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

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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

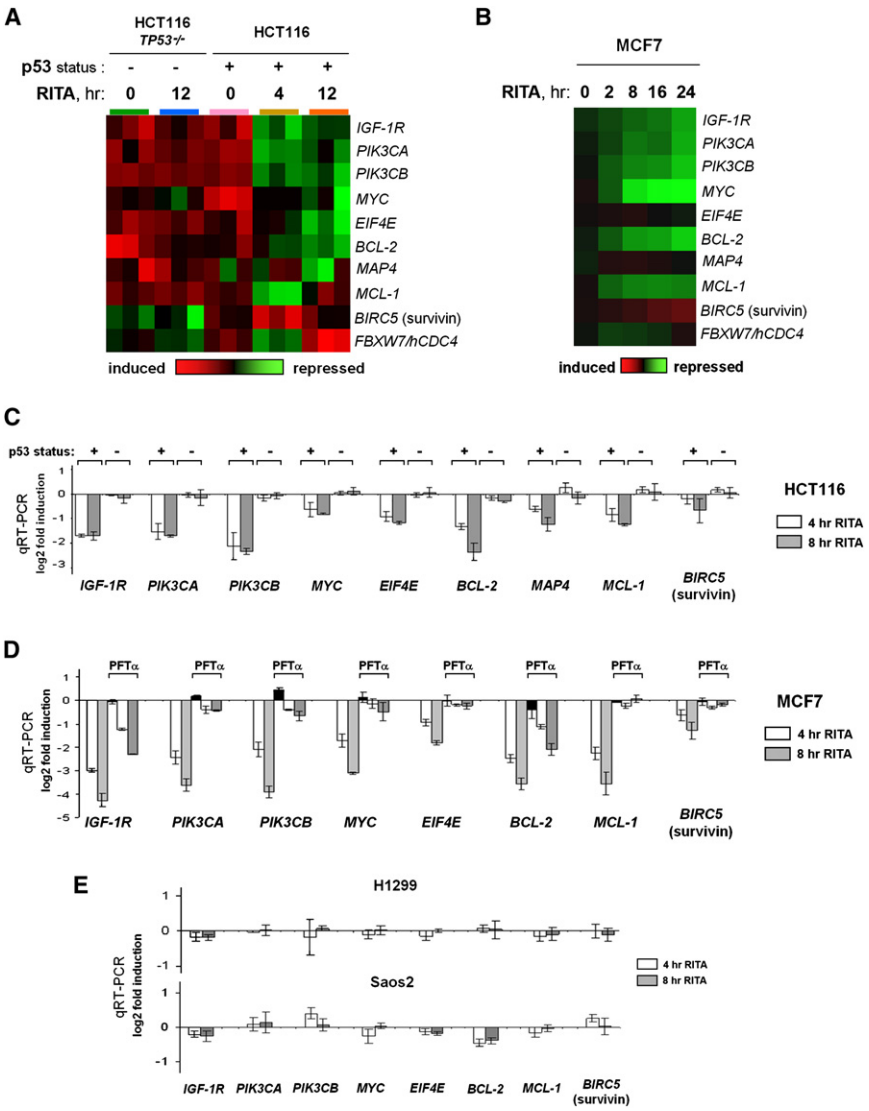

Figure 1. p53-Induced Transcriptional Repression of a Set of Oncogenes

(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).

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 downstream 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

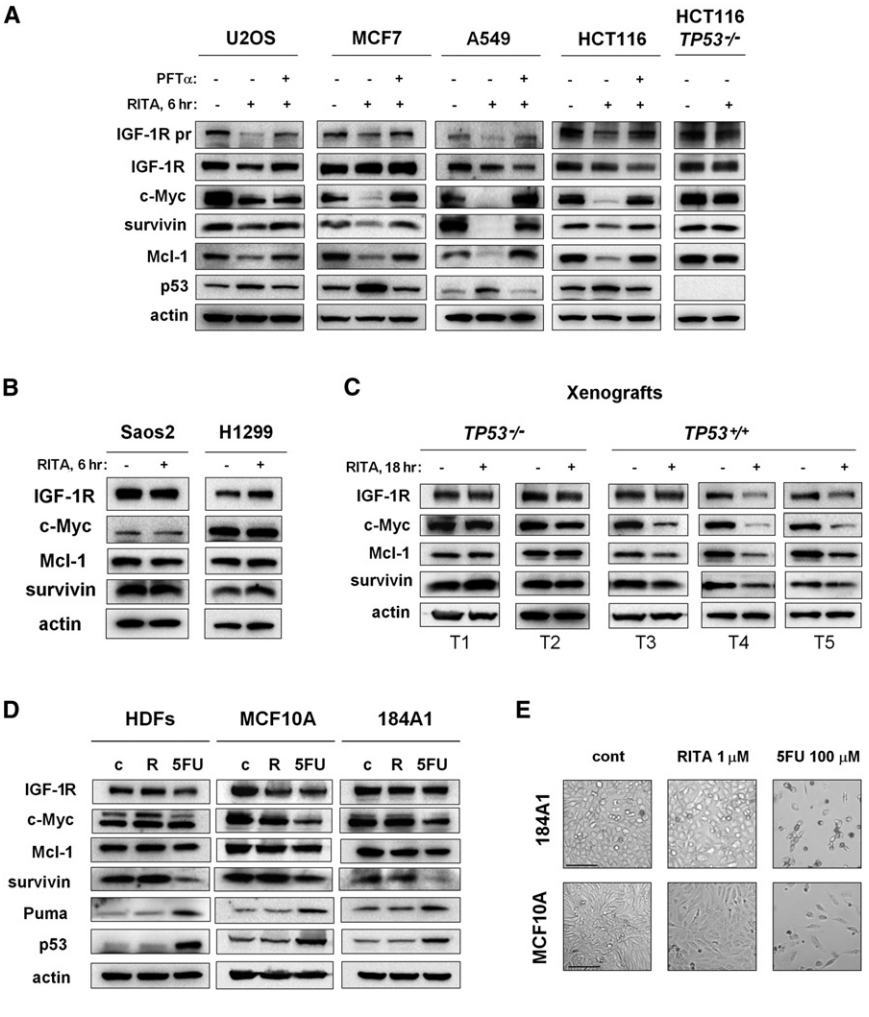

Figure 2. p53-Dependent Downregulation of the Oncoproteins c-Myc, IGF-1R, Mcl-1, and Survivin In Vitro and In Vivo

(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.

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 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

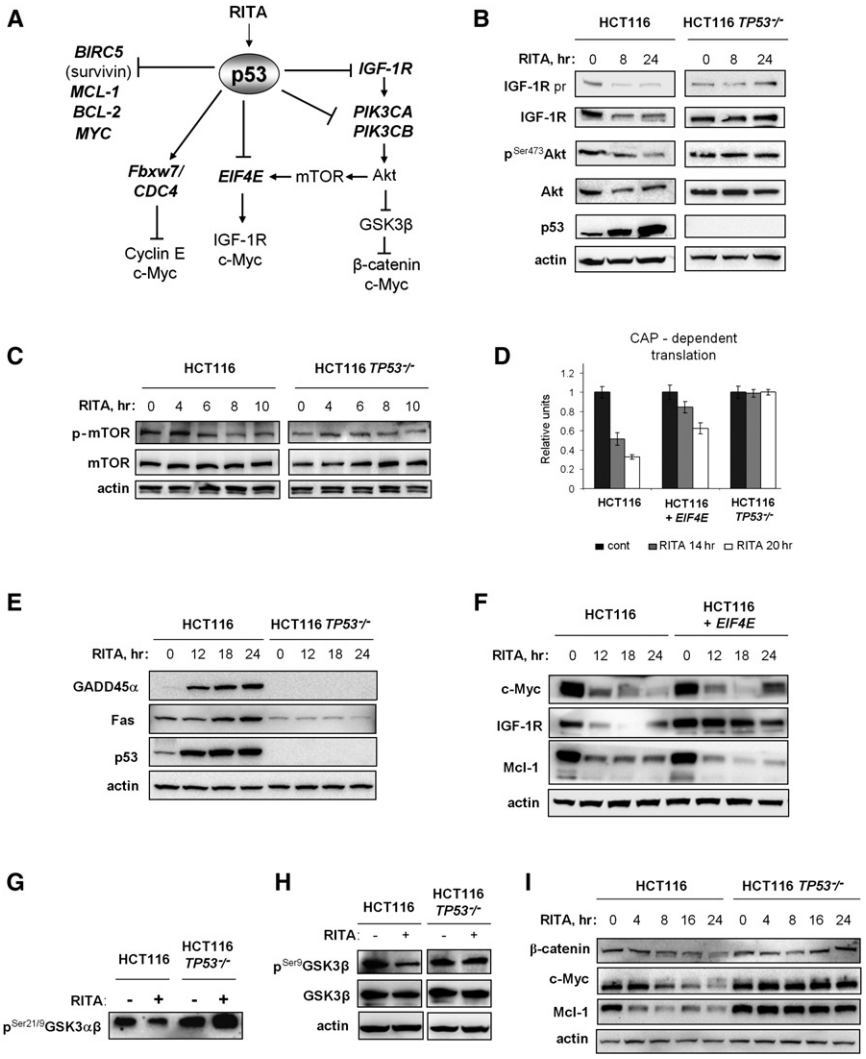

Figure 3. p53-Dependent Inhibition of the Akt Pathway upon RITA Treatment

(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).

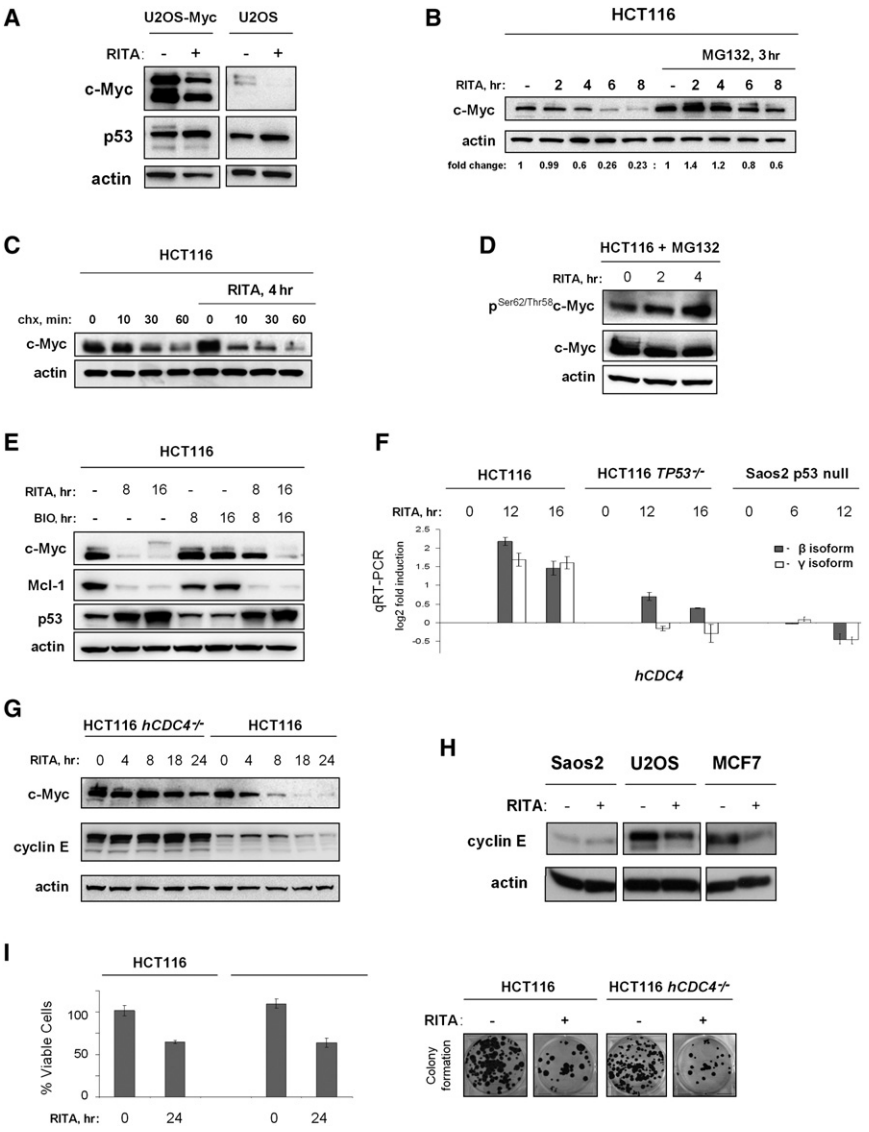

Figure 4. Reactivation of p53 by RITA Induces Proteasomal Degradation of c-Myc via Activation of GSK3β and Fbxw7/hCdc4

(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).

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 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

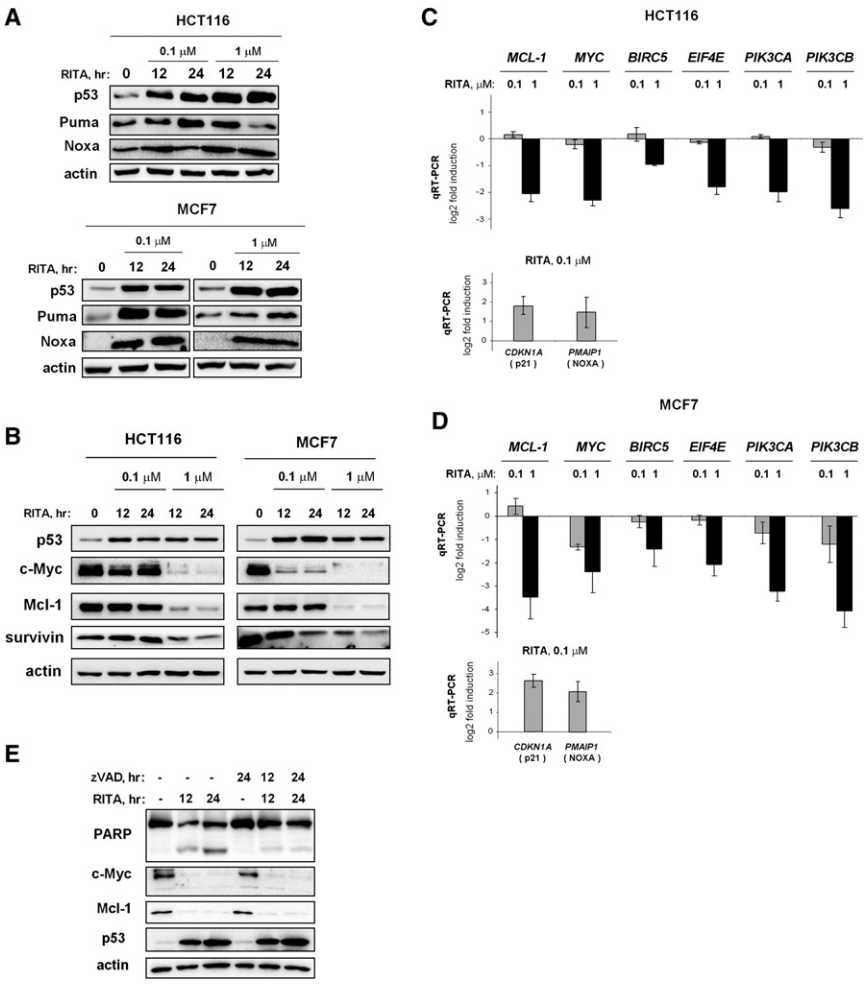

Figure 5. Dose-Dependent Effect of RITA on the Transcriptional Activation of Proapoptotic Genes and the Repression of Oncogenes

(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).

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).

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.

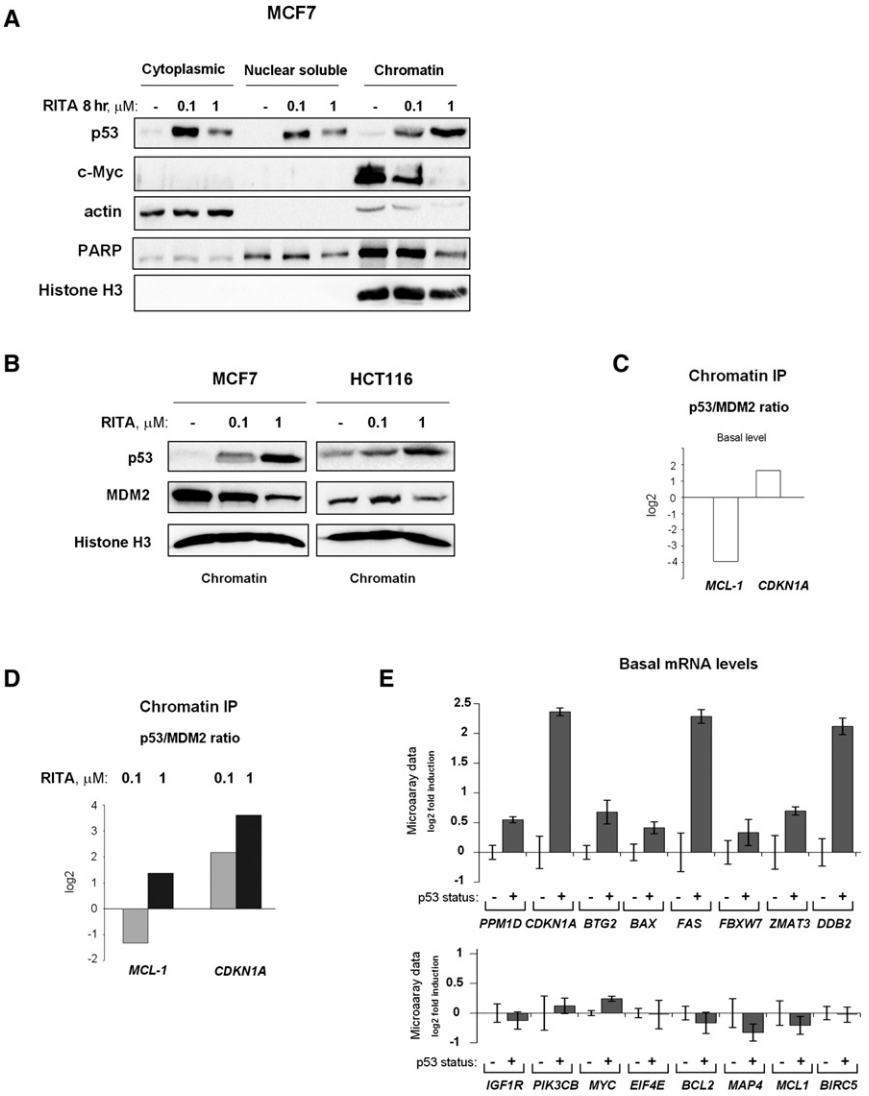

Figure 6. Dose-Dependent Differences in the Subcellular Distribution of p53 Correlate with Distinct Transcriptional Programs Induced by p53

(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).

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 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 nultin3a, 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

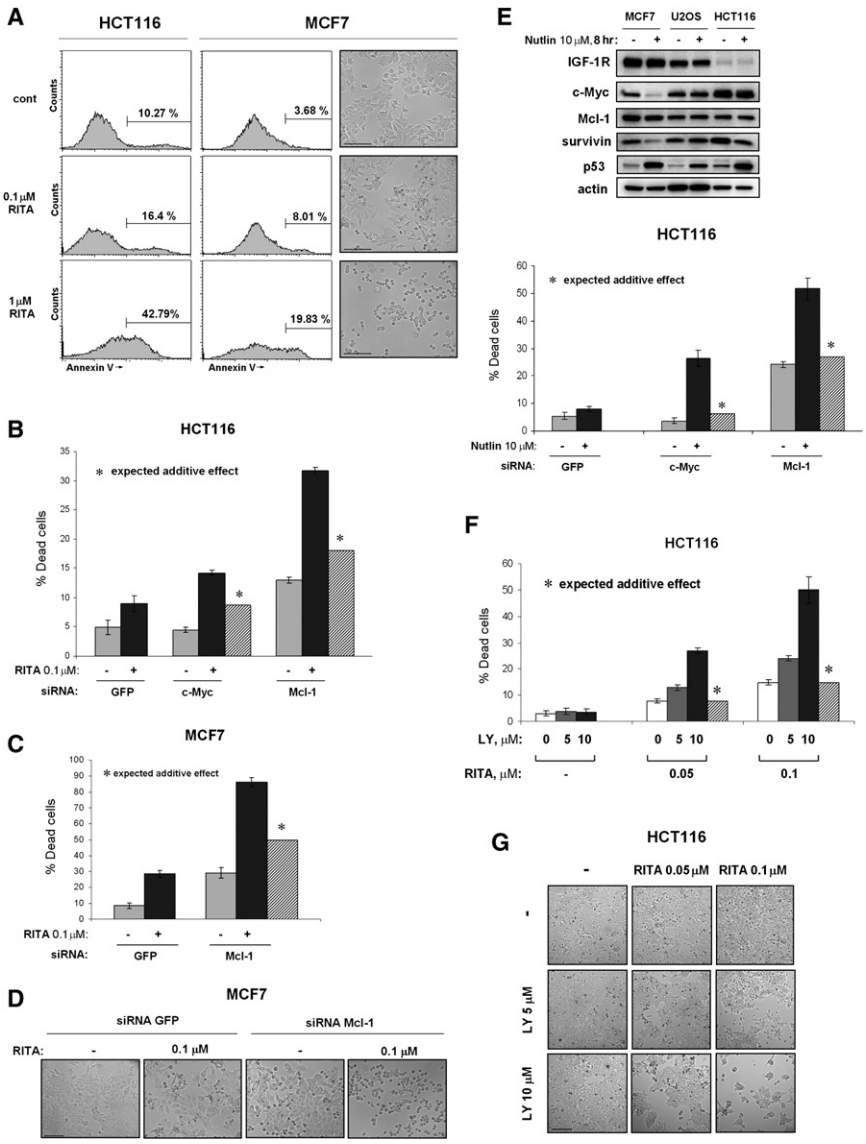

Figure 7. Inhibition of Oncogenes Plays a Significant Role in Apoptosis Induction by Pharmacologically Activated p53

(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.

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 experiments 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

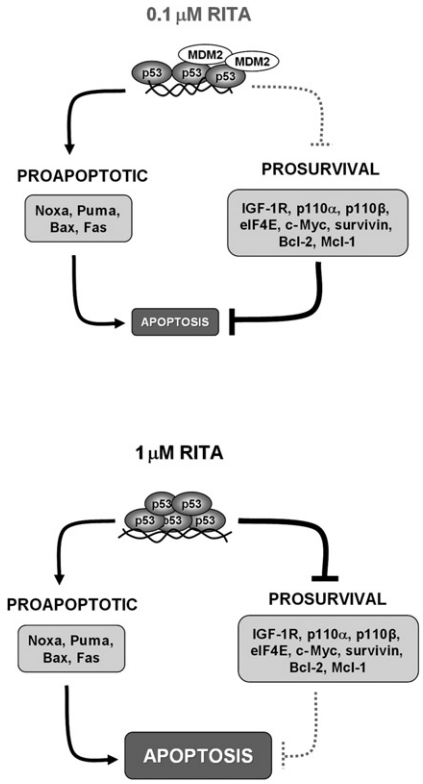

Figure 8. Model: Two Branches of the p53 Transcriptional Program Are Required for Efficient Apoptosis Induction

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 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 expression 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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