, was prevented by JNK inhibitor (Figure 4e).

Thus, we concluded that JNK is a crucial player downstream of ROS in the molecular pathway leading to the synthetic lethality upon p53 activation combined with TrxR inhibition.

JNK activated by ROS contributes to the induction of DDR and converts p53 into an efficient inhibitor of oncogenes

Next, we focused on elucidating the mechanisms by which JNK enhances p53-induced apoptosis. Since JNK has been shown to mediate the UV-induced γH2AX [30], we assessed the role of JNK in γH2AX accumulation. JNK

inhibitor SP600125, as well as JNK depletion by siRNA, markedly reduced γH2AX (Figures 4d-f and Supplementary Figure S5B), implying that JNK is the critical kinase mediating DDR. In addition, JNK mediated p53 phosphorylation at Ser33 (Figures 4d-e, 4g and Supplementary Figure S5D).

Strikingly, the p53-dependent downregulation of several oncogenes, anti-apoptotic factor Mcl-1 and p53 inhibitors Wip1 and MdmX (Figure 4b, Supplementary Figure S1B), was rescued by NAC, resveratrol and JNK inhibitor (Figures 4c-e and Supplementary Figures S5A-B). Further evidence of the crucial role of ROS and JNK in oncogene downregulation by p53 was provided by the experiments in which we used the combination of the low dose of RITA with auranofin. While Wip1 expression was increased upon the low dose of RITA, its induction was partially prevented by auranofin (Figure 4e). Moreover, the combination of low dose of RITA and auranofin led to a dramatic downregulation of MdmX. Notably, auranofin-mediated downregulation of Wip1 and MdmX was rescued by JNK inhibitor (Figure 4e).

The JNK-dependence of Wip1 and MdmX downregulation was further supported by the JNK depletion experiments (Figure 4g and Supplementary Figure S5D).

Interestingly, the p53 level was largely unaffected by the JNK inhibitor, as well as the induction of its target genes Puma and Noxa (Figuress 4c-d and Supplementary Figures S5A-B).

Next, we assessed whether the observed inhibition of oncogenes occurs on mRNA level. In line with our previous results [15], RT-qPCR experiments demonstrated that pharmacological activation of p53 led to a decreased mRNA level of a set of oncogenes, including *MCL1*, Wip1-

encoding *PPM1D*, *MDM4* (MdmX), as well as *PIK3CA* and *PIK3CB*, encoding catalytic subunits of PI3 kinase, and translation factor *EIF4E* (Figure 4i). The rescue of oncogene inhibition by JNK inhibitor corroborated the key role of JNK (Figure 4i). In addition, we have previously shown that *MCL1*, *PIK3CA*, and *PIK3CB* are not downregulated by the low dose of RITA or nultin; however, their inhibition converts the p53-mediated growth arrest into apoptosis [15, 31].

Therefore, we concluded that the induction of ROS upon RITA leads to the activation of JNK which mediates the phosphorylation of H2AX at Ser139, phosphorylation of p53 at Ser33 and the inhibition of the expression of a set of pro-survival oncogenes by p53, conferring apoptosis induction.

Next, we addressed the question whether and how the repression of *PPM1D*, downstream of JNK, contributes to the synthetic lethal effect.

Inhibition of Wip1 by p53 promotes the induction of γH2AX

p53 activation is opposed by Wip1, an oncogene which removes inactivating phosphorylation marks from Mdm2 and activating phosphorylation marks from p53, p14/p19[ARF] and checkpoint kinases [32], and dephosphorylates γH2AX [33, 34], thereby attenuating DDR. Moreover, Wip1 is a p53 target gene which serves as one of the critical determinants of the biological outcome [34, 35]. These data and our present results showing that Wip1 activation was induced by nutlin and low dose of RITA (Supplementary Figures S6D-E and Figure 4e, respectively), but was repressed by 1 μM RITA in JNK-dependent manner, prompted us to test how inhibition of Wip1 contributes to p53 activity.

We found that the decline of Wip1 mRNA and protein levels upon RITA strongly correlated with γH2AX induction (Figures 5a-b).

Inhibition of Wip1 plays a role in DDR induction upon RITA, since its depletion by shRNA in combination with the low dose of RITA significantly increased the level of γH2AX, comparable with the level induced by 1 μM RITA (Figure 5c).

The negative contribution of Wip1 to DDR was supported by a significantly reduced induction of γH2AX by RITA upon Wip1 overexpression, as assessed by Western blotting (Figure 5d).

Wip1 depletion enhances the transactivation function of p53 and contributes to the synthetic lethality

We reasoned that the enhancement of DDR resulting from Wip1 down-regulation could facilitate the p53 transcriptional activity leading to a more robust response. To assess this notion, we analyzed the p53 transcriptional response upon Wip1 silencing in MCF7 cells treated with the low dose (0.1 μM) RITA for 4 and 16 hours using microarray analysis.

Wip1 depletion *per se* only weakly affected the expression of p53 target genes (Figure 6a). Low dose of RITA induced several p53 targets including *GPRC5A* [7], *TNFRSF10B* (KILLER/DR5), *FAS*, *RPRM*, and *ENC1* (PIG10) [36]; however, their induction was not observed at a late time point. Unsupervised clustering analysis indicated similarity of the profiles obtained at 16h of treatment with control samples (Figure 6a).

Notably, Wip1 depletion by shRNA facilitated the induction of several

p53 targets by the low dose of RITA and rescued their decline at 16h (Figure 6a). This indicates that the inhibition of Wip1 conferred sustainability to p53 response.

qPCR experiments confirmed that upon Wip1 silencing the expression of p53 targets *FAS*, *GDF15* and *BTG2* was substantially induced, further elevated upon 0.1 μM RITA and lasted longer (Figure 6c, upper panel), confirming that the inhibition of Wip1 leads to a sustained p53-mediated transactivation.

However, Wip1 silencing did not facilitate the downregulation of the pro-survival genes (Figure 6b and Figure 6c, lower panel), which are inhibited by 1 μM RITA in a p53-dependent manner [15]. In line with these data, ectopic expression of Wip1 did not prevent RITA-induced downregulation of Mcl-1 (Figure 5d). Further, we found that Wip1 overexpression did not relieve the repression of *PIK3CA, PI3KCB* and *IGF-1R* (Figure 6d). These results suggest that in contrast to p53 transactivation function, p53-mediated inhibition of oncogenes is less tightly regulated by Wip1.

Next, we addressed whether inhibition of Wip1 contributes to the biological response triggered by p53. Indeed, Wip1 depletion promoted the apoptosis induced by both 0.1 and 1 μM RITA, as shown in Figure 6e and Supplementary Figures S6A-B

As shown in Figure 4e and Supplementary Figure S6E, auranofin partially prevented the induction of Wip1 by low RITA or nutlin in U2OS cells as well as triggered the downregulation of Mdm2 and MdmX and apoptosis (Supplementary Figures S6E-F). We observed a prominent induction of cell death associated with the increase of γH2AX, p53 and decrease of MdmX in

the Wip1-silenced cells upon auranofin treatment (Supplementary Figures S6C-D). Induction of cell death by auranofin in Wip1-depleted cells was comparable with the extent of apoptosis upon nutlin/auranofin combination, which underscores the crucial role of Wip1

Taken together, our data demonstrate that the inhibition of Wip1 significantly contributes to the synthetic lethal effects upon p53 activation and TrxR inhibition.

Discussion

Elucidation of the molecular mechanisms governing the cellular responses elicited by p53 is still a challenge in the p53 field, limiting the effective harnessing of p53 activity for cancer treatment [5]. Our present study revealed the crucial role of ROS and activated JNK in triggering p53's pro-apoptotic function in cancer cells upon pharmacological activation of p53. Further, we elucidated the JNK-mediated mechanisms which play a role in this process and identified a set of key factors downstream of p53 affected by JNK that confer cell death outcome.

Our previous study established that the inhibition of anti-apoptotic factor Mcl-1, as well as catalytic subunits of PI3 kinase, translational factor eIF4E and p53 inhibitor MdmX, all well-known oncogenes and a high-priority anti-cancer targets [37, 38, 39, 40], is crucial for the robust apoptosis induction by p53 [15]. However, it remained unclear which factors control the inhibition of survival genes by p53. Here, we demonstrated that the ablation of this set of oncogenes by p53 is JNK-dependent.

Despite the extensive efforts, we still have much to learn about the molecular mechanisms of p53-mediated transcriptional repression [36]. p21 has been implicated in the p53-mediated repression of a number of genes [36]. However, p21 is depleted upon p53 activation by RITA, which contributes to the induction of apoptosis [14], ruling out the possibility that the observed transcriptional repression is p21-dependent.

p53 might play a direct role in the transcriptional repression of several oncogenes, including *EIF4E*, *PPM1D* and *MDM4*, as evidenced by p53 binding to its consensus motif within a short distance from the transcriptional starting site of these genes (Supplementary Table S2), identified using p53 chromatin immunoprecipitation coupled to deep sequencing (ChIP-seq) described in [7]. The binding of p53 to *MYC* and *MCL1* promoters we have previously described [15, 31]. However, we can not rule out an indirect transcriptional inhibition of oncogenes by p53 which might be attributed to the p53-regulated microRNAs or other p53-upregulated factors.

We have previously found that p53-activated gene *PPM1D*, whose product Wip1 inhibits p53 signalling [32], is repressed upon RITA [35]. However, the mechanism of this intriguing phenomenon has not been identified. Our present study implicates JNK as a crucial factor which can convert p53 from a transactivator to a repressor of Wip1.

Based on these results, we suggest a model illustrating how pharmacological release of p53 from the Mdm2 complex, combined with the inhibition of TrxR, results in the excessive accumulation of ROS which leads to further p53 activation by JNK (Figure 6f). In turn, active p53 induces pro-oxidant genes, such as PUMA and PIGs, increasing ROS and further feeding

activating signals to JNK and thus, itself. p-JNK converts p53 to an efficient inhibitor of pro-survival oncogenes Mcl-1, eIF4E and PI3 kinase, which contributes to apoptosis induction. Further, two p53's own inhibitors, Wip1 and MdmX, are repressed in p-JNK-dependent manner, which amplifies p53-activation. Establishment of the JNK-p53 positive feedback loop and the inhibition of p53-Wip1 negative feedback loop result in the enhanced and sustained p53 activation, which produces a robust apoptotic outcome, leading to the effective elimination of cancer cells.

Molecular pathways by which reconstituted p53 becomes pro-apoptotic selectively in malignant tumors are a subject of a debate [2, 3, 41, 42, 43]. Reinstatement of p53 efficiently eliminates advanced lung carcinoma cells whereas leaving early lesions unperturbed. This is due to the amplified stress-activated MAPK, which engages the MDM2 inhibitor p19ARF (p14ARF in humans), in turn activating p53 [2, 3]. Notably, another *in vivo* study has shown that JNK is required for p53 induction upon oncogene activation [43].

Our data suggest that elevated ROS in malignant tumors might provide an activating signal to p53 via JNK leading to the enhanced and sustained p53 activity. It is tempting to speculate that this may constitute a basis for the selective elimination of advanced cancers by the reinstated p53, observed in mouse models [2, 3]. The relative contribution of the ROS/MAPK pathway in oncogenic signaling and preferential suppression of malignant tumors by p53 is an interesting subject for future studies.

Our results demonstrating that pharmacological activation of p53 in combination with TrxR inhibition and ROS induction confers synthetic lethality, could be an important consideration for the clinical application of p53-

reactivating drugs. Indeed, we showed that growth arrest/senescence by nutlin, which is now being tested in clinic, could be converted to apoptosis upon the low dose of TrxR inhibitor auranofin. Furthermore, auranofin is a FDA approved drug for the treatment of rheumatoid arthritis; therefore repositioning such old drug for cancer therapy through the combination strategy as shown in this study will save the time and the cost for developing more effective cancer treatment approaches.

One of the important biochemical differences between normal and cancer cells is a decreased capability of cancer cells to buffer high ROS levels. Our study suggests that dual targeting of p53 and the cellular antioxidant system might allow to maximally exploit the p53-mediated tumor suppression as a therapeutic strategy.

Materials and Methods

Cell culture. Colon carcinoma cell lines HCT116 and HCT116 TP53-/- were kindly provided by B. Vogelstein, John Hopkins University, USA. p14ARF negative HCT116, HCT116 TP53-/-, breast carcinoma MCF7, osteosarcoma U2OS cells were grown under standard conditions; mammary epithelial cell line MCF10A and 184A1 were obtained from Serhiy Souchelnytskyi, Karolinska Institutet. MCF10A cells were kept in medium containing 50% of MEBM (Clonetics), 50% of Nutrient Mixture F-12 HAM (Sigma) and 5% horse serum supplemented with MEGM SingleQuot (Clonetics); 184A1 cells were kept in MEGM complete medium supplemented with 5% horse serum.

Plasmids, shRNA and siRNA. Plasmids encoding FLAG-Wip1 and shRNA for Wip1 were kindly provided by René H. Medema, Utrecht, Netherlands. ATM and p53 siRNA were from Santa Cruz Biotechnology. Custom siRNA targeting both JNK1 and JNK2 [44] was synthesized by Thermo Scientific Dharmacom. Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) and siRNA was transfected using HiPerFect (Qiagen) according to manufacturer's instructions.

Drug treatments. RITA was obtained from the National Cancer Institute (NCI). Nutlin and caffeine from Calbiochem were used at 10 μM and 4 mM, respectively. Resveratrol (BIOMOL International) was used at 1 μM. Pifithrin-α, SP600125, neocarzinostatin, cisplatin (CDDP), H_2O_2 and *N*-Acetyl-L-cysteine (NAC) (all from Sigma) were used at 10 μM, 40 μM, 200 ng/ml, 50 μM, 400 μM and 5 mM, respectively. Pan-caspase inhibitor Z-VAD-FMK (R&D Systems) and KU55933 (Tocris Bioscience) were used at 10 μM. Nordihydroguaiaretic acid (NDGA), a kind gift from O. Rådmark and Doxorubicin from B. Zhivitovsky, (both from Karolinska Institutet) were used at 10 and 2 μM, respectively.

Molecular and cell biology assays. Western blotting was performed according to a standard procedure. The following antibodies were used: anti-p53 (DO-1), PARP-1/2, Mcl-1, c-Myc, phospho-p53 (Ser15), phospho-p53 (Ser33), phospho-JNK (Cell Signaling), phospho-histone H2AX (Ser139) (Millipore), ATM (Abcam), Wip1 (Bethyl laboratories), Noxa, Puma (Calbiochem), β-actin (Sigma). After transfer membranes were cut to detect

several proteins on the same membrane; in Fig. 3 and Fig. S3, the proteins were detected in the following order: (1) Wip1, p-JNK (30 minutes exposure), γH2AX; (2) MdmX, p-S15-p53, actin, Puma; (3) PARP, p-S33-p53, Mcl1, Noxa; (4) p53.

Alkaline comet assay, FACS analysis of propidium iodide (PI)-stained cells and qPCR were performed as described [45] and [14]. FACS analysis of FITC-Annexin V and PI (from BD Biosciences) double-stained cells was performed according to the manufacture's protocols. Detection of activated caspases by FAM-FLICA[TM] Poly-Caspase Detection Kit from ImmunoChemistry TECHNOLOGIES was performed using FACS according to the manufacture's protocol..

Primers are described in Table S3.

ROS measurement. ROS were measured as in [21]. Briefly, after treatment with different compounds, cells were washed once with serum-free medium and incubated with 10 μM 2',7'-Dichlorodihydrofluorescein diacetate (DCF-DA) in serum-free medium for half an hour under standard conditions; then cells were washed twice with PBS, trypsinized, harvested and washed another two times with PBS. The samples were sorted on Becton Dickinson FCAScan using FL1-H channel, and analyzed by CellQuest software 4.0.2.

Microarray analysis. Systematic clustering of gene expression data was performed with CRC clustering method [46]. When applied to 2000 10-point profiles with the largest fold change, the method gives about ten high quality clusters with more than 40 profiles in them, and with the gene lists of the

clusters enriched in GO terms at P-value < 0.002. To compare gene expression profiles under different treatments, we plot the mean expression profile of the clustered genes in one case and the mean profile of the same (not clustered) genes in other cases. This allowed detecting of conserved and variable features.

p53 ChIP-seq. The detailed description of p53 ChIP-seq experiment is provided in [7]; the analysis of p53 binding to inhibited genes was performed as described in [31].

In vitro **assay of TrxR1 activity**. In this inhibition analysis, 50 nM wild type TrxR1 (24.0 U/mg) was reduced by 150 µM NADPH in TE buffer (pH 7.5) and then RITA at defined concentration ranging from 0.1 µM to 200 µM was incubated with the reduced enzyme in the dark at room temperature for 60 min. DMSO instead of RITA was used as control. Small aliquots of RITA-inhibited enzyme were taken out at 60-min time point for measuring the DTNB reduction activity and the NADPH-reduced enzyme was used as control. After TrxR1 activity was inhibited, 500 µl RITA-inhibited TrxR1 (50nM) was applied onto TE-equilibrated NAP-5 column (GE Healthcare Life Sciences, Uppsala, Sweden) and then eluted with 1 ml TE buffer. TrxR1 activities using DTNB (2.5 mM), human Trx1 (20 µM) and juglone (50 µM) as substrates were measured after desalting. DTNB reduction was measure at 412 nm (extinction coefficient of 13,600 M^{-1} cm^{-1}) by adding the desalted enzyme respectively into the DTNB reaction mixture (200 µl) contained 2.5 mM DTNB, 300 µM NADPH and 4.5 nM enzyme in TE buffer (pH7.5). Trx-coupled insulin assay

was performed by measuring the NADPH consumption at 340nm (extinction coefficient of 6,220 M^{-1} cm^{-1}) and reaction system contains 20 μM human Trx1, 160 μM insulin, 300 μM NADPH and 9 nM enzyme in TE buffer (pH 7.5). The NADPH oxidase activity was monitored as the decrease at 340 nm (extinction coefficient of 6,220 M^{-1} cm^{-1}) for 60 min. The reaction system (200 μl) contains 50 μM juglone, 200 μM NADPH and 4.5 nM enzyme in TE buffer (pH 7.5). Enzymatic reactions and measurements were performed with 10-sec time interval reads at 25 °C using a VersaMax microplate reader (Molecular Devices, USA), with the reaction mixtures without enzyme serving as reference. Activity measurements were performed in triplicate and analyzed with the Prism 5 software (GraphPad, USA).

Acknowledgements

Supplementary information is available at Cell Death and Differentiation's website.

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FIGURE LEGENDS

Figure 1 Different kinetics of gene expression upon pharmacologically activated p53. (a) Systematic clustering analysis of gene expression profiles revealed distinct kinetics of transcription of several gene clusters upon treatment with different doses of RITA or nutlin for 0, 2, 4, 6, 8, 10, 12, 16, 20, 24 hours in MCF7 cells. The plots show mean profile (bold) and RMS deviation (grey) for genes, found to have clustered profiles in 1 μM RITA. (b) Heatmaps of representative p53 target genes differentially regulated by 0.1 and 1 μM RITA in MCF7 cells. Values are normalized to untreated control.

Figure 2 p53- and dose-dependent induction of DDR by RITA in cancer cells, but not in non-tumorigenic cells. (a) MCF7 and HCT116 cells were treated with 0.1 and 1 μM RITA for indicated periods and levels of p53, γH2AX and p-S15-p53 were detected by immunoblotting. Actin was used as a loading control. (b) RITA did not induce γH2AX in non-tumorigenic MCF10A and 184A1 cells as assessed by immunoblotting. (c) Wild type p53-expressing cells HCT116 and p53-null HCT116 p53-/-, H1299, Saos-2 cells were treated with 1 μM RITA for indicated periods (upper and lower panels); HCT116 cells were pretreated with pifithrin-α for 2 hours and treated with 1 μM RITA for 12 hours (middle panel). Proteins were analyzed by Western blotting. (d) HCT116 cells were treated with 1 μM RITA or doxorubicin for 12 hours and DNA strand breaks were assessed by comet assay. Right panel, quantification of cells containing strand breaks (mean±SEM, n=3).

Figure 3 Induction of ROS leads to DDR and p53-dependent apoptosis. (a) Dose-dependent inhibition of the ability of TrxR1 to reduce 5,5'-Dithiobis (2-Nitrobenzoic Acid) (DTNB) and thioredoxin (Trx), but not its NADPH oxidase activity by RITA, as measured in *in vitro* assay using purified TrxR1. (b) ROS were measured in MCF7 cells treated for 6 hours with 0.1 and 1 μM RITA (upper left panel), nutlin or 400 μM H_2O_2 (upper right panel), and 5 μM TrxR inhibitor auranofin as a positive control (lower panel). (c) Antioxidants NAC and resveratrol prevent the induction of ROS by 1 μM RITA. (d) Antioxidants NAC (upper panel) and resveratrol (lower panel) inhibited the induction of γH2AX and PARP cleavage by RITA as analyzed by Western blotting. (e) MCF7 and HCT116 cells were treated with 2 μM auranofin or 5 μM nutlin, or their combination (with or without NAC pretreatment) for 48 hours; cells were photographed before proceeding to crystal violet staining. (f) HCT116 cells were treated with 2 μM auranofin, 10 μM Nutlin, or 0.1 μM RITA, or their combination (with or without NAC pretreatment) for 48 hours; then cells were harvested and proceeded to double staining with Annexin V and propidium iodide (PI) followed by FACS analysis (mean±SEM, n=3). PI only: necrotic and/or late apoptotic cells; PI + Annexin V: apoptotic cells; Annexin V only: early apoptotic cells; * expected additive effect. (g) U2OS cells were treated with 1 μM auranofin or 0.05 μM RITA, or their combination (with or without NAC or 10 μM SP600125 pre-treatment) for 48 hours and assessed as in (f) (mean±SEM, n=3); * expected additive effect.

Figure 4 ROS-mediated activation of JNK contributes to the p53-mediated apoptosis, DDR and transcriptional repression of oncogenes. (a) Dose-

dependent induction of p-JNK, p-Ser33-p53, p-Ser15-p53, γH2AX, PARP cleavage and inhibition of Wip1, Mcl-1 and MdmX by RITA as assessed by Western blotting. (b) HCT116 and HCT116 p53-/- cells were treated with 1 μM RITA; analyzed as in (a). (c) Pretreatment with NAC for 6h prevented the induction of p-JNK, γH2AX, PARP cleavage and inhibition of Wip1, Mcl-1 and MdmX by RITA as analyzed by immunoblotting. (d) JNK inhibitor SP600125 prevented the induction of p-JNK, p-Ser33-p53, γH2AX, PARP cleavage and inhibition of Wip1, Mcl-1 and MdmX by RITA, as assessed by Western blotting. (e) 10 μM SP600125 blocked the induction of p-JNK, p-Ser33-p53, γH2AX, PARP cleavage and inhibition of MdmX and Wip1 by combination treatment with 0.05 μM RITA and 1 μM auranofin for 24h, as assessed by Western blotting.(f, g) Depletion of JNK by siRNA prevented the induction of γH2AX (f), p-Ser3-p53 and inhibition of Wip1 and MdmX (g), analyzed by immunoblotting. (h) Inhibition of JNK by siRNA prevented apoptosis induction by RITA, as measured by FACS of PI-stained cells. (i) SP600125 blocked the repression of *MCL1, PPM1D, PIK3CA, PIK3CB, EIF4E and MDM4* (MdmX) mRNA upon RITA treatment as assessed by qPCR (mean±SEM, n=3) in HCT116 (12 hours treatment) and MCF7 (8 hours treatment) cells.

Figure 5 Inhibition of Wip1 promotes the induction of γH2AX upon RITA treatment. (a) Wip1 mRNA was repressed after 8 hours treatment with 1 μM RITA, but not 0.1 μM RITA, as assessed by qPCR (mean±SEM, n=3). (b) Downregulation of Wip1 protein level correlated with the induction of γH2AX upon RITA treatment as analyzed by immunoblotting. (c) MCF7 and U2OS cells stably transfected with empty vector shRNA or Wip1 shRNA were

treated with 0.1 and 1 μM RITA for indicated periods and γH2AX was assessed as in (b). (d) HCT116 and U2OS cells transfected with either empty vector or FLAG-Wip1 were treated with 1 μM RITA for indicated times. Proteins were detected by Western blotting.

Figure 6 Depletion of Wip1 confers a sustained transcriptional activation of p53 target genes, but does not facilitate transrepression. (a,b) Microarray analysis of MCF7 cells with (indicated in violet) or without (indicated in grey) Wip1 depletion by shRNA, treated with 0.1 μM RITA or DMSO for indicated time points revealed that p53-mediated transactivation was enhanced by Wip1 silencing. (c) Wip1 downregulation led to the increased induction of p53-activated genes (upper panel) but did not augment the repression of pro-survival genes by p53 (lower panel) upon low dose of RITA as analyzed by qPCR (mean±SEM, n=3). Insert demonstrates the efficiency of Wip1 depletion, as assessed by immunoblotting. (d) MCF7 cells transfected with either empty vector or FLAG-Wip1 were treated with 1 μM RITA or DMSO for 8 hours and mRNA levels of *PIK3CA*, *PIK3CB* and *IGF-1R* were assessed by qPCR (mean±SEM, n=3). (e) MCF7 cells stably transfected with shWip1 or control shVector were treated with 0.1 and 1 μM RITA or DMSO for 24 hours, and cells were stained with Annexin V followed by FACS analysis (mean±SEM, n=3, * $P < 0.05$, ** $P < 0.01$, by two-tailed t test). (f) Model of the synthetic lethality upon activation of p53 and inhibition of TrxR. Inhibition of TrxR lead to the accumulation of ROS and activation of JNK, facilitating p53 function upon its release from Mdm2. In turn, activated p53 induces pro-oxidant genes, which increases the level of ROS, further activating JNK, and

thus, p53. Activated JNK converts p53 from an activator to an inhibitor of Wip1 and MdmX, therefore amplifying p53 activity. Transcriptional repression of Mcl-1, eIF4E and PI3K abolishes survival signaling, contributing to apoptosis induction. Thus, the dual targeting of p53 and TrxR (i.e, by RITA) leads to the robust apoptosis.

Figure 1

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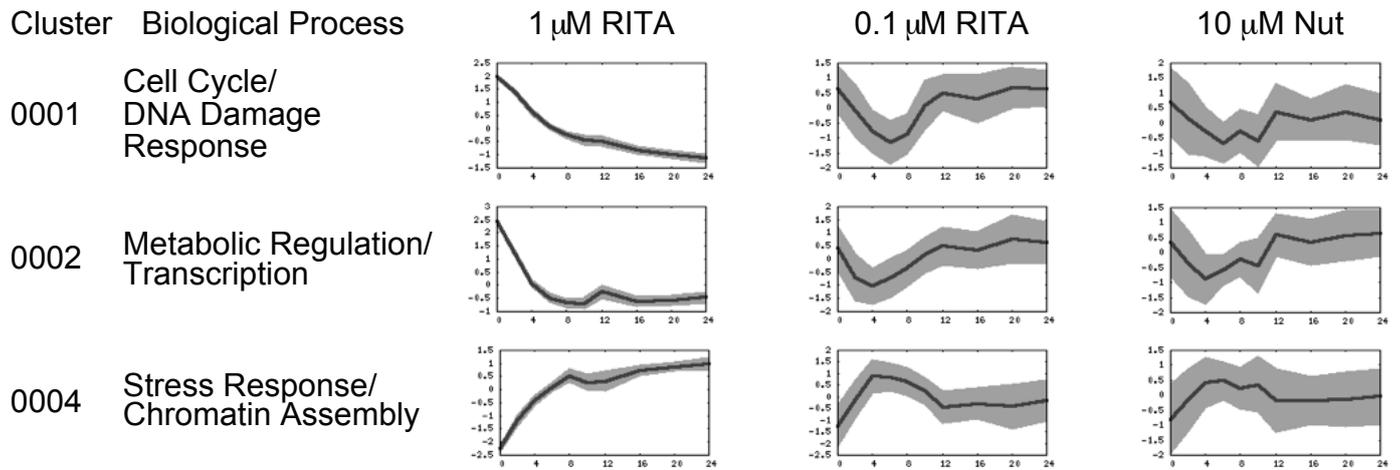


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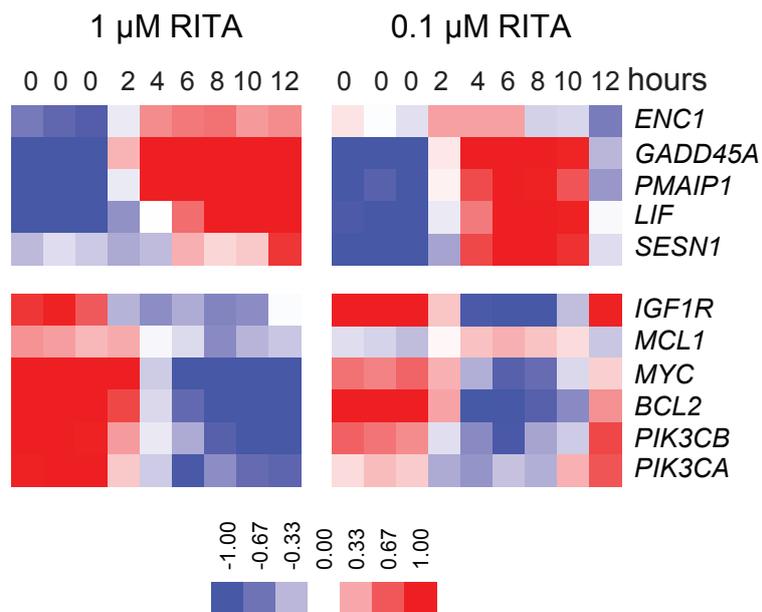
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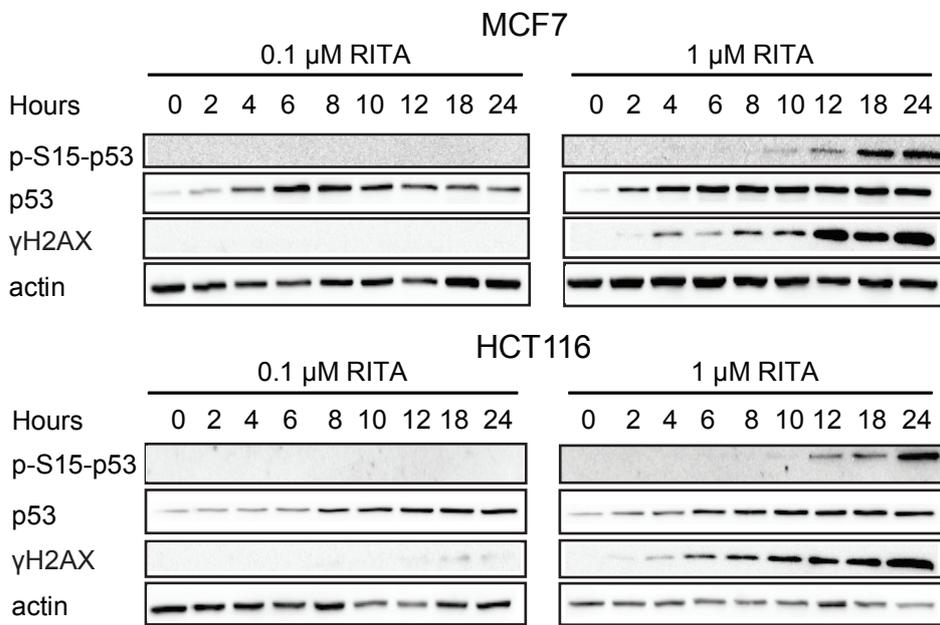


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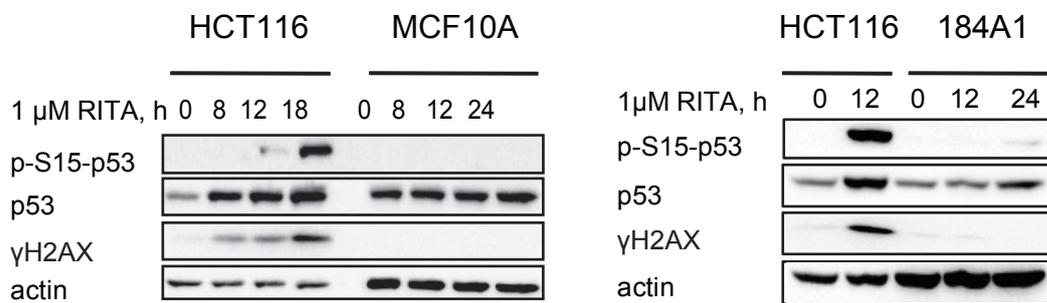
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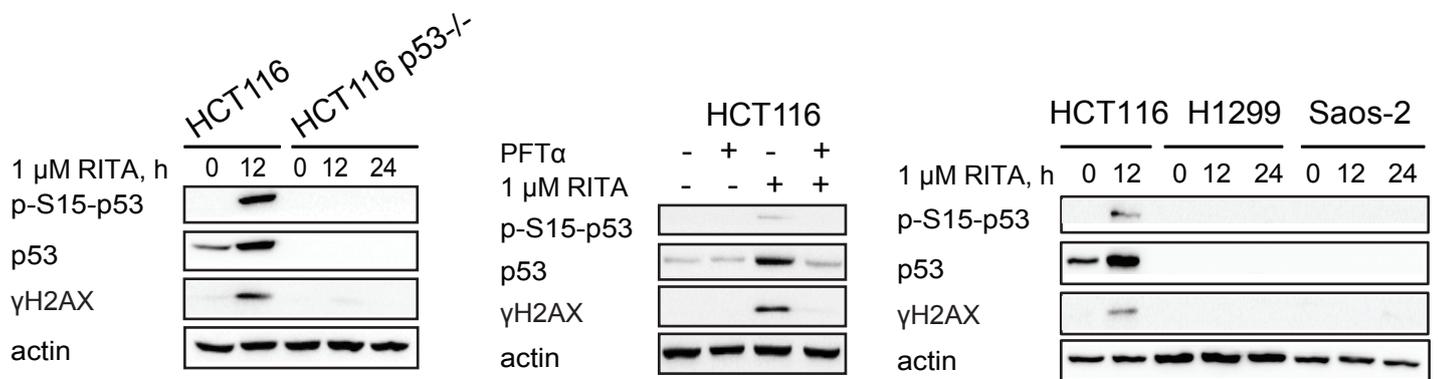
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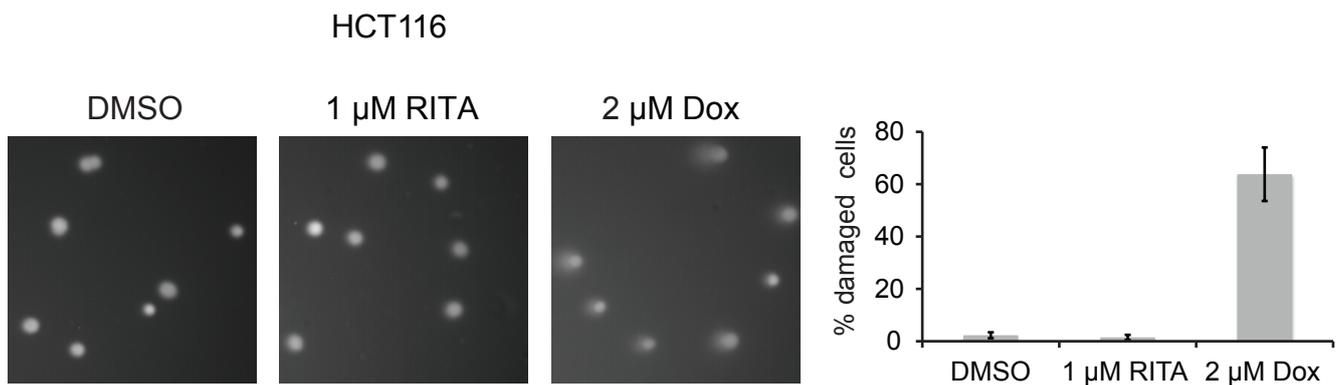
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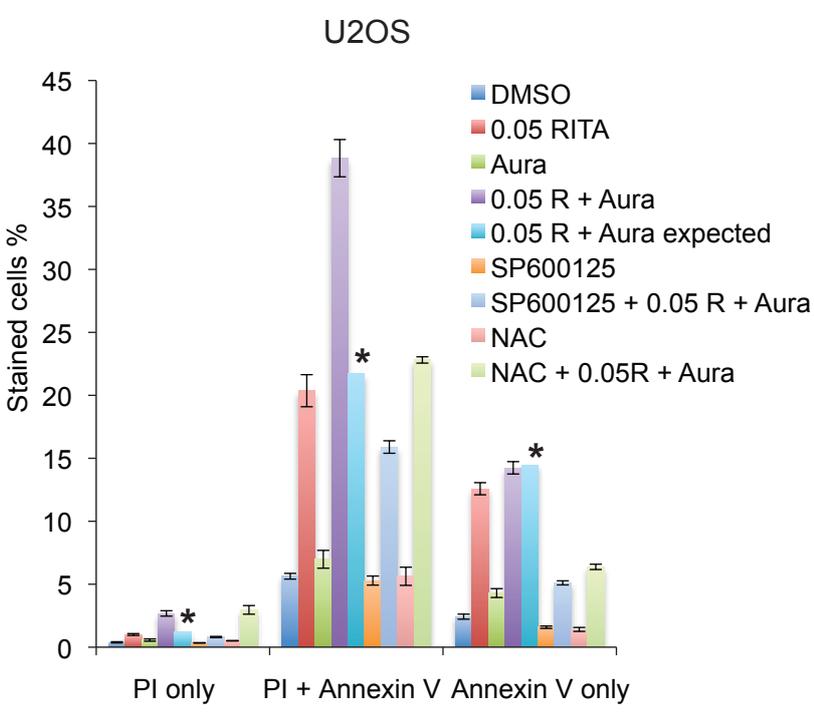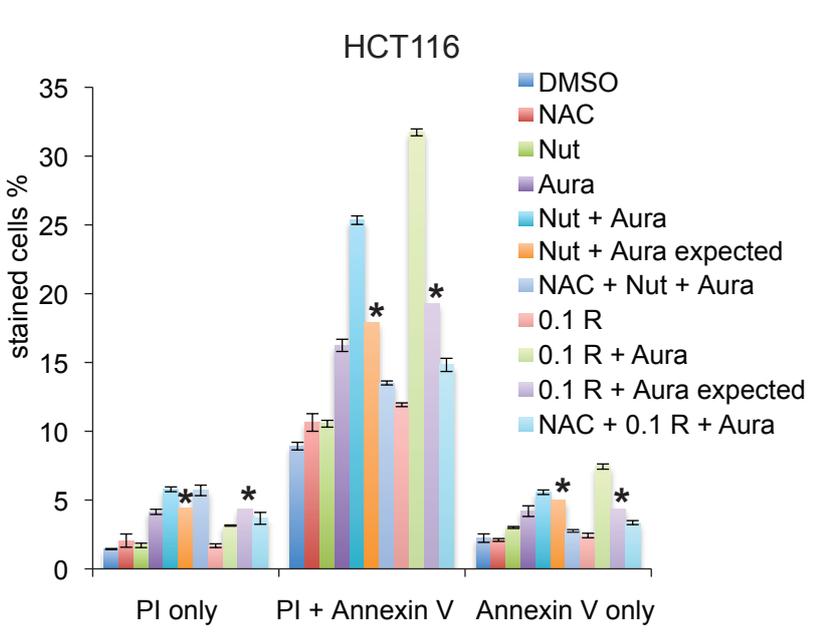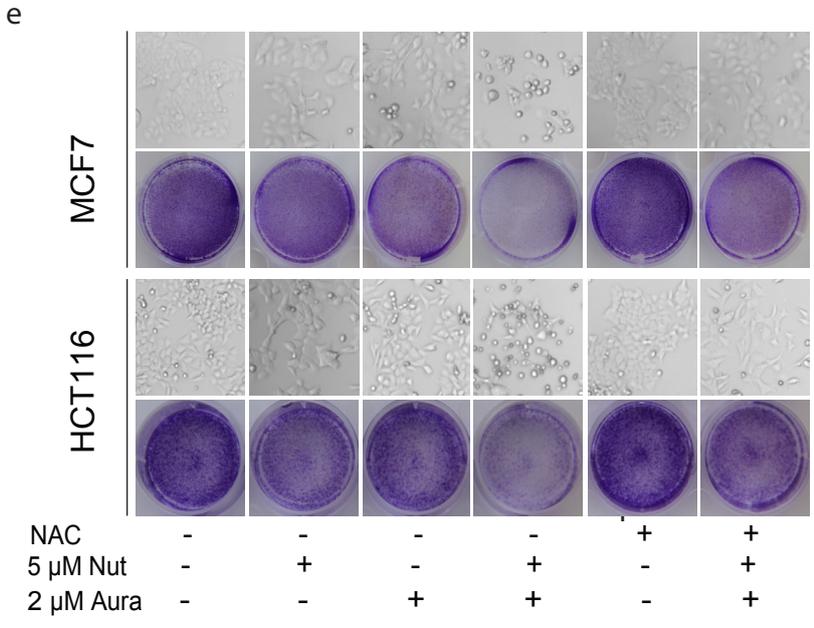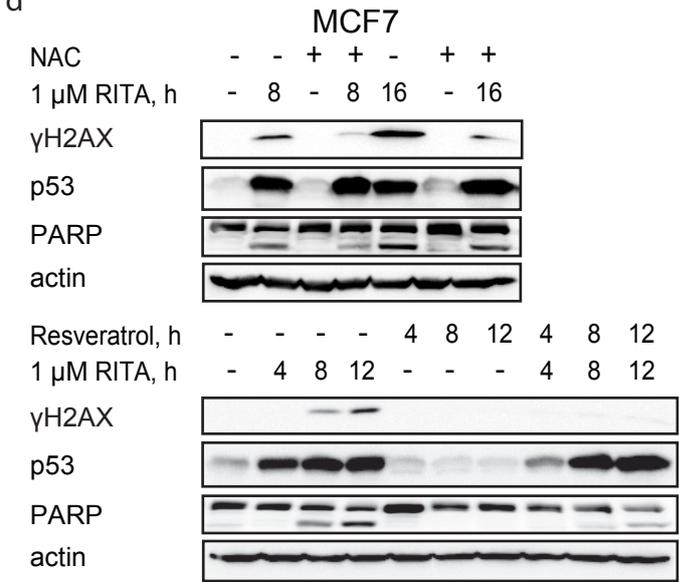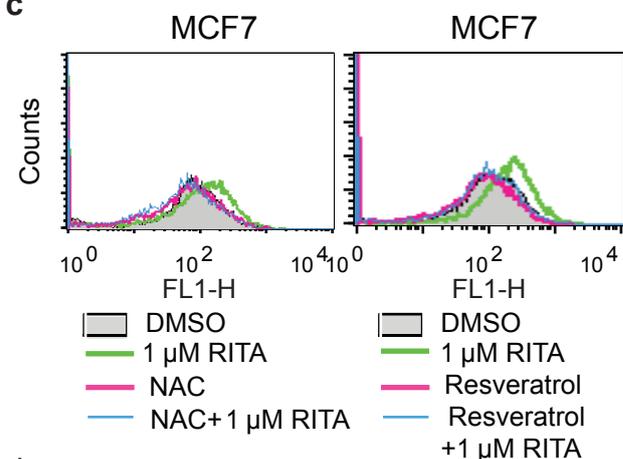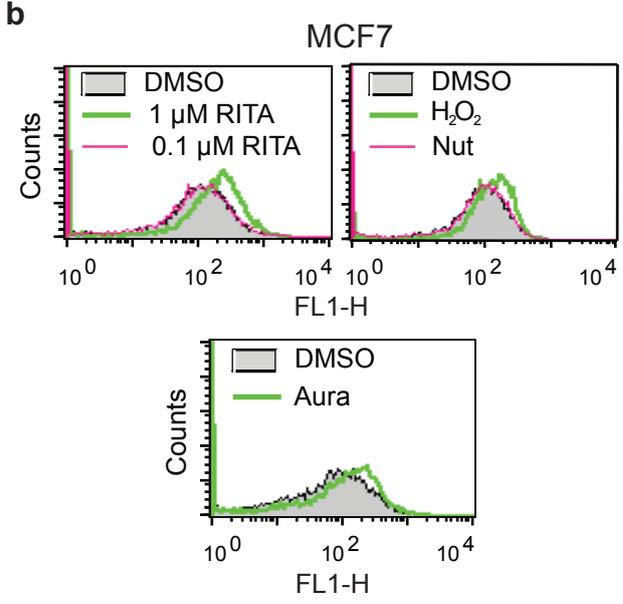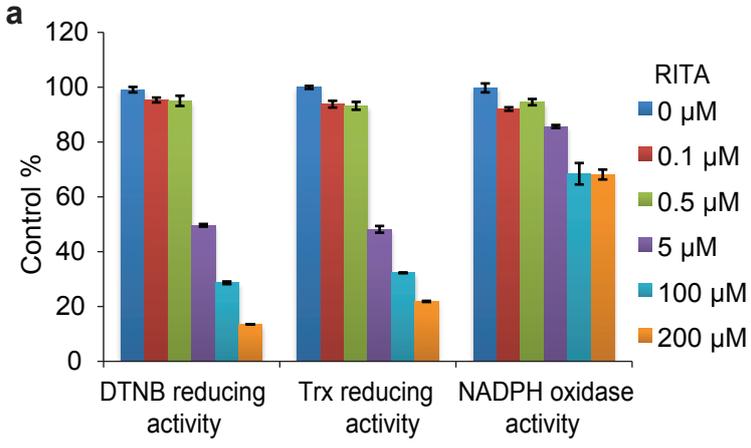
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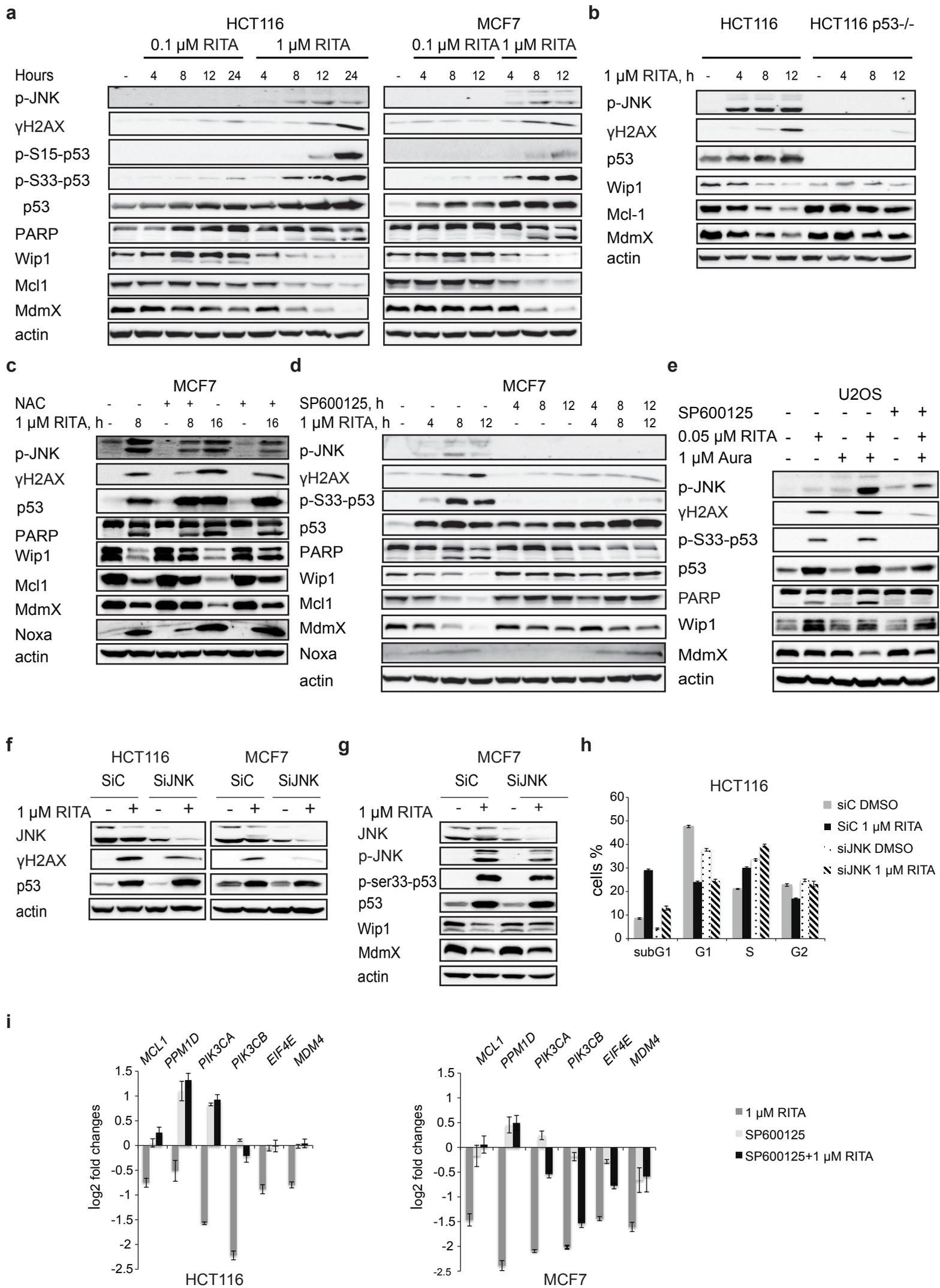
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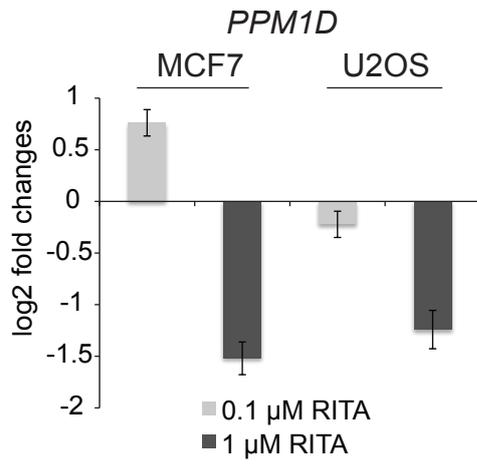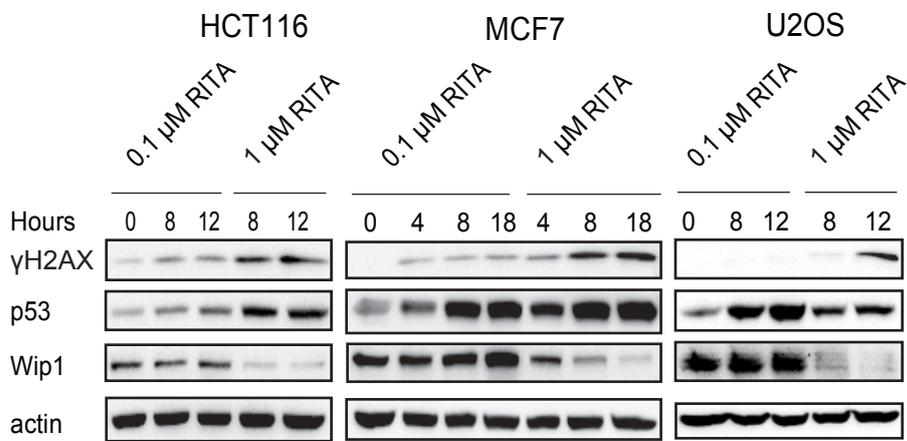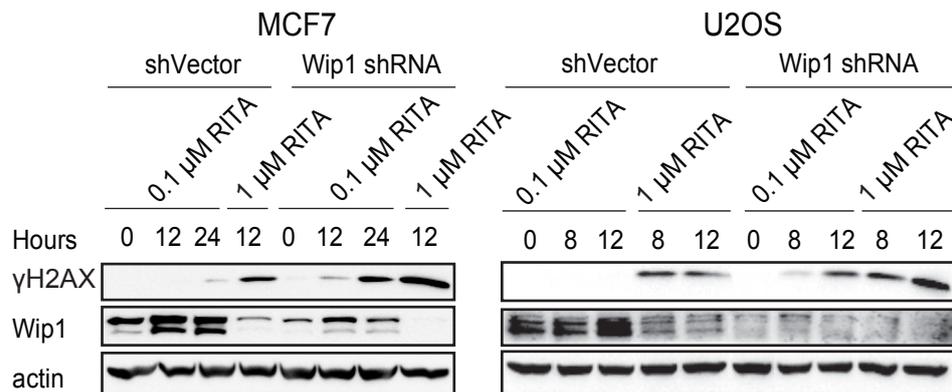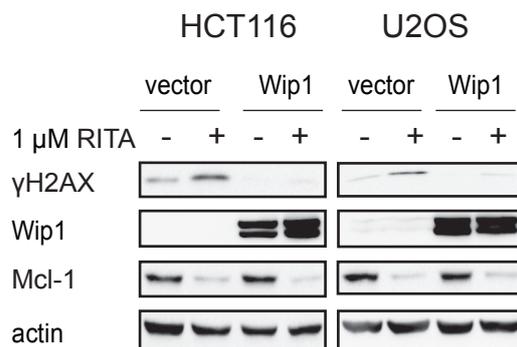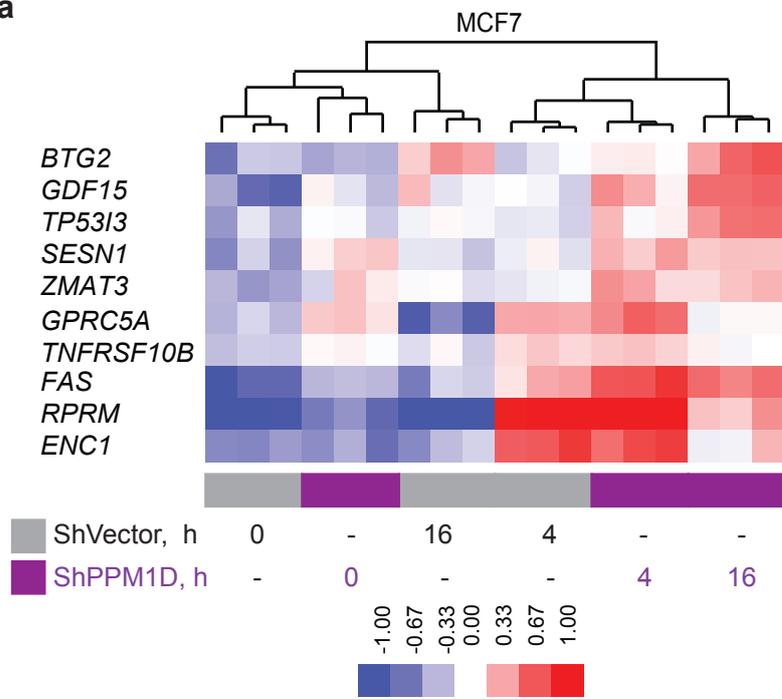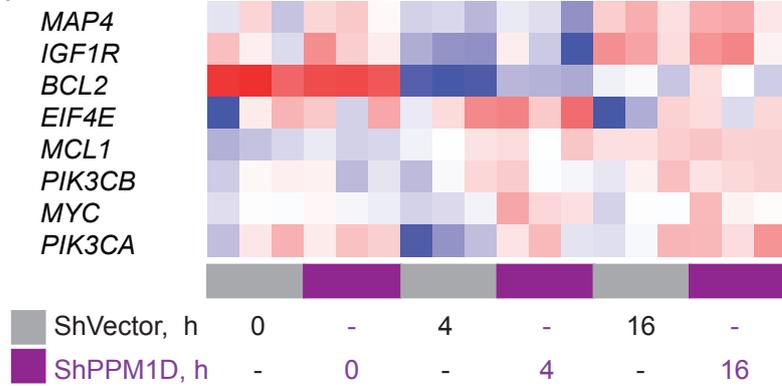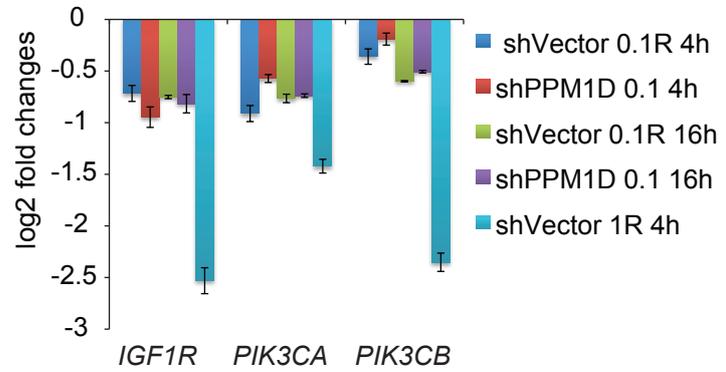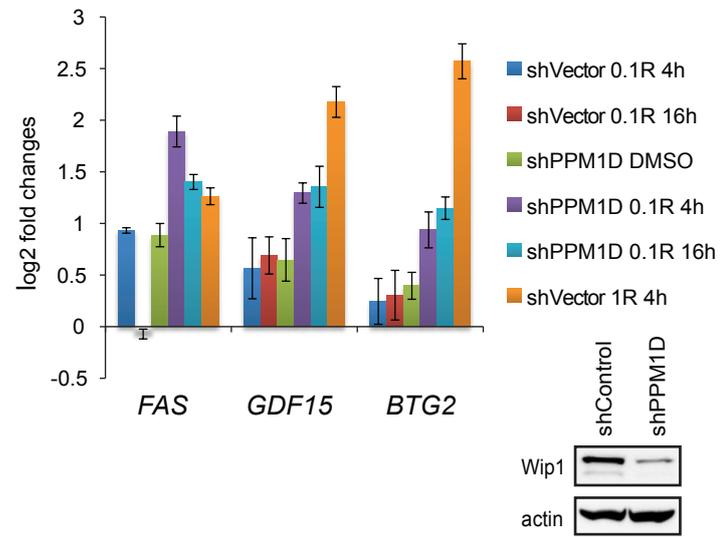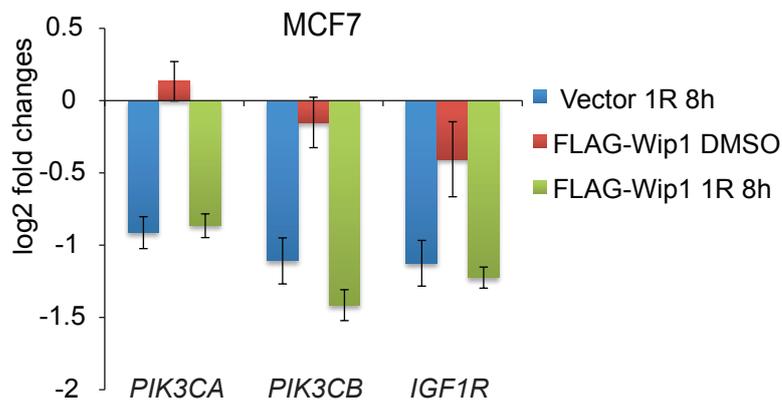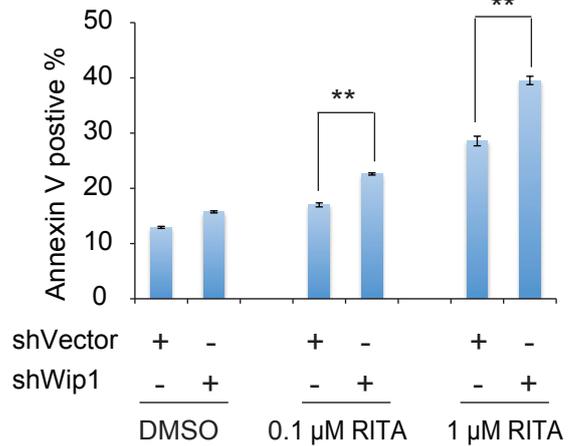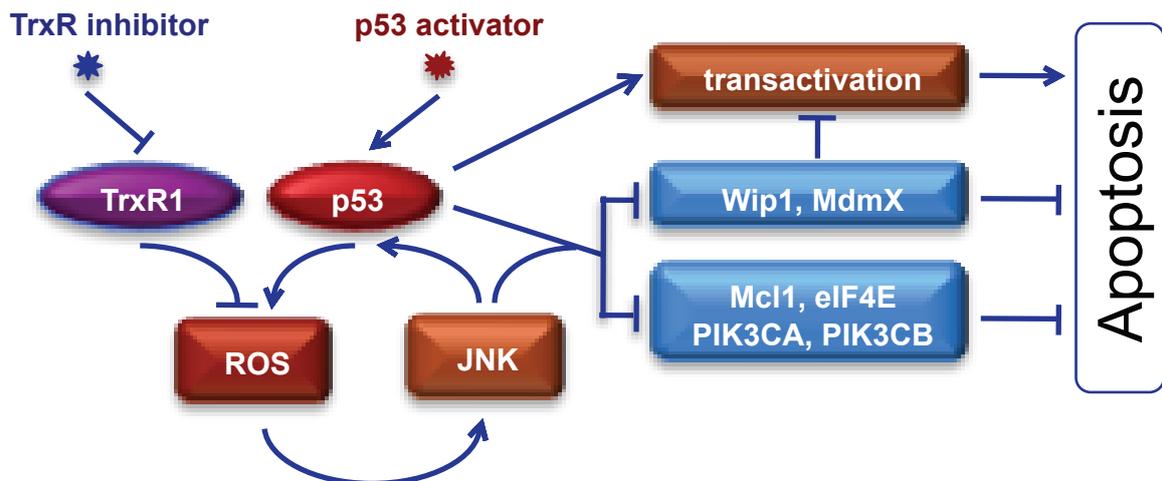
Figure 6

Supplemental Figure and Table Legends

Figure S1 Induction of DNA damage response by RITA. (A) Dose-dependent induction of DNA damage signaling in U2OS cells. U2OS cells were treated with 0.1 and 1 μM RITA for indicated time points and protein levels of p53, H2AX phosphorylated at ser139 (γH2AX) and p53 phosphorylated at ser15 (p-S15-p53) were detected by immunoblotting. Actin was used as a loading control. (B) p53-dependent induction of γH2AX and down-regulation of MdmX and Wip1 upon RITA treatment. U2OS cells were transfected with either control siRNA or siRNA against p53 for 48 hours and then treated with 1 μM RITA for 12 hours; protein levels were analyzed by Western blotting. (C) Apoptosis induced by high dose of Nutlin is p53 independent. HCT116 p53+/+ and p53-/- cells were treated with indicated dosages of nutlin and RITA for 48 hours; cells were stained by propidium iodide (PI) and analyzed by FACS. (D) Apoptosis-independent induction of DNA damage signaling upon RITA treatment. HCT116 cells were pretreated with pan-caspase inhibitor Z-VAD-fmk (10 μM) for 2 hours, followed by 1 μM RITA treatment for 8 hours; protein levels were analyzed by western blotting. (E) RITA did not induce detectable strand breaks as analyzed by pulse-field electrophoresis. HCT116 cells were treated with 1 μM RITA for indicated time points and DNA double-strand breaks were analyzed by pulse-field electrophoresis. Ionizing radiation (5Gy) was used as a positive control for DNA damage. (F) HCT116 cells transfected with siRNA against ATM were treated with 1 μM RITA for indicated time points and protein levels were assessed by Western blotting. siRNA against GFP was used as a control. (G) HCT116 cells were pretreated with ATM

inhibitor KU55933 (10 μM) for 2 hours followed by 12 hours treatment with 1 μM RITA. Neocarzinostatin (200 ng/ml) was used as a positive control for DNA damage signaling induction. (H) HCT116 cells were pretreated with caffeine for 2 hours followed by 8, 12 and 16 hours treatment with 1 μM RITA. Protein levels were assessed by Western blotting.

Figure S2 Induction of ROS leads to DDR and p53-dependent apoptosis. (A) ROS were measured in HCT116 cells treated for 6 hours with 0.1 and 1 μM RITA (left panel), nutlin or 400 μM H_2O_2 (middle panel), and 5 μM auranofin as a positive control (right panel), (B) Anti-oxidants NAC and resveratrol prevent the induction of ROS by 1 μM RITA. (C) Anti-oxidants NAC and resveratrol inhibited the induction of γH2AX and PARP cleavage by RITA as analyzed by Western blotting. (D) HCT116 cells were pretreated with resveratrol or NDGA for 2 hours, followed by RITA treatment for 24 hours and apoptosis was assess by FACS analyzed of PI stained cells. (E) ROS were measured in MCF10A cells treated with 1 μM RITA for 6h. (F) FACS analysis of MCF10A cells treated with 1 μM RITA for 48h followed by PI staining.

Figure S3 Statistical analysis of the ROS induction by different treatments. (A) MCF7 cells were treated and analyzed by FACS as described in Figure 3b and 3c; data from three independent experiments are presented as mean ± SEM, n=3. (B) HCT116 cells were treated and analyzed by FACS as described in Supplementary Figure S2A and S2B; data from three independent experiments are presented as mean ± SEM, n=3. (C) MCF10A

cells were treated and analyzed as in Supplementary Figure S2E; data from three independent experiments are presented as mean ± SEM, n=3. * P<0.05, ** P < 0.01, by two-tailed t test.

Figure S4 Anti-oxidants rescued the growth inhibition induced by RITA, Cisplatin or the combination treatment of 5 μM Nutlin and 2 μM Auranofin. (A) HCT116 and MCF7 cells were pretreated with antioxidants resveratrol or NDGA for 2 hours, followed by RITA treatment for 24 hours and stained with crystal violet. (B) HCT116 cells were pretreated with NAC, resveratrol or NDGA, followed by cisplatin treatment for 48 hours and assessed by crystal violet staining. (C) HCT116 cells were treated with 5 μM Nutlin, 2 μM Auranofin or their combination with or without NAC pretreatment. 36 hours later, cells were harvested and proceeded to FACS analysis for detection of activated caspases according to manufacture's instructions.

Figure S5 ROS-mediated activation of JNK contributes to the p53-mediated apoptosis and inhibition of oncogenes. (A) Pretreatment with resveratrol for 2h prevented the induction of p-JNK, γH2AX, PARP cleavage and inhibition of Wip1, Mcl-1 and MdmX by RITA as analyzed by immunoblotting. (B) JNK inhibitor SP600125 prevented the induction of p-JNK, p-Ser33-p53, γH2AX, PARP cleavage and inhibition of Wip1, Mcl-1 and MdmX by RITA, as assessed by Western blotting. (C) SP600125 partially rescued the growth inhibition by 1 μM RITA (24 hours) as assessed by crystal violet staining. (D) Depletion of JNK by siRNA prevented the induction of p-ser33-p53 and repression of MdmX.

Figure S6 Depletion of Wip1 promoted cell death by RITA and auranofin. (A,B) MCF7 and U2OS cells stably transfected with empty vector shRNA or shWip1 were treated with 0.1 and 1 μM RITA or DMSO for 48 hours (A) or 16 hours (B), and cell death was assessed by Trypan blue staining (mean±SEM, n=3, * P<0.05, ** P < 0.01, by two-tailed t test). (C,D) MCF7 cells stably transfected with empty vector shRNA or shRNA against Wip1 were treated with 10 μM nutlin, 2 μM auranofin, or their combination for 48 hours (C) or 24 hours (D); microscopy pictures were taken before crystal violet staining (C); Protein levels were assessed by immunoblotting (D). (E,F) U2OS cells were treated with 10 μM nutlin, 1 μM auranofin, or their combination for 24 hours (E) or 48 hours (F); protein levels were detected by Western blotting (E); images were taken under the microscope (F).

Table S1 The detailed information of 10 best GO classifications (with lowest P value) in each cluster as analyzed in Fig. 1A.

Table S2 Chromosomal coordinates of p53-occupied sites in vicinity of inhibited genes upon 1 μM RITA and 10 μM nutlin as analyzed by ChIP-seq.

Table S3 List of Primers for qPCR.

Figure S1

Figure S2

Figure S3

A

B

C

Figure S4

A

Resveratrol	−	−	+	+
1 μM RITA	−	+	−	+

| HCT116 | | | | |
| MCF7 | | | | |

NDGA	−	−	+	+
1 μM RITA	−	+	−	+

B

HCT116

NAC	−	−	+	+
CDDP	−	+	−	+

Resveratrol	−	−	+	+	−	−
NDGA	−	−	−	−	+	+
CDDP	−	+	−	+	−	+

C

HCT116

cells % with activated caspases

Legend:
- DMSO
- NAC
- 5 μM Nut
- 2 μM Aura
- 5 μM Nut +2 μM Aura
- 5 μM Nut +2 μM Aura expected
- NAC + 5 μM Nut +2 μM Aura

Figure S5

A

HCT116

Resveratrol, h	-	-	-	-	8	12	24	8	12	24
1 μM RITA, h	-	8	12	24	-	-	-	8	12	24

MCF7

Resveratrol, h	-	-	-	-	4	8	12	4	8	12
1 μM RITA, h	-	4	8	12	-	-	-	4	8	12

p-JNK

γH2AX

p-S15-p53

p-S33-p53

p53

PARP

Wip1

Mcl1

MdmX

Puma

Noxa

actin

B

HCT116

SP600125, h	-	-	-	8	12	8	12
1 μM RITA, h	-	8	12	-	-	8	12

p-JNK

γH2AX

p-S33-p53

p53

PARP

Wip1

Mcl1

MdmX

Puma

Noxa

actin

C

HCT116

SP600125

DMSO   1 μM RITA

MCF7

SP600125

DMSO   1 μM RITA

D

HCT116

	SiC		SiJNK	
1 μM RITA, 12h	-	+	-	+

JNK

p-JNK

p-ser33-p53

p53

MdmX

actin

Figure S6

A

MCF7

shVector	+	-	+	-	+	-
shWip1	-	+	-	+	-	+
	DMSO		0.1 µM RITA		1 µM RITA	

B

U2OS

shVector	+	-	+	-	+	-
shWip1	-	+	-	+	-	+
	DMSO		0.1 µM RITA		1 µM RITA	

C

MCF7 shVector

DMSO   Nut   Aura   Nut+Aura

MCF7 shWip1

DMSO   Nut   Aura

D

MCF7

	shVector				shWip1		
10µM Nut	-	+	-	+	-	+	-
2 µM Aura	-	-	+	+	-	-	+

Wip1
γH2AX
p53
MdmX
actin

E

U2OS

10 µM Nut, 24h	-	+	-	+
1 µM Aura, 24h	-	-	+	+

MdmX
Wip1
Mdm2
p53
actin

F

U2OS

10 µM Nut, 48h	-	+	-	+
1 µM Aura, 48h	-	-	+	+

Table S1

10 best GO classifications in each cluster

Cluster 0001	GO Term	p-value
GO:0007049	cell cycle	1.071e-10
GO:0005634	nucleus	1.240e-10
GO:0051726	regulation of cell cycle	1.595e-10
GO:0044428	nuclear part	1.791e-10
GO:0022402	cell cycle process	2.144e-10
GO:0022403	cell cycle phase	3.073e-10
GO:0034960	cellular biopolymer metabolic process	3.964e-10
GO:0006974	response to DNA damage stimulus	4.462e-10
GO:0044260	cellular macromolecule metabolic process	5.680e-10
GO:0031981	nuclear lumen	2.077e-09
Cluster 0002	**GO Term**	**p-value**
GO:0034960	cellular biopolymer metabolic process	2.765e-15
GO:0044260	cellular macromolecule metabolic process	5.721e-15
GO:0043283	biopolymer metabolic process	1.03e-12
GO:0043170	macromolecule metabolic process	1.501e-12
GO:0006350	transcription	9.407e-12
GO:0003677	DNA binding	1.783e-11
GO:0034961	cellular biopolymer biosynthetic process	3.009e-11
GO:0043284	biopolymer biosynthetic process	4.263e-11
GO:0005634	nucleus	5.515e-11
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid	1.653e-10
Cluster 0004	**GO Term**	**p-value**
GO:0042221	response to chemical stimulus	1.503e-07
GO:0050896	response to stimulus	2.831e-06
GO:0042493	response to drug	3.799e-06
GO:0009636	response to toxin	7.636e-06
GO:0006334	nucleosome assembly	1.555e-05
GO:0000786	nucleosome	2.910e-05
GO:0034622	cellular macromolecular complex assembly	3.394e-05
GO:0034728	nucleosome organization	3.528e-05
GO:0034621	cellular macromolecular complex subunit organization	3.862e-05
GO:0031497	chromatin assembly	4.791e-05

Table S2
Chromosomal coordinates of p53-occupied sites in vicinity of inhibited genes upon 1 µM RITA treatment are shown. Red color indicates the p53-bound fragments occupied also upon 10 µM nutlin treatment. Higher score indicates better fit to the consensus binding site.

	Genomic Location of the p53 sites						Identification of p53 consensus motifs within p53-bound fragments using p53MH and p53scan			
Gene Symbol	chr	start	end	Distance to transcription starting site	Area of p53 peaks	Ratio p53/IgG peaks	p53MH motif	p53MH score, %	p53scan motif	p53scan score
EIF4E	4	100069098	100069283	91	9	4.794543465	CGTCCCGCGG AGT CCCCCAGTCA	17.78		
PPM1D	17	55997504	55997750	34756	15	2.557089848	ATGCATGCAC AGAAGTTCCC AAACCAGCAT	68.85		
PPM1D	17	56012688	56013497	18970	35	1.917817386	TGCCTTGTAC CTGAACCAC AAGCCAGTCC	74		
PPM1D	17	56014831	56015321	17260	28	4.63472535	TTGCCTGAAC CTGGGGGGTTCC AGGCCAGCCT	59.29		
PPM1D	17	56018058	56019203	13788	92	2.346802854	CTGCCAGTTC CCAGGCCTC AACCTGGAAT	45.13		
PPM1D	17	56024832	56025490	7147	25	2.493162602	ACTCCAGCCT GGGCAATA CAGCAAGACC	67.14		
PPM1D	17	56031199	56035770	72	1172	3.919163404	GGCCCAGCTC TCGC GGACAAGTCC	78.07	GGACAAGTCC AGACATCGCG	6.7282734
PPM1D	17	56049058	56049263	16738	10	2.636998906	TAGCTCGTTT TT TCACTTGCCA	70.69		
PPM1D	17	56050708	56051429	18908	32	2.434152836	ATCCTTGTGT TGCACA TGGCAGGATT	54.74		
PPM1D	17	56056468	56056675	24275	11	2.876726079	TCCCATGATA TCG TTACTTGCCT	64.2		
MDM4	1	202744987	202745524	6815	86	27.8083521	CTGCATGTTA . CAACATGCCC	86.93	CTGCATGTTA CAACATGCCC	9.2341985
MDM4	1	202751934	202752467	21	22	2.004990904	GCCCTCGTGT GA GGCCGTGTGG	33.03		
MDM4	1	202760110	202760356	1002	10	3.515998541	TTCCCTGATG . TGGCTTGTTA	57.13		
MDM4	1	202760558	202761247	415	212	14.58911083	AGACATGTTC . CAACATGTTG	93.52	AGACATGTTC CAACATGTTG	12.382569
MDM4	1	202782409	202782748	8802	13	4.474907234	TAATTAGCCT CATCTA AAACAAGTAA	61.99		

Table S3

Primers used in the study

Primers (5'-3')		Sequence
qRT-PCR		
PPM1D	F	ATACCTGAACCTGACTGAC
	R	CTCCTCCAGTGACTTGAC
GDF15	F	CAGAGCTGGGAAGATTCG
	R	GTCTTGCAAGGCTGAGC
BTG2	F	GAGGCTTAAGGTCTTCAGC
	R	TCCATCTTGTGGTTGATGC
FAS	F	TCTGCCATAAGCCCTGT
	R	GTCTGTGTACTCCTTCCCT
PIK3CA	F	TATGATGCAGCCATTGACC
	R	ATGAAACAGTTGTCCATCGT
PIK3CB	F	TTGGATCTTCGGATGTTGC
	R	AGGGCATCTTTGTTGAAGG
IGF1R	F	CCATTCTCATGCCTTGGTCT
	R	TGCAAGTTCTGGTTGTCGAG
EIF4E	F	CATATAGGGAGGGTATACAAGGA
	R	CTATGAGAATACTCAGAAGGTGTC
MCL1	F	TGGCTAAACACTTGAAGACC
	R	GTCCTCTACATGGAAGAACTC
MDM4	F	GGTTACCTCTGAGGATGAGTG
	R	ATCCTTCCTCAAGGCCC
GAPDH	F	TCATTTCCTGGTATGACAACG
	R	ATGTGGGCCATGAGGT

Clinical Cancer Research

Dual Targeting of Wild-Type and Mutant p53 by Small Molecule RITA Results in the Inhibition of N-Myc and Key Survival Oncogenes and Kills Neuroblastoma Cells *In Vivo* and *In Vitro*

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Cancer Therapy: Preclinical

Dual Targeting of Wild-Type and Mutant p53 by Small Molecule RITA Results in the Inhibition of N-Myc and Key Survival Oncogenes and Kills Neuroblastoma Cells *In Vivo* and *In Vitro*

Mikhail Burmakin[1], Yao Shi[1], Elisabeth Hedström[1], Per Kogner[2], and Galina Selivanova[1]

Abstract

Purpose: Restoration of the p53 function in tumors is a promising therapeutic strategy due to the high potential of p53 as tumor suppressor and the fact that established tumors depend on p53 inactivation for their survival. Here, we addressed the question whether small molecule RITA can reactivate p53 in neuroblastoma and suppress the growth of neuroblastoma cells *in vitro* and *in vivo*.

Experimental Design: The ability of RITA to inhibit growth and to induce apoptosis was shown in seven neuroblastoma cell lines. Mechanistic studies were carried out to determine the p53 dependence and the molecular mechanism of RITA-induced apoptosis in neuroblastoma, using cell viability assays, RNAi silencing, co-immunoprecipitation, qPCR, and Western blotting analysis. *In vivo* experiments were conducted to study the effect of RITA on human neuroblastoma xenografts in mice.

Results: RITA induced p53-dependent apoptosis in a set of seven neuroblastoma cell lines, carrying wild-type or mutant p53; it activated p53 and triggered the expression of proapoptotic p53 target genes. Importantly, p53 activated by RITA inhibited several key oncogenes that are high-priority targets for pharmacologic anticancer strategies in neuroblastoma, including N-Myc, Aurora kinase, Mcl-1, Bcl-2, Wip-1, MDM2, and MDMX. Moreover, RITA had a strong antitumor effect *in vivo*.

Conclusions: Reactivation of wild-type and mutant p53 resulting in the induction of proapoptotic factors along with ablation of key oncogenes by compounds such as RITA may be a highly effective strategy to treat neuroblastoma. *Clin Cancer Res; 19(18); 5092–103. ©2013 AACR.*

Introduction

Neuroblastoma (NB) belongs to the most challenging oncologic diseases of childhood. Despite intensive multimodal therapy, often resulting in good immediate response in many children, high-risk neuroblastoma frequently acquires therapy resistance with fatal clinical outcome (1). There is a strong need to develop novel targeted strategies that inhibit specific neuroblastoma pathways and key molecules for its growth and progression.

Among the diversity of genetic variations in neuroblastoma, *MYCN* amplification, leading to overexpression of the transcription factor N-Myc, is a genetic hallmark of the disease and an independent marker of dismal prognosis (1, 2). Selective targeting of N-Myc in neuroblastoma cells

using different approaches showed encouraging results and provides a promising treatment strategy (3). In addition, several other oncogenes have been implicated in neuroblastoma tumorigenesis, invasion, and dissemination and are regarded as targets for therapy (4). Among others, these include *PPM1D*, which encodes oncogenic phosphatase Wip1 (wild-type p53 induced phosphatase 1), increased expression of which is likely to be associated with 17q gain, a predictor of poor prognosis (5). Recent studies have shown a correlation between high expression of antiapoptotic factors Mcl-1 and Bcl-2 and resistance to therapy in neuroblastoma (6). Mcl-1 depletion via RNA interference induced apoptosis in neuroblastoma cell lines and sensitized them to cytotoxic chemotherapy, suggesting that Mcl-1, as well as Bcl-2, might be promising targets for neuroblastoma treatment (6, 7).

Notably, chemotherapy-resistant neuroblastoma often express p53 inactivated by a point mutation (8–10). p53 is the potent tumor suppressor, which halts tumor progression by inducing apoptosis or cell-cycle arrest (11). p53 is inactivated in the majority of human tumors, either by point mutation of the gene or via its inhibitors, mainly MDM2 and MDMX. MDM2 ubiquitinates p53 and marks it for destruction by the proteasome, thus keeping p53 at bay

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Translational Relevance

There is a strong need for novel target-specific therapeutic approaches to treat high-risk neuroblastoma. Restoration of p53 is a promising strategy to treat cancer. Several compounds reactivating p53 are currently being tested in clinical trials. Unlike chemotherapy regimens which kill healthy cells along with tumor cells, leading to severe side effects, target-specific drugs spare normal cells, and have the potential to be well-tolerated therapies, which will enable patients with cancer to live longer and have an improved quality of life. Here we report that reactivation of p53 by target-specific molecule RITA triggers ablation of key factors crucial for neuroblastoma survival, including N-Myc, the driving oncogene in neuroblastoma. Inhibition of oncogenes by p53 may thus constitute a new therapeutic approach for high-risk neuroblastomas. The capability of p53 to target several oncogenes might allow p53-based therapies to cope with the daunting challenge of therapy—multiple genetic abnormalities in individual cancers. With no current satisfactory strategy for treatment of high-risk neuroblastoma, it would be highly relevant to implement this strategy in the clinic.

in the absence of stress (11). MDMX is a paralog of MDM2 required for the efficient inhibition of p53 by MDM2, but it can also suppress p53 function independently of MDM2, therefore maximal activation of p53 requires inhibition of both MDM2 and MDMX (12). Multiple studies provided evidence of the crucial role of p53 for tumor suppression, as well as for response to anticancer therapy in different types of cancer including high-risk neuroblastoma (13).

p53 dysfunction in neuroblastoma has been linked to *MDM2* amplification and Wip1 activation (5, 14), as well as to homozygous deletions of *CDKN2A*, encoding MDM2 inhibitor p14ARF (15). Moreover, N-Myc inactivates p53 by inducing the expression of MDM2 (16), which in turn upregulates N-Myc (17). p53 mutations occur very seldom in neuroblastoma, but in cell lines established at relapse p53 mutations are more frequent, implicating mutant p53 in the development of therapy-resistant phenotype (8, 9).

Albeit inactive, the p53 protein is expressed in cancers, leading to the idea of p53 reactivation to combat cancer (18). Moreover, *in vivo* studies in animal models showed that re-instatement of p53 has much more profound tumor suppressor effects in aggressive, metastatic tumors (19, 20). These data greatly encouraged us to explore the effect of p53-reactivating molecules in neuroblastoma.

Several p53-reactivating molecules have been developed and at least 2 of them are currently being tested in clinical trials: MDM2 inhibitor nutlin3a discovered by Hoffmann La Roche (21) and the mutant p53-reactivating compound PRIMA-1^{MET}/APR-246, identified by us (22). Nutlin3a has

been shown to activate p53-dependent growth suppression in neuroblastoma carrying wild-type (wt) p53 *in vitro* and *in vivo* (23, 24). Evidence that defects in effector molecules downstream of p53 are remarkably rare in neuroblastoma leads further support to the strategy to restore the function of p53 in neuroblastoma (25).

However, recent studies show that treatment with nutlin3a creates a selective pressure for p53 mutations in neuroblastoma and other types of cancer leading to nutlin3a resistance, which in some cases contributes to multidrug resistance (26, 27). Thus, it might be beneficial to develop therapies which will simultaneously reactivate wild-type and mutant p53.

We have identified a small molecule RITA which binds to the N-terminus of p53 and induces a conformational change blocking its interaction with MDM2, leading to the robust induction of apoptosis in cancer cells of different origin *in vitro* and *in vivo*, without apparent toxic effects (28–32). Notably, RITA can also reactivate mutant p53, probably because RITA treatment impinges on p53 conformation (31). Furthermore, reactivation of p53 by RITA leads to the ablation of survival signaling in cancer cells via downregulation of Myc, Bcl-2, Mcl-1,Wip-1, MDMX, and other oncogenes (30, 33). Taken together, these data inspired us to test whether RITA is capable of restoring wild-type and mutant p53 activity in neuroblastoma.

Here, we report that RITA triggers robust apoptosis in different neuroblastoma lines, including the ones with mutant p53. RITA-activated p53 induces the expression of its proapoptotic target genes such as PUMA and Noxa and also a rapid and substantial downregulation of several key survival factors in neuroblastoma, including N-Myc, Aurora kinase A, MDM2, MDMX, Wip1, and Mcl-1. Notably, RITA efficiently suppressed the growth of human neuroblastoma xenografts in mice.

Materials and Methods

Cell lines

Neuroblastoma cell lines used in this study and the status of p53 and N-Myc in these lines is indicated in Table 1. SKN-BE(2) and SHEP cells were maintained in RPMI 1640

Table 1. p53 status and N-Myc amplification status in the cell lines used in the study

Cell line	p53 status	N-Myc amp
SH-SY5Y	wt	−
SK-N-BE(2)	C135F	+
SK-N-AS	wt[a]	−
SKN-FI	M246R	−
SK-N-DZ	wt	+
IMR-32	wt	+
SHEP	wt	−

p53 status is indicated according to ref. 35.
[a]C-terminal homozygous deletion.

medium, all other cell lines were maintained in Dulbecco's Modified Eagle Medium. Plasmid DNA and siRNA transfections were conducted with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Plasmid encoding p53shRNA was kindly provided by A. Jochemsen (The Netherlands).

Growth suppression assays

For long-term viability assay, 100,000 cells were seeded in 12-well plates, treated with RITA for 2 weeks and stained with crystal violet. For short-term viability assay, 3,000 cells/well were plated in a 96-well plate, treated with RITA for 48 hours, and cell viability was assessed using proliferation reagent WST-1 (Roche) according to the manufacturer's instructions. TUNEL assay was conducted as we previously described (34). Fluorescence-activated cell sorting (FACS) analysis of the propidium iodide–stained cells was conducted as in ref. 29.

Antibodies and Western blotting

The following primary antibodies were used: rabbit polyclonal anti-p53 CM1 was from Novocasta; antibodies for p53 (DO-1, FL393), PARP (H-250), Mcl-1 (S-19), N-Myc (C-19), MDM2 (SMP14), Bax (N-20), Bcl-2 (C-2), from Santa Cruz. Anti-actin (AC15) from SIGMA and anti-p21 (Cip1/waf1) from Nordic Biosite. Antibodies for Noxa and PUMA (Ab-1) from Calbiochem, anti-MDMX (S403) and anti-Wip1 antibodies from Bethyl. Immunoblotting was conducted according to standard procedures.

Chemicals

Pifithrin-α (PFTα), a kind gift from A. Gudkov (USA), was used at 10 μmol/L 2 hours before RITA treatment. The proteasomal inhibitor MG132 was used at a concentration of 20 μmol/L. RITA was obtained from National Cancer research Institute (USA).

Quantitative real-time reverse transcriptase (RT)–PCR

Total RNA was extracted and purified with an RNeasy kit (QIAGEN) using the manufacturer's protocol. RNA (5 μg) was reverse transcribed using a SuperScript First-Strand RT-PCR kit (Invitrogen). Real-time PCR was conducted with SYBR green reagent (Applied Biosystems) according to the manufacturer's protocol. Primers used for real-time RT-PCR were as we previously described (29, 30).

Co-immunoprecipitation

Neuroblastoma cells were treated with 1 μmol/L RITA and harvested after 24 hours. Lysates (500 μg) were precleared with Protein A agarose beads and rabbit immunoglobulin G (Santa Cruz Biotechnologies) before immunoprecipitation with anti-p53 antibody FL-393 conjugated to agarose beads (Santa Cruz Biotechnologies). Beads were washed 5 times with IP buffer (50 mmol/L Tris, pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP-40). Bound proteins were detected by Western blotting using MDM2 and MDMX antibodies.

In vivo experiments

The Northern Stockholm Animal Ethical Committee approved all animal studies and animal care was in accordance with the Karolinska Institutet guidelines. SKN-DZ cells (3×10^7) were injected subcutaneously on the left and right flanks of 6- to 8-week-old female severe combined immunodeficient (SCID) mice. Xenografts seemed palpable 7 days after inoculation, at which time the treatment was started. The mice were treated by intraperitoneal injections twice daily with injection of 200 μL solution containing 10 mg/kg of RITA and 5% dimethyl sulfoxide (DMSO) in PBS or 5% DMSO in PBS for a period of 18 days. Xenograft volumes were measured every day. Animals were sacrificed on the last day of treatment; tumors were extracted, weighted, and photographed. Body weight of mice was measured before and after treatment.

Results

RITA inhibits the growth of neuroblastoma cells

We have previously shown that p53 reactivating compound RITA prevents p53/MDM2 interaction, induces p53 accumulation and activation, and triggers apoptosis in tumor cells of a different origin *in vitro* and *in vivo* (28–32). Here, we tested the effects of RITA in 7 neuroblastoma cell lines, differing in N-Myc and p53 status (Table 1).

Treatment with RITA efficiently suppressed the growth of neuroblastoma cell lines expressing wild-type p53 in a dose-dependent manner, as detected by cell-proliferation assay (Fig. 1A). These include 2 cell lines with amplified N-Myc, SKN-DZ, and IMR32. Furthermore, a long-term viability assay showed that treatment with RITA purged the entire population of neuroblastoma cells, leaving virtually no alive cells after several days of treatment (Fig. 1B).

In addition to the activation of the wtp53 activity, RITA can also restore the activity of mutant p53 in human tumor cells of different origin (31). In line with these results, we found that RITA efficiently inhibited the growth of SKN-BE (2) cells, which express C135F p53 mutant and SKN-FI, carrying M246R p53 mutant, as assessed in short- and long-term viability assays (Fig. 1A and B). In addition, the growth of SKN-AS cell line carrying p53 truncated at its very C-terminus, but retaining partial p53 activity (35), was also inhibited by RITA.

Thus, RITA efficiently suppressed the growth of neuroblastoma cells, carrying both wild-type and mutant p53, with or without N-Myc amplification.

RITA induces apoptosis in neuroblastoma cells

Nutlin3a, an inhibitor of p53/MDM2 interaction, induces a pronounced growth arrest and senescent phenotype in neuroblastoma cells (23). However, we did not observe senescent cells upon treatment with RITA. Microscopy analysis of cell morphology revealed the induction of cell death by RITA in all cell lines tested (Fig. 2A). Furthermore, we detected DNA fragmentation, the hallmark of apoptosis using TUNEL assay (Fig. 2B). Activation of caspases, manifested as induction of PARP cleavage, served as an additional proof of apoptosis. Using immunoblotting,

Figure 1. RITA inhibits the growth of neuroblastoma cells. A, B, inhibition of growth of 7 neuroblastoma lines by RITA, as assessed using short-term cell-proliferation assay (48 hr, A) and by long-term viability assay (2 weeks, B).
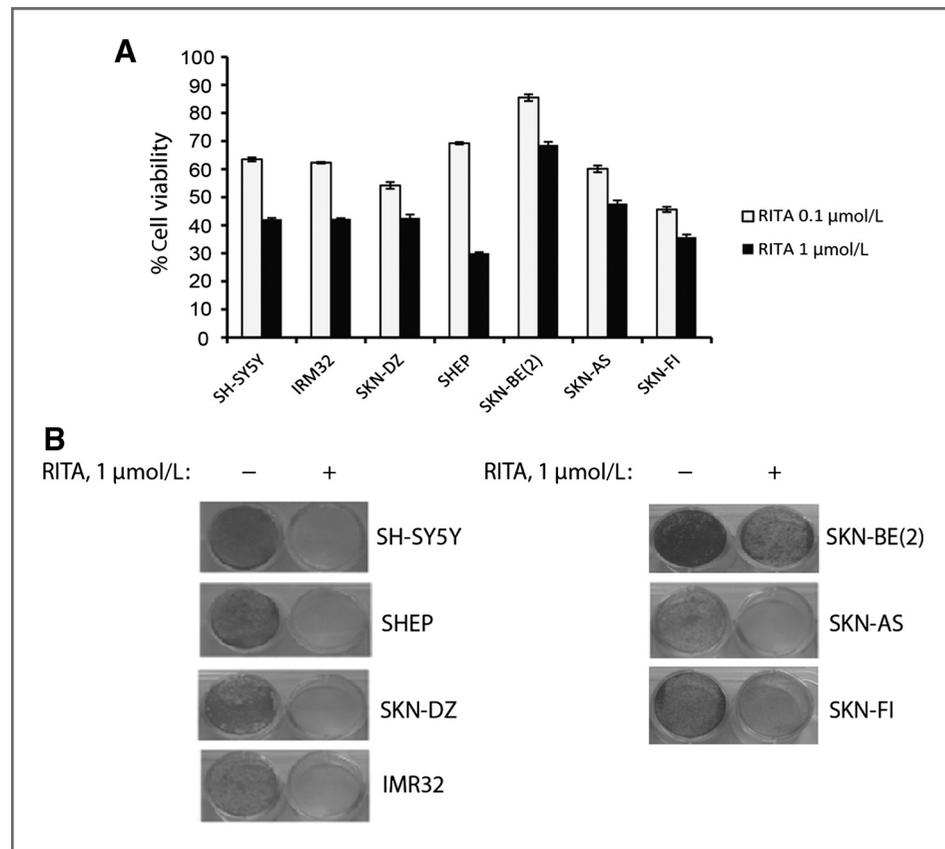
we showed the induction of PARP cleavage upon RITA in several neuroblastoma lines (Fig. 2C). Appearance of fragmented DNA, another indication of apoptosis, was observed upon FACS analysis of propidium iodide–stained SKN-BE(2) cells (Fig. 2D) and, as shown later, in SHEP and SKN-DZ cells. Taken together, our results strongly suggest that RITA induces neuroblastoma cell death via apoptosis.

RITA disrupts the interaction between p53 and MDM2/MDMX

We have previously shown that RITA induces apoptosis due to disruption of the p53/MDM2 complex (28), and also found similar inhibitory effect on the p53/MDMX complex. In line with these results, we found that RITA significantly decreased the complex formation between p53 and MDM2, as well as between p53 and MDMX, as assessed by co-immunoprecipitation assay (Fig. 3A). These data suggest that in wild type p53 cells the induction of apoptosis upon treatment with RITA is due to the inhibition of interaction between p53 and its negative p53 regulators MDM2 and MDMX.

Apoptosis induced by RITA is p53-dependent

To assess whether apoptosis induced by RITA is p53 dependent, we used 2 different approaches. First, we silenced p53 either by stably expressing p53shRNA in SHEP and SKN-DZ cells or by transient depletion of mutant p53 in SKN-BE(2) cells by pSUPER shp53 transfection. The silenc-

ing of p53 prevents apoptosis induction by RITA, as shown using a short-term viability assay (Fig. 3B, top), FACS analysis (Fig. 3C). Second, we assessed the p53 dependence by using chemical inhibitor of p53 transcriptional function, small molecule PFTα (36). Inhibition of p53 by PFTα before administration of RITA protects SHEP cells from apoptosis (Fig. 3B, bottom left). PFTα also rescued SKN-BE(2) cells carrying mutant p53 (Fig. 3B, bottom right). In addition, as shown below, PARP cleavage in SKN-BE(2) cells was rescued by p53 depletion. Taken together, our results show that apoptosis induced by RITA in neuroblastoma cell lines is triggered by p53. Thus, we set out to explore in more detail the mechanisms of p53-induced apoptosis.

p53 induced by RITA activates the expression of its proapoptotic targets

As expected, we observed the induction of p53 protein levels upon treatment with RITA in all neuroblastoma cell lines, except SKN-BE(2), carrying mutant p53 (Fig. 3D). Moreover, p53 accumulation upon RITA treatment resulted in the induction of p53 targets, the key proapoptotic factors PUMA, Noxa, and Bax, as well as CDK inhibitor p21 (Fig. 3D). These data are in line with the prevention of RITA-mediated apoptosis by RNAi-mediated silencing of p53 and the inhibitor of p53 transcriptional activity PFTα and suggest that p53 activated by RITA is transcriptionally active.

Furthermore, according to qPCR analysis, the expression of several p53 target genes was induced, including proapoptotic
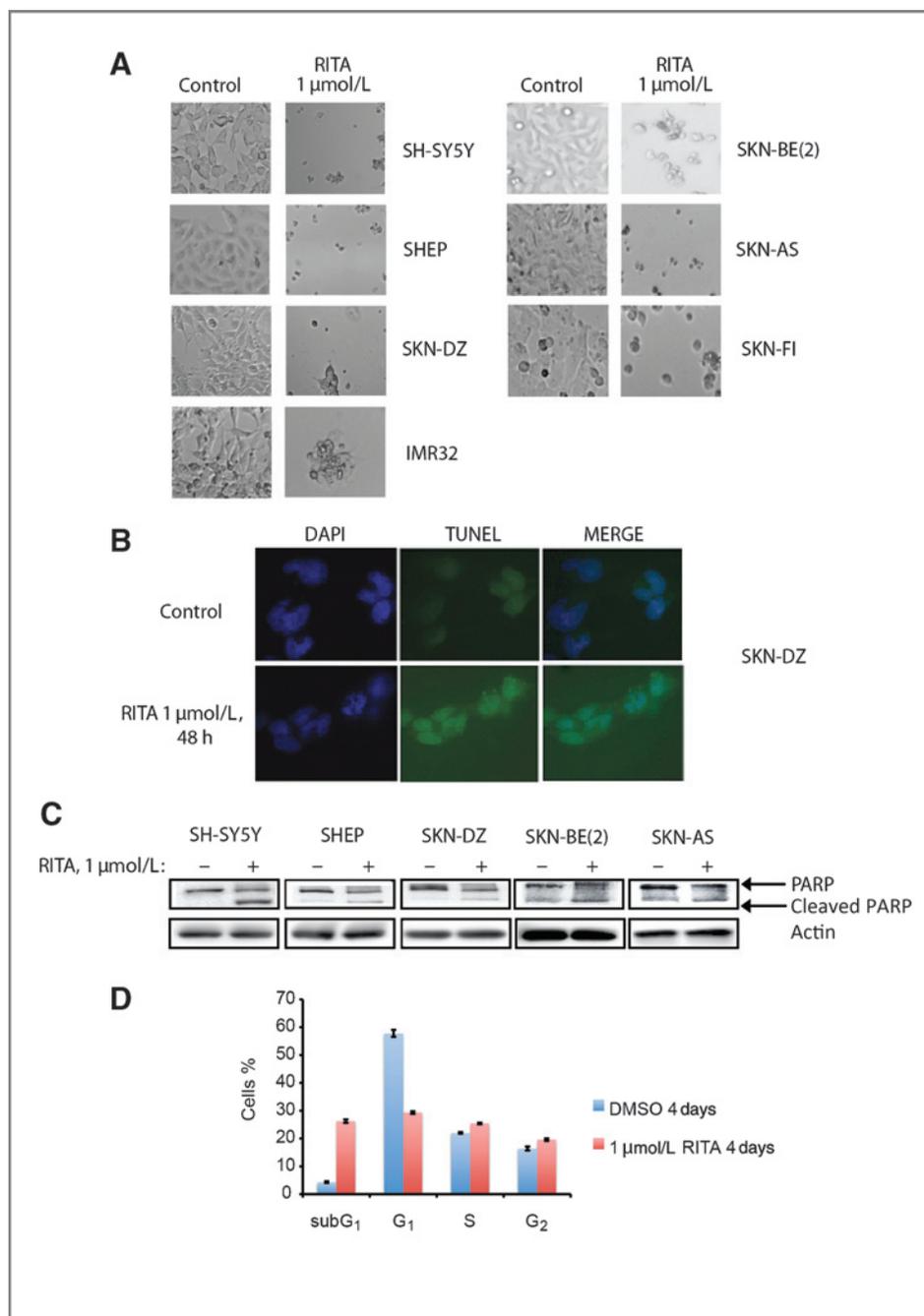
Figure 2. RITA induces apoptosis in neuroblastoma cells. A, induction of cell death in neuroblastoma cell lines was assessed by microscopy analysis. Cells were treated with RITA or DMSO as a control for 48 hours, except SKN-BE(2) cells, which were treated for 4 days, and images were taken under microscope. B, induction of DNA fragmentation by RITA was detected using TUNEL assay in SKN-DZ cells after 48 hours of RITA treatment. C, induction of PARP cleavage upon RITA treatment was assessed by immunoblotting. D, induction of apoptosis in SKN-BE(2) cells upon 4 days treatment with RITA as assessed by FACS analysis of propidium iodide–stained cell.

Bax and *BBC3* (encoding PUMA), as well as *CDKN1A* gene encoding CDK inhibitor p21 (Fig. 3D, bottom).

p53 inhibits the expression of N-Myc and several other oncogenic factors important for neuroblastoma growth

Recently we reported a potent inhibition of crucial oncogenes by p53 *in vitro* and *in vivo* upon reactivation by RITA, which includes Mcl-1, Bcl-2, c-Myc, cyclin E, and β-catenin (30), as well as MDM2, MDMX, and Wip1 encoded by *PPM1D* (33). We found that the inhibition of oncogenes by

p53 reduces the cell's ability to buffer proapoptotic signals and elicits robust apoptosis (30). Thus, we decided to test whether p53 reactivation by RITA can inhibit oncogenes which play important role in neuroblastoma development, including N-Myc, Wip1, Mcl-1, and Bcl-2 (3, 5–7), as well as p53 inhibitors MDM2 and MDMX.

Analysis of protein levels of N-Myc in 3 cell lines carrying *MYCN* amplification, SKN-DZ, SKN-BE(2), and IMR32, revealed a strong downregulation of N-Myc upon RITA (Fig. 4A, top). Downregulation of N-Myc was p53-dependent, as evidenced by a rescue of N-Myc, albeit incomplete,
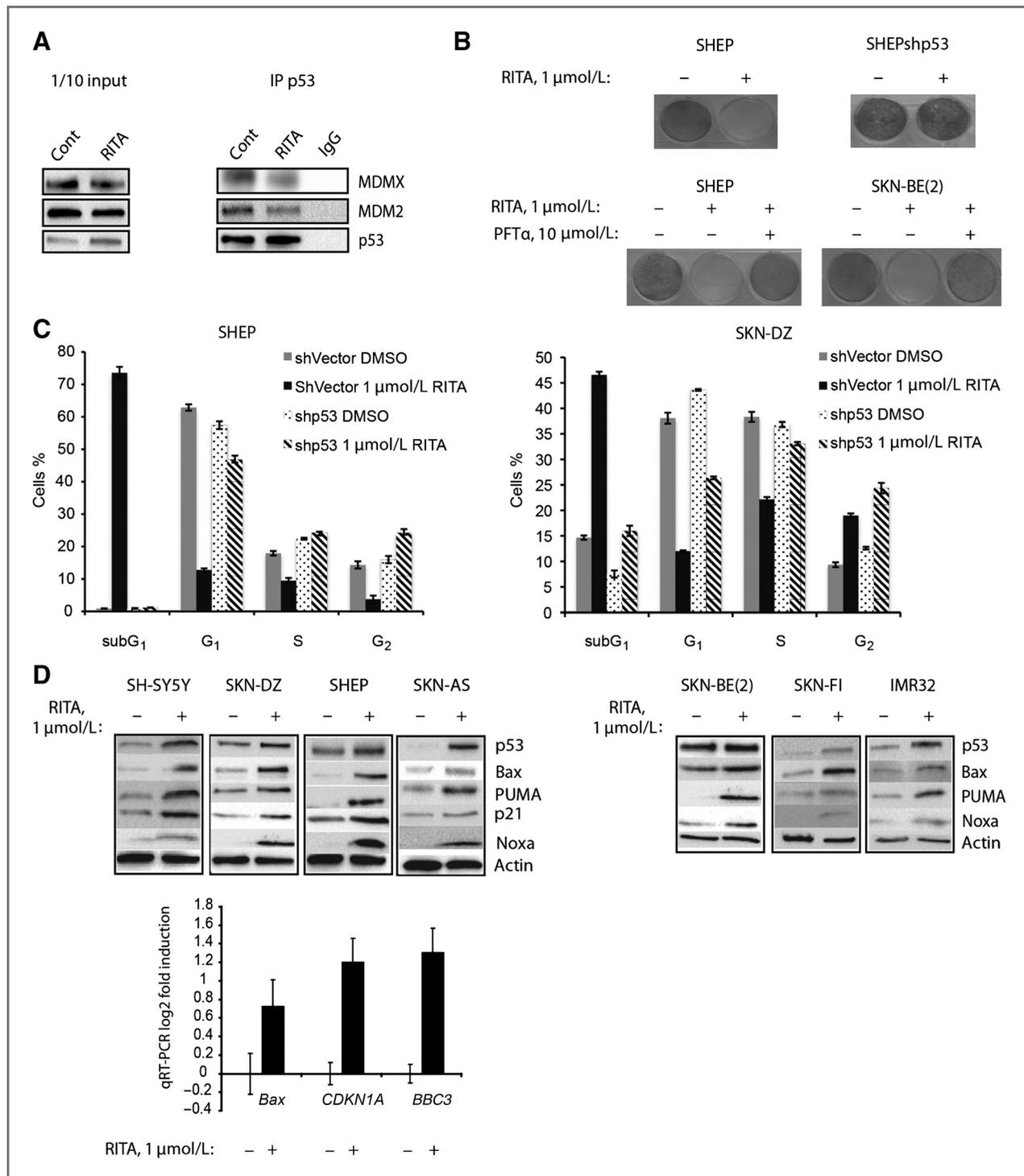
Figure 3. Induction of apoptosis in neuroblastoma cell by RITA is p53 dependent. A, RITA disrupts the interaction between p53 and MDM2/MDMX, as detected by co-immunoprecipitation in SKN-DZ cells followed by Western blotting. B, depletion of p53 by shRNA protects SHEP cells from RITA-induced cell death, as detected by short-term viability assay (top). Inhibition of p53 by pretreatment with PFTα prevents growth suppression by RITA in SHEP and SKN-BE(2) cells, as assessed using short-term viability assay (bottom). C, rescue of apoptosis induced by RITA upon p53 silencing in SHEP (left) and SKN-DZ (right) cell lines as analyzed by FACS of propidium iodide–stained cells. D, induction of p53 and its targets upon 24 hours of RITA treatment, as detected by immunoblotting (top). RITA induces the expression of p53 target genes encoding Bax, Puma (*BBC3*), and p21 (*CDKN1A*) in SKN-DZ cells, as detected by qPCR (bottom).
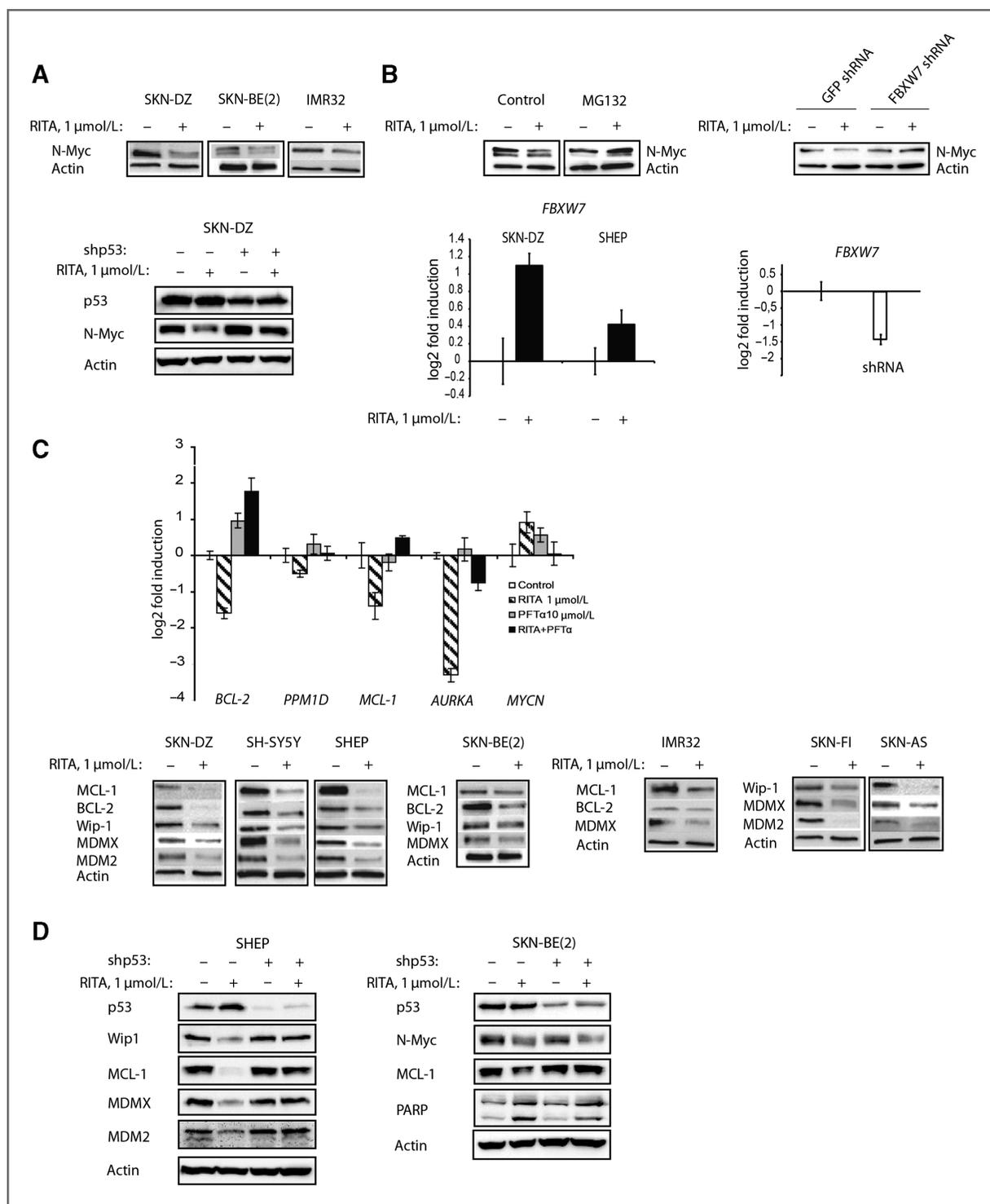
Figure 4. p53 reactivated by RITA inhibits crucial oncogenes in neuroblastoma cells. A, decrease of N-Myc protein level in SKN-DZ, SKN-BE(3), and IMR32 cells upon RITA treatment as detected by immunoblotting (top). Partial rescue of N-Myc in SKN-DZ cells upon inhibition of p53 by shRNA as assessed by Western blotting (bottom). B (top left), pretreatment with proteasome inhibitor MG132 rescues downregulation of N-Myc protein level by RITA; (top right) depletion of *FBXW7* by shRNA prevented downregulation of N-Myc by RITA, as assayed by immunoblotting; (bottom left) induction of *FBXW7* mRNA level upon RITA treatment, as detected by qPCR; (bottom right) shRNA decreased the level of *FBXW7* mRNA as detected by qPCR. C, downregulation of several oncogenes in neuroblastoma cells upon RITA treatment on mRNA and protein level; (top) transcriptional repression of *BCL-2*, *PPM1D*, *MCL-1*, and *AURKA*, but not *MYCN* upon RITA treatment, as assessed by qPCR. Downregulation of these genes was p53 dependent, because it was rescued by pretreatment with p53 inhibitor PFT-α; (bottom) downregulation of survival oncogenes in neuroblastoma cells upon 24 hours of RITA treatment as detected by immunoblotting. D, effect of p53 silencing on downregulation of survival oncogenes in SHEP (left; 8 hours of RITA treatment) and in SKN-BE(2) cells (right; 3 days of RITA treatment).

upon partial silencing of p53 in SKN-DZ cells (Fig. 4A, bottom).

Pretreatment by MG132 rescued N-Myc level upon RITA, suggesting that the decline of N-Myc protein is proteasome dependent (Fig. 4B, top left). In addition, we did not detect a decrease of N-Myc mRNA levels by qPCR (Fig. 4C, top). It has been shown that Fbxw7 E3 ligase ubiquitinates N-Myc and triggers its proteasomal degradation (37). Therefore, we tested whether downregulaiton of N-Myc is dependent on Fbxw7. Indeed, silencing of the Fbxw7 expression by shRNA prevented N-Myc decline upon RITA (Fig. 4B, right). Moreover, qPCR analysis showed the induction of Fbxw7 mRNA upon RITA treatment (Fig. 4B, bottom left), in line with Fbxw7 being the p53 target gene (30).

Furthermore, we observed the p53-dependent transcriptional repression of *AURKA* gene, encoding Aurora kinase A (Fig. 4C, top), which we recently identified as a novel p53 target gene (38). It is possible that the transcriptional repression of *AURKA* encoding Aurora kinase, known to oppose Fbxw7-mediated degradation of N-Myc (37), might also contribute to the degradation of N-Myc upon RITA.

Moreover, in our set of neuroblastoma cell lines p53 activated by RITA triggered a potent decrease of protein levels of several oncogenes implicated in high-risk neuroblastoma, including Bcl-2, Mcl-1, and Wip-1 (Fig. 4C, bottom). In addition, we observed downregulation of the p53 inhibitor MDMX, which cooperates with MDM2 in p53 inhibition. Consistent with downregulation of MDM2 by RITA in other cell types (39), RITA treatment triggered a decline of MDM2 level (Fig. 4C, bottom).

Because p53 activated by RITA has been shown to be a potent transcriptional repressor of a number of genes, including p53 target genes *Bcl-2* and *Mcl-1* (30) and we have recently found that p53 can repress *PPM1D* encoding Wip1 (33), we addressed the question whether p53-mediated downregulation of these oncogenic factors in neuroblastoma is conferred on mRNA level. qPCR analysis showed that the treatment of cells with RITA lead to a decreased levels of *Bcl-2*, *Mcl-1*, and *PPM1D* mRNA (Fig. 4C, top). In contrast, *MDM4* and *MDM2* were not decreased (data not shown). This is in line with our published data that p53 activated by RITA induces degradation of MDMX in Wip1–dependent manner, along with decline of MDM2 (33). The transcriptional repression of oncogenes was p53 dependent, as it was rescued by the p53 inhibitor (Fig. 4C, top) and on protein level by RNAi-mediated silencing of p53 in wild-type and mutant p53 expressing cells SHEP and SKN-BE(2), respectively (Fig. 4D). However, in mutant p53 expressing SKN-BE(2) cells, N-Myc levels were not rescued by p53 silencing (Fig. 4D, right). It is possible that in SKN-BE(2) cells other mechanisms might contribute to N-Myc downregulation.

Strong antitumor effect of RITA in SKN-DZ xenografts in mice

The most rigorous test for the antitumor effect of novel compounds which could predict their potency as possible anticancer drugs is the assessment of their effects *in vivo*. To study the effects of RITA *in vivo*, we used SKN-DZ xenografts grown in SCID mice. Upon formation of palpable tumors, we injected intraperitonealy 10 mg/kg of RITA or vehicle twice daily. RITA treatment significantly suppressed the growth of neuroblastoma *in vivo*, resulting in a 2-fold decrease in the volume of SKN-DZ xenografts and decrease of the weight of tumors (Fig. 5A–C, left). The substantial reduction of tumor volume caused by RITA was not followed by body weight loss (Fig. 5C, right), suggesting the absence of systemic toxicity. Notably, treatment with RITA decreased microvascular density in some tumors, probably due to the downregulation of N-Myc, known to have strong proangiogenic function (ref. 3; Fig. 5B). Indeed, we observed downregulation of N-Myc, along with the p53 target antiapoptotic factor Mcl-1, in xenograft tumors treated with RITA (Fig. 5D).

Discussion

The relapse and chemoresistance in cancers, including neuroblastoma, is often associated with inactivation of the p53 tumor suppressor. Elegant studies in mice show that reinstatement of p53 causes regression of aggressive metastatic tumors (19, 20). This makes pharmacologic rescue of p53 an attractive strategy to combat cancer. Several compounds are currently undergoing clinical trials: JnJ-26854165 (Johnson & Johnson), PXn727 and PXn822 (Priaxon), RG7112/nutlin3a (F. Hoffmann–la Roche), and PRIMA-1^{MET}/Apr-246 identified by us (22). High attrition rate of novel drugs observed during later stages of clinical trials due to unfavorable pharmacokinetics or toxicity demand the search for novel compounds targeting p53.

Rescue of wild-type p53 in neuroblastoma by nutlin3a has been reported (23, 24), supporting the idea that reactivation of p53 by small molecules could be a good strategy to combat neuroblastoma. Nutlin3a is highly selective: sensitivity to nutlin-3a was highly predictive of absence of p53 mutation (25). However, recent study shows that continuous treatment with nutlin-3a confers selective pressure for p53 mutations, resulting in resistance (27). Moreover, p53-mutated nutlin-3a–resistant neuroblastoma cells display an MDR phenotype (26). Emergence of nutlin3a-resistant clones via *de novo* p53 mutations was observed also in osteosarcoma and colon carcinoma (27). Expression of mutant p53 in neuroblastoma is known to result in establishment of a MDR phenotype (10), thus it is imperative that anticancer drugs and/or their combinations be developed that target both wild-type and mutant p53.

In this study, we report that the small molecule RITA causes disruption of p53/MDM2 and MDMX complex and induces apoptosis in a set of neuroblastoma cell lines. However, in contrast with nutlin-3a, which does not inhibit the growth of mutant p53-expressing neuroblastoma (23), RITA can reactivate mutant p53 in neuroblastoma cell lines.

In our previous study we have shown that RITA binds to the N-terminal domain of p53 and induces a conformational change which propagates from the N-terminus to the core and C-terminal domain. This prevents the binding to
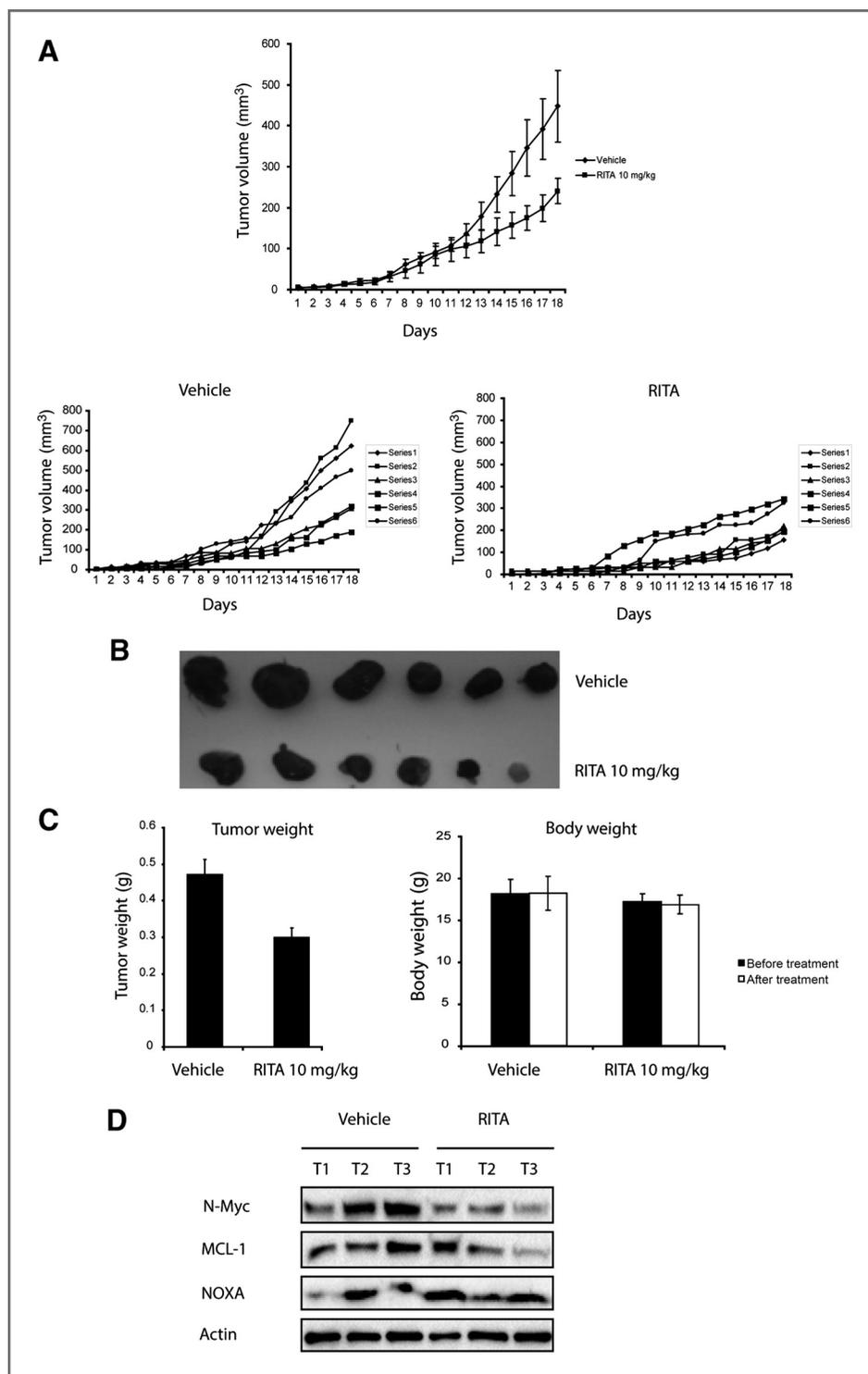
Figure 5. Antitumor effect of RITA in SKN-DZ xenografts in mice. A (top), growth of SKN-DZ tumor xenografts *in vivo* upon injection of 10 mg/kg RITA twice daily in comparison to vehicle treatment; (bottom) growth curves of individual tumors upon RITA or vehicle treatment. B, pictures taken from excised SKN-DZ tumors treated or nontreated with RITA. C (left), comparison of the weight of SKN-DZ tumors treated and nontreated with RITA; (right), body weight of mice before and after treatment with RITA. D, treatment with RITA decreased the protein level of N-Myc and MCL-1 *in vivo*, as assessed by immunoblotting.

p53 of several inhibitors, including MDM2, iASPP, Parc, and E6-AP (28, 32). These observations imply that RITA treatment may affect the global folding of the p53 protein and thus might also affect the folding of mutant p53. Indeed, we have found that a broad range of p53 mutants were reactivated by RITA, including several hot spot mutants (31). Taken together with this study, our results promote the idea of developing compounds capable of simultaneously targeting wild type and mutant p53. This type of compounds should reduce the chance of emergence of *de novo* resistance and enhance clinical success. Indeed, in line with our data on the ability of RITA to reactivate mutant

p53 in neuroblastoma, recent study using UKF-NB-3 neuroblastoma cells as a model does not suggest p53 mutations being the mechanism of acquired resistance to RITA, in contrast to nutlin3a (41). Interestingly, several p53-binding molecules that rescue mutant p53 have been shown to activate the function of wild-type p53 as well. These include CDB3 (42), SCH529074 (43), CP-3139 (44), and PRIMA-1^MET/Apr-246 (45). At least some of them seem to inhibit the p53/MDM2 interaction via induction of a conformational change (43), although in most cases the mechanism remains elusive and awaits a detailed investigation.

Amplification of the *MYCN* gene predicts poor prognosis and resistance of neuroblastoma to therapy. Inhibition of N-Myc is therefore regarded as a promising approach for the development of targeted therapies (3). Here, we have identified p53 as a potent inhibitor of N-Myc expression in neuroblastoma. We found that p53 activated by RITA induced the expression of its target Fbxw7, which has a critical function in proteasomal degradation of the N-Myc protein (37). Moreover, we showed that p53 represses the transcription of the antagonist of Fbxw7-mediated degradation of N-Myc, Aurora A (37). Aurora A is a negative prognostic factor and a potential therapeutic target in neuroblastoma (46), which, according to our recent study, is a *bona fide* p53 target (38). In addition, RITA treatment leads to the decrease of MDM2, which upregulates N-Myc (17). Taken together, our data suggest that reactivation of p53 by RITA causes inhibition of N-Myc via induction of its E3 ligase Fbxw7. This might be further facilitated by transcriptional repression of Aurora A and inhibition of MDM2.

It is possible that additional mechanisms of N-Myc inhibition by RITA might exist, as we did not detect N-Myc rescue upon mutant p53 silencing in SKN-BE(2) cells. For example, inhibition of TrxR1 by RITA might play a role (47). We would like to note, however, that the mutant p53 silencing by 4 different RNAi constructs caused SKN-BE (2) cell death, limiting our analysis. We speculate that the survival of SKN-BE(2) cells might depend on mutant p53 expression, due to gain-of-function of mutant p53. This limitation precludes a more vigorous analysis of N-Myc regulation by p53 in SKN-BE(2) cells.

Our study reveals the ability of p53 to unleash the transcriptional repression of several major survival factors in neuroblastoma. Our data suggest that the repression of Bcl-2 and Mcl-1, reducing the cancer cell's ability to buffer proapoptotic signal, might contribute to the robust induction of apoptosis in neuroblastoma by pharmacologically reactivated p53.

Another factor downregulated in neuroblastoma cells by RITA-reactivated p53 is Wip1, encoded by the *PPM1D* gene at 17q, whose gain is associated with poor prognosis in neuroblastoma (5). Wip1 interferes with the DNA damage response and p53 activation by dephosphorylating crucial effectors, thus conferring resistance to standard treatments. It is overexpressed in different cancers and is important for the survival of tumor stem cells, which makes the development of Wip1 inhibitors an attractive strategy for therapy (48). The multitude of oncogenes, inhibited by

RITA-reactivated p53 creates a robust p53 response. It might allow p53 to cope with the daunting challenge of anticancer therapy–multiple genetic abnormalities in individual cancers. Because tumors are often "addicted" to the oncogenes, such as increased expression of N-Myc, Wip1, Aurora A, Bcl-2, or Mcl-1, their inhibition might be an essential component of anticancer therapies targeting p53. Thus, the ability of reactivated p53 to inhibit several key oncogenes in neuroblastoma adds a new dimension to the mechanism of tumor suppression upon p53 activation by small molecules.

RITA efficiently inhibited the growth of neuroblastoma tumor xenografts without the apparent toxicity. Notably, the morphology of tumors suggests that reactivation of p53 by RITA is able to inhibit the growth of tumors' blood vessels, in line with inhibition of potent proangiogenic factor N-Myc and previous studies suggesting that p53 can affect the transcription of several genes involved in angiogenesis (49). The effect of RITA on tumor blood vessels is very interesting and will be investigated further. Although we did not attempt to maximize the therapeutic response *in vivo*, it is conceivable that the dosing regimen and the schedule of treatment could be improved, for example, by the administration of higher dose (50–100 mg/kg, shown previously to be safe in mice; ref. 50).

In conclusion, we showed that RITA is efficient and potent activator of both wild-type and mutant p53 and inducer of p53-dependent apoptosis in neuroblastoma *in vitro* and *in vivo*. Ablation of oncogenes driving neuroblastoma, in particularly, N-Myc, by pharamacologicaly reactivated p53 might be a very important factor for future application of p53-based therapy in neuroblastoma. Our study provides further support for the notion of using molecules reactivating p53 to combat neuroblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Burmakin, Y. Shi, P. Kogner, G. Selivanova
Development of methodology: M. Burmakin, Y. Shi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Burmakin, Y. Shi, E. Hedström
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Burmakin, Y. Shi, E. Hedström, G. Selivanova
Writing, review, and/or revision of the manuscript: M. Burmakin, Y. Shi, P. Kogner, G. Selivanova
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Selivanova
Study supervision: G. Selivanova

Grant Support

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