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Report

Multiple p53-independent gene silencing mechanisms define the cellular response to p53 activation

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The cellular response to Nutlin-3, a small-molecule inhibitor of the p53 repressor MDM2, varies widely among human cancer-derived cell types. Whereas HCT116 colorectal carcinoma cells display sustained cell cycle arrest, BV173 leukemia cells undergo rapid apoptosis and other cell lines show an intermediate response. We found that the expression of the p53 target genes *p21, 14-3-3*σ and the microRNA *miR-34a* correlates tightly with the cell fate choice adopted. All three genes were strongly induced in arresting cells, but silenced in cells undergoing Nutlin-3-induced apoptosis. In contrast, key apoptotic p53 target genes were equally expressed in arresting and apoptotic cells. Interestingly, we establish that *miR-34a* cooperates with *p21* and *14-3-3*σ to override the apoptotic signals generated by p53 activation. Strikingly, p53 binding to chromatin and p53-mediated recruitment of certain coactivators to all three target loci does not vary among cell types. Instead, the cell type-specific silencing of these genes is due to enhanced *p21* mRNA degradation, *14-3-3*σ promoter DNA methylation and reduced processing of the *miR-34a* primary transcript. Thus, p53-independent events regulating expression of protein-coding genes and microRNAs within the network can define the cellular outcome of p53 activation.

Introduction

Deciphering how complex gene networks determine cell behavior is of paramount importance for the biomedical sciences, as disruption of regulatory interactions within key networks is a hallmark of many disease states, including cancer. The p53 transcription factor enables cells to undergo cell cycle arrest, apoptosis or senescence in response to a variety of forms of cellular stress, which generates a selective pressure to inactivate this protein during tumor growth.[1] In the absence of stress, cells express low levels of p53 protein due to rapid turnover mediated by the E3-ubiquitin ligase MDM2.[2] Induction of cell cycle arrest by p53 is achieved mostly by transcriptional activation of the cell cycle inhibitors *p21* and *14-3-3*σ.[3,4] The molecular events that lead to p53-dependent apoptosis are more complex. p53 induces expression of numerous apoptotic genes involved in the mitochondrial apoptotic pathway and the death-receptor pathway, but there is also evidence for transcriptionally-independent functions of p53 in apoptosis.[5]

p53-inducible microRNAs constitute a newly recognized class of p53 target genes.[6] miRNAs are small non-coding RNAs that post-transcriptionally regulate gene expression. miRNAs are transcribed as primary transcripts (pri-miRNAs) and then processed into mature miRNAs by the sequential action of the ribonucleases Drosha and Dicer. Mature miRNAs are incorporated into RISC (RNA-induced Silencing Complex) to inhibit expression of target mRNAs by varied mechanisms.[7] Recently, *miR-34a* was identified as a transcriptional target of p53. The precise function of *miR-34a* in the p53 network is unclear at this point, as some reports implicate *miR-34a* in cell cycle arrest whereas others propose that *miR-34a* promotes apoptosis (reviewed in ref. 6).

What determines a particular cellular outcome after p53 activation? This question is of paramount importance in the clinical arena: understanding how a cell decides whether or not to die in response to p53 activation will enable us to envision therapeutic strategies to harness this pathway for selective elimination of cancer cells. The choice of cellular response to p53 activation seems to be influenced by a number of variables, including but not restricted to p53 post-translational modifications, p53 cofactors and the action of parallel signaling pathways.[8] Recently, a small-molecule inhibitor of the p53-MDM2 interaction, known as Nutlin-3, has been developed.[9] Nutlin-3 mimics three hydrophobic amino acids of p53 required for MDM2 binding, thus behaving as a competitive inhibitor that activates p53 in the absence of genotoxic stress. Intriguingly, Nutlin-3 treatment induces p53-dependent cell cycle arrest, rather than apoptosis, in most cell types tested.[10] Here, we report that expression levels of *p21, 14-3-3*σ and *miR-34a* correlate tightly with the cellular response evoked by Nutlin-3 and we demonstrate that *miR-34a* plays an anti-apoptotic role in cooperation with *p21* and *14-3-3*σ. Most importantly, our mechanistic studies show that p53 target gene activity is ultimately defined by p53-independent regulatory events such as enhanced *p21* mRNA turnover, *14-3-3*σ promoter DNA methylation and pri-miR-34a processing.

Results and Discussion

Distinct responses to p53 activation by Nutlin-3 correlate with differential expression of p21 and 14-3-3σ. In order to study

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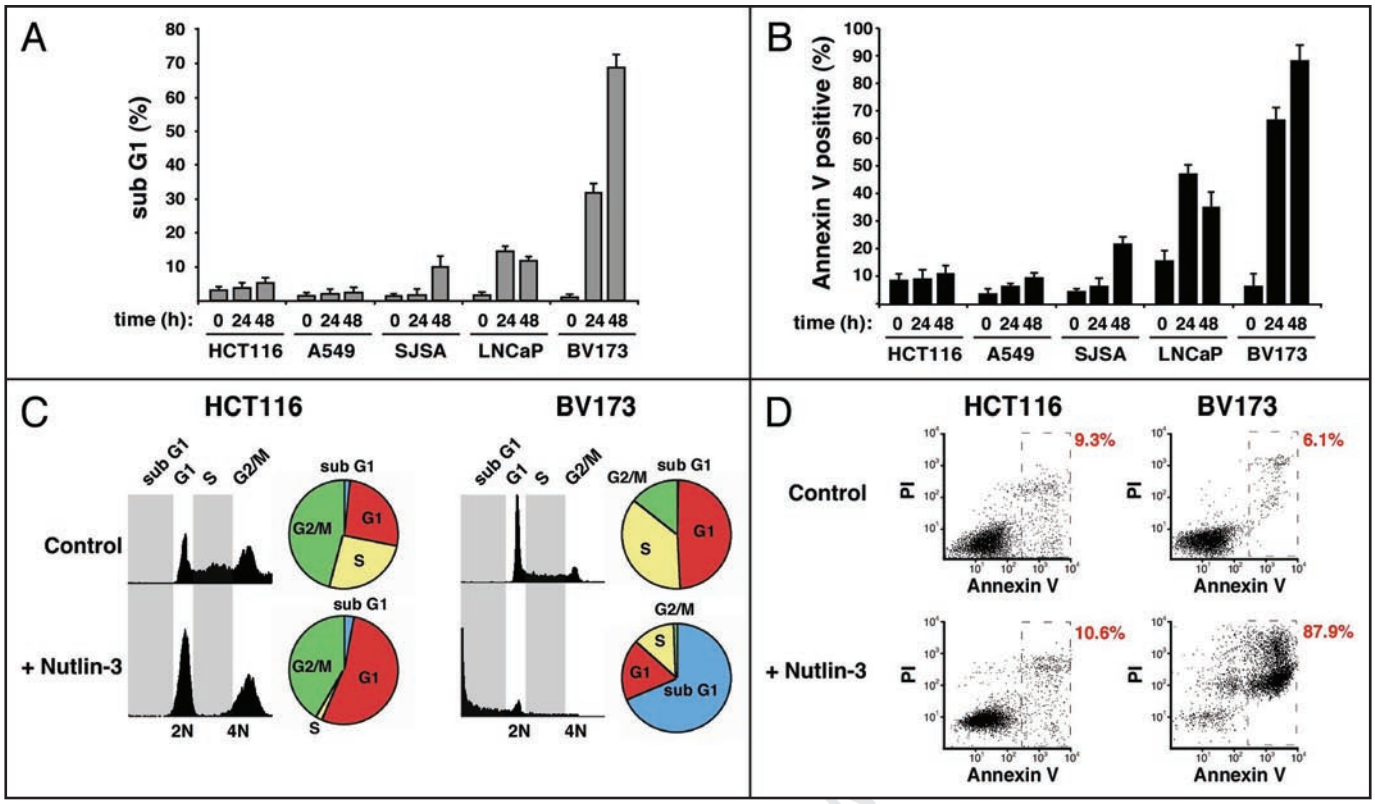

Figure 1. Cell type-specific responses to Nutlin-3. Cultures were treated with Nutlin-3 for the indicated time points before analysis of DNA content (A and C) and apoptotic index (B and D). Average levels of DNA degradation and phosphatidyl-serine exposure from three independent experiments are displayed for all five cell-lines (A and B). Raw data from one representative experiment is shown for HCT116 and BV173 cells (C and D, 48 h treatment).

mechanisms of cell type-specific responses to p53 activation, we analyzed the cellular response to Nutlin-3 treatment in a panel of cell lines expressing wild type p53. Using FACS analysis of DNA content and phosphatidyl-serine exposure, we observed varied responses after identical treatment (Fig. 1). At one end of the spectrum, HCT116 colorectal carcinoma cells show negligible signs of apoptosis even at 48 h post-treatment. DNA content profiles indicate that HCT116 cells undergo cell cycle arrest in G_1 and G_2/M with depletion of cells in S phase. In sharp contrast, chronic myelogenous leukemia (CML) BV173 cells undergo rapid apoptosis, as seen by increased sub G_1 population and Annexin V staining. A549 lung cancer cells respond similarly to HCT116 cells, whereas SJSA osteosarcoma cells and LNCaP prostate cancer cells show an intermediate response. Expectedly, Nutlin-3 produced no effects on HCT116 $p53^{-/-}$ and K562 cells (CML, p53 null) (data not shown).

We hypothesized that differential expression of p53 target genes may drive the response adopted by different cell types. To test this, we analyzed expression of diverse p53 target genes at the mRNA and protein levels. We began with Q-RT-PCR analysis of HCT116 and BV173 cells, which revealed important differences between the two cell types (Fig. 2A). Interestingly, BV173 cells display significantly lower levels of *p21* and *14-3-3σ* mRNAs both before and after Nutlin-3 treatment. These differences are clearly gene-specific, as the mRNAs for *PUMA* and *PIG3* reach similar levels upon induction in both cell types. Several other p53 target genes, including *MDM2, BTG2, GADD45a* and *FAS/APO1* also showed equivalent expression (Suppl. Fig. 1). Importantly, Western Blots revealed that Nutlin-3 treatment leads to equivalent p53 stabilization and PUMA induction in both cell types, yet accumulation of p21 and 14-3-3σ proteins is only observed in HCT116 cells (Fig. 2B). Of note, concurrent activation of p21, 14-3-3σ and PUMA in HCT116 cells results in cell cycle arrest, suggesting that the apoptotic program is overridden in this scenario and that the dosage of cell cycle inhibitors could define the cellular response to Nutlin-3. This notion is further supported by analysis of other cell lines (Fig. 2C). A549 cells, which respond similarly to HCT116 cells, express both p21 and 14-3-3σ. SJSA and LNCaP cells, which show an intermediate response, express p21 but not 14-3-3σ. Furthermore, a detailed kinetics analysis indicates that the delayed onset of apoptosis in SJSA cells correlates with decreased p21 expression over time (Suppl. Fig. 2). In summary, our analysis establishes a strong correlation between expression of p21 and 14-3-3σ and the cellular response adopted.

Lack of p21 and 14-3-3σ sensitizes colorectal carcinoma cells to Nutlin-3-induced apoptosis. The anti-apoptotic effects of p21 and 14-3-3σ have been previously established when analyzing the cellular response to genotoxic p53-activating agents.[11] To define the role of these two cell cycle inhibitors in cell fate choice upon non-genotoxic activation of p53, we employed HCT116 cells where both genes have been knocked-out.[11] After prolonged Nutlin-3 treatment, parental HCT116 cells remain arrested and show low levels of cell death (Fig. 2D). In contrast, *$p21^{-/-}$ 14-3-3$σ^{-/-}$* cells show reduced arrest and increased apoptosis, thus confirming the notion that p21 and 14-3-3σ play a protective role against Nutlin-3-induced apoptosis. However, the level of apoptosis in the double knock-out cells did not reach that observed for BV173 cells, which lead us to hypothesize the existence of additional anti-apoptotic factors.

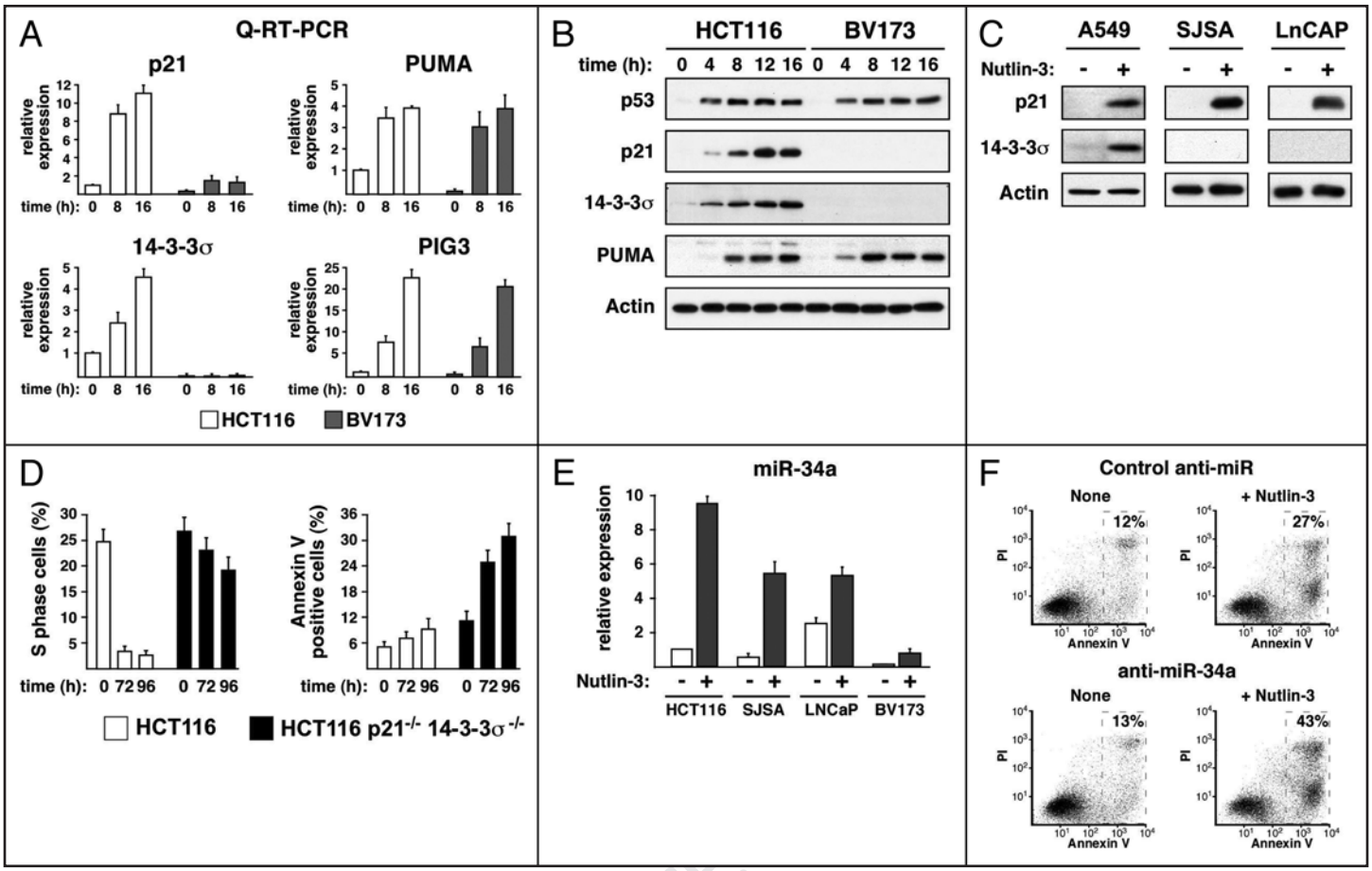

Figure 2. p21, 14-3-3σ and miR-34a protect cells from the apoptotic effects of Nutlin-3. (A) Q-RT-PCR analysis reveals differential expression of *p21* and *14-3-3σ* in HCT116 versus BV173 cells. Cultures were treated with Nutlin-3 for the indicated times before RNA extraction and RT-PCR. 18s rRNA was used for normalization. (B) Western Blot analysis showing cell type-specific accumulation of p21 and 14-3-3σ proteins after Nutlin-3 treatment. (C) Western Blot showing induction of p21 and/or 14-3-3σ expression in Nutlin-3-treated (24 h) A549, SJSA and LNCaP cells. (D) Comparative analysis of parental versus double knock-out HCT116 cells demonstrates the anti-apoptotic effects of p21 and 14-3-3σ during Nutlin-3 treatment. (E) Strong correlation between overall miR-34a expression and the cellular response to Nutlin-3 among cancer cell types. Cells were treated with Nutlin-3 for 16 h before extraction of small RNAs and Taqman assays. U6 snRNA was used as normalization control. (F) Knock-down of miR-34a increases the apoptotic effects of Nutlin-3 in *p21*$^{-/-}$ *14-3-3*$^{-/-}$ HCT116 cells. Control or anti-miR34a LNA-oligonucleotides were transfected 24 h prior to Nutlin-3 addition. Apoptotic indexes were performed at 96 h post-treatment. Error bars indicate standard deviations over three independent experiments.

miR-34a cooperates with p21 and 14-3-3σ in cell fate choice to Nutlin-3 treatment. Recently, miR-34a was identified as a p53-inducible miRNA, but its role in cell fate choice has not been defined. Using Taqman assays, we measured miR-34a levels in several cell types (Fig. 2E). As expected, miR-34a was induced by Nutlin-3 in all p53-expressing cell lines. However, important differences in overall miR-34a levels were evident, with HCT116 cells showing the highest expression and BV173 cells the lowest. Clearly, miR-34a levels correlate negatively with the apoptotic efficacy of Nutlin-3, which led us to hypothesize that miR-34a may also play anti-apoptotic roles. To test this, we utilized Locked-Nucleic Acid (LNA) technology to decrease endogenous miR-34a activity in HCT116 *p21*$^{-/-}$ *14-3-3*$\sigma^{-/-}$ cells. Transfection of anti-miR-34a, but not of control oligonucleotides, enhanced Nutlin-3-induced apoptosis as seen by increased numbers of Annexin V-positive cells (27% to 43%, Fig. 2F). Overall, we conclude that p21, 14-3-3σ and miR-34a collaborate to suppress the p53-dependent apoptotic program in Nutlin-3-treated cells.

These results indicate that determining the expression levels of p21, 14-3-3σ and miR-34a in a tumor sample could have prognostic value in terms of Nutlin-3 response. However, it is likely that additional factors also contribute to the heterogeneous responses to the drug. Recent reports suggest that cell lines expressing higher levels of MDM2 seem to be more susceptible to Nutlin-3-induced apoptosis.[10] Other reports postulate that the levels of MDM4, a p53 repressor acting independently of MDM2, can also influence the cellular response to the drug.[12-14] Furthermore, cancer cells employ different strategies to block the apoptotic machinery,[15] which could determine the efficacy of apoptotic genes induced by Nutlin-3-activated p53. Overall, we can hypothesize that a 'molecular signature' capable of predicting the response to Nutlin-3 would include information on the status of p21, 14-3-3σ, miR-34a, MDM2, MDM4, as well as several components of the apoptotic machinery.

p53-dependent induction of p21 is suppressed at post-transcriptional steps in BV173 cells. Having defined a role for *p21*, *14-3-3σ* and *miR-34a* in cell fate choice upon Nutlin-3 treatment, we inves-

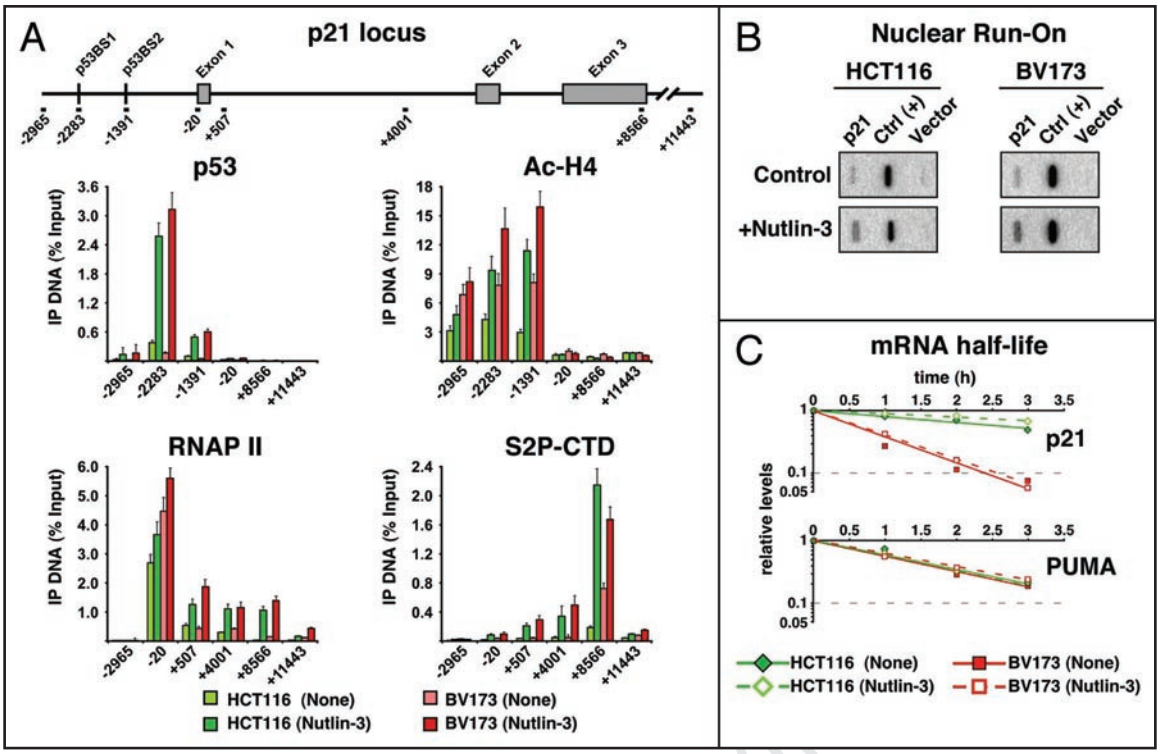

Figure 3. p21 induction is suppressed at post-transcriptional steps in BV173 cells. (A) ChIP analysis of the *p21* locus indicates that p53 binding, histone acetylation and RNAP II activation is equivalent in HCT116 and BV173 cells. Cells were treated with Nutlin-3 for 8 h before protein extract preparation. After immunoprecipitation with the indicated antibodies, ChIP-enriched DNA was analyzed by Q-PCR using the amplicons shown on top. Amplicon number indicates relative position to the transcription start site (base pairs). Error bars indicate standard deviations over three independent experiments. (B) Nuclear run-on assays demonstrate that *p21* mRNA is synthesized at similar rates in both HCT116 and BV173 cells. (C) mRNA half-life assays indicate that the *p21* mRNA half-life is much longer in HCT116 cells (~195 min) than in BV173 cells (~43 min).

tigated the mechanisms driving cell type-specific expression of these genes. Influenced by our past work on stimulus-specific transcriptional regulation of p53 target genes,[16-18] we hypothesized that differential accumulation of *p21* mRNA in HCT116 versus BV173 cells could be due to transcriptional events. To test this, we performed Chromatin Immunoprecipitation (ChIP) analysis of the *p21* locus (Fig. 3A). We found that induced levels of chromatin-bound p53 were equivalent in both cell types. Transcriptional activation by p53 involves recruitment of histone acetyl-transferases (HATs) to the promoter region of target genes.[19] Importantly, ChIP assays detected high levels of acetylated histone H4 (Ac-H4) upstream of the *p21* promoter in both cell types, suggesting that p53-mediated recruitment of HATs is not impaired in BV173 cells. Transcriptional activation of *p21* occurs at post-initiation steps and involves conversion of paused RNAP II into the elongating form of the enzyme.[16] Accordingly, significant amounts of pre-loaded RNAP II were detected at the *p21* proximal promoter in both HCT116 and BV173 cells. Upon Nutlin-3 treatment, a significant increase in RNAP II occupancy was observed at the intragenic region in both cell types (amplicons +507, +4001 and +8566), indicative of RNAP II activation. RNAP II elongation is more easily visualized when using antibodies against phosphorylated Serine 2 within the C-terminal domain repeats of its largest subunit (S2P-CTD), a modification that accumulates toward the 3' end of genes. Our ChIP analysis showed that S2P-CTD signal is effectively induced in both HCT116 and BV173 cells, suggesting that the *p21* gene is transcriptionally activated to similar extent in both cell types.

To confirm that the *p21* locus was transcriptionally active in BV173 cells, we performed nuclear run-on assays. These experiments detected de novo synthesis of *p21* mRNA in both HCT116 and BV173 cells, with the transcription rate increasing about 3-fold upon Nutlin-3 treatment (Fig. 3B). Based on these results, we hypothesized that differences in steady-state *p21* mRNA levels could be due to effects on mRNA turnover. To test this, we performed mRNA half-life assays, which demonstrated that *p21* mRNA half-life is drastically shorter in BV173 cells (~43 minutes) than in HCT116 cells (~195 minutes) (Fig. 3C). In contrast, *PUMA* mRNA half-life is identical in both cell types, thus revealing the action of gene-specific mechanisms. Importantly, mRNA half-life for *p21* and *PUMA* is similar before and after Nutlin-3 treatment, indicating that the differences in mRNA stability are not dependent on p53 activity. In summary, these results indicate that repression of *p21* expression in BV173 cells occurs at post-transcriptional steps, in a p53-independent process likely involving enhanced mRNA degradation. Additional post-transcriptional mechanisms silencing p21 expression, such as translation inhibition or enhanced protein degradation, can not be discarded at this point.

Despite significant advances in the elucidation of post-transcriptional regulatory mechanisms, not much is known about their role in cell fate choice upon p53 activation. Several studies have identified cis-regulatory elements in the 3' UTR of the *p21* mRNA, as well as RNA binding proteins recognizing these sequences. For example, the RNA binding protein HuR binds to and stabilizes the *p21* mRNA

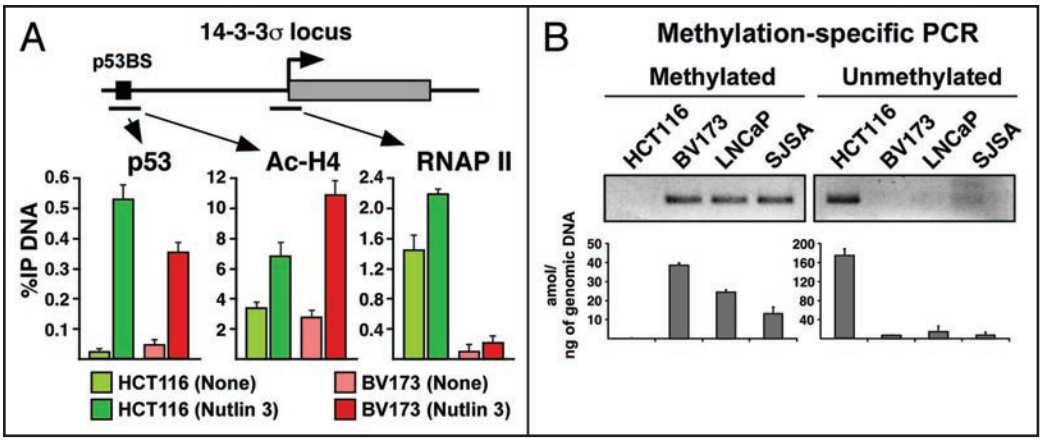

Figure 4. The *14-3-3σ* promoter DNA is methylated in several cancer cell types. (A) ChIP analysis of the *14-3-3σ* locus reveals equal binding of p53 and histone acetylation in HCT116 and BV173 cells, but absence of RNAP II in the latter. (B) Methylation-specific PCR demonstrates that the *14-3-3σ* promoter DNA is hypermethylated in BV173, LNCaP and SJSA cells. Genomic DNA was analyzed by PCR with primer pairs recognizing the alternative methylation states. Reactions were analyzed by agarose electrophoresis (top) and Q-PCR (bottom).

during myogenic differentiation.[20] In contrast, binding of the AUF1 protein to the *p21* 3' UTR leads to its destabilization.[21] Future work will be required to identify cis-elements and trans-acting factors affecting *p21* mRNA stability in different cancer cell types and, consequently, their impact on the efficacy of p53-based therapies.

14-3-3σ is repressed via promoter DNA methylation. Next, we investigated the mechanism mediating differential expression of *14-3-3σ*. *14-3-3σ* is an intronless gene harboring a p53 response element ~1.8 kb upstream of the transcription start site (Fig. 4A). As for *p21*, ChIP assays detected equivalent levels of p53 and increased histone acetylation at the *14-3-3σ* regulatory region in HCT116 and BV173 cells (Fig. 4A). However, RNAP II occupancy at the proximal promoter is greatly reduced in BV173 cells, suggesting that a repressive event downstream of p53 binding and histone acetylation is taking place at the *14-3-3σ* locus in these cells. It has been reported that *14-3-3σ* transcription is silenced in some cancers via aberrant DNA methylation of its promoter region.[22,23] To investigate this possibility, we performed methylation-specific PCR assays. Results in Figure 4B indicate that the *14-3-3σ* promoter is indeed methylated in BV173, LNCaP and SJSA cells (none of which expresses 14-3-3σ), but not in HCT116 cells.

Silencing of tumor suppressor genes via promoter DNA methylation is a frequent event during cancer development. The mechanism driving *14-3-3σ* promoter methylation remains to be elucidated. A recent report demonstrated that the protein kinase IKKα shields *14-3-3σ* from DNA methylation, as *IKKα*$^{-/-}$ keratinocytes display repressive histone modifications and recruitment of DNA methyltransferases to this locus.[24] It will be interesting to determine if differential IKKα activity contributes to cell type-specific silencing of *14-3-3σ* and, consequently, to the apoptotic efficacy of Nutlin-3.

Differential expression of miR-34a is due to differences in pri-miRNA processing. Lastly, we investigated the mechanism driving differential expression of miR-34a in HCT116 versus BV173 cells. miR-34a is encoded by its own transcription unit containing p53 binding sites within the first exon (Fig. 5A). ChIP analysis revealed that Nutlin-3 induces a significant increase in chromatin-bound p53 and histone acetylation at the 5' end of the gene in both cell types (Fig. 5A). Although basal levels of total RNAP II are significantly higher in HCT116 cells, Nutlin-3 treatment leads to equivalent levels of total RNAP II in both cell lines. Most importantly, similar levels of S2P-CTD are detected at the 3' region in Nutlin-3-treated HCT116 and BV173 cells. These results mimic those obtained for the *p21* locus, and prompted us to investigate if differences in miR-34a levels could be due to post-transcriptional events. To test this, we designed Q-PCR amplicons to measure total pri-miR-34a (both unprocessed and processed, labeled α in Fig. 5B) or only the unprocessed form (labeled β). We also employed Taqman assays to measure the abundance of mature, fully processed miR-34a (γ). Analysis of total pri-miR-34a supports the notion that the *miR-34a* locus is transcriptionally activated in both cell types, as BV173 cells accumulate significant amounts of this RNA species upon Nutlin-3 treatment. Interestingly, although BV173 cells accumulate somewhat less total pri-miR-34a than HCT116 cells, they carry significantly more of the unprocessed pri-miRNA. This suggests that pri-miRNA processing is impaired in BV173 cells, and, accordingly, they express much less of the mature form of miR-34a (γ).

Little is known about the regulation of miRNA expression and activity. Most pri-miRNAs are transcribed by RNAP II and are likely to be regulated by combinatorial usage of transcription factors as observed for protein-coding genes. Here, we extend the finding that miR-34a is a p53-inducible miRNA by showing that p53 binds to the *miR-34a* locus, mediates histone acetylation and stimulates RNAP II activity (none of this was observed in HCT116$^{-/-}$ cells, data not shown). Interestingly, our analysis indicates that cell type-specific differences in miR-34a abundance can be attributed to unequal processing of its pri-miRNA. Recent reports reveal the prevalence of regulated pri-miRNA processing.[25,26] For example, the levels of several mature miRNAs are upregulated >1000-fold during mouse development without increase in the levels of the corresponding pri-miRNAs.[26] Regulated pri-miRNA processing could involve editing of the pri-miRNA by adenosine deaminases,[27] or even blockade of Drosha-mediated cleavage by RNA binding proteins.[28] Our results indicate that studies aimed at elucidating the precise mechanism(s) mediating cell type-specific processing of miR-34a, and perhaps other p53-inducible miRNAs, could lead to the identification of additional factors affecting cell fate choice to p53 activation.

Final remarks. As our understanding of the mechanisms driving cellular responses to various stimuli increases, so does our capacity to artificially manipulate specific molecular machineries to provoke a desired outcome. This is particularly evident in cancer biology, where the identification of precise molecular perturbations driving cancerous growth has led to effective therapies, as in the treatment of CML patients with Bcr-Abl inhibitors. Finding ways to activate p53-

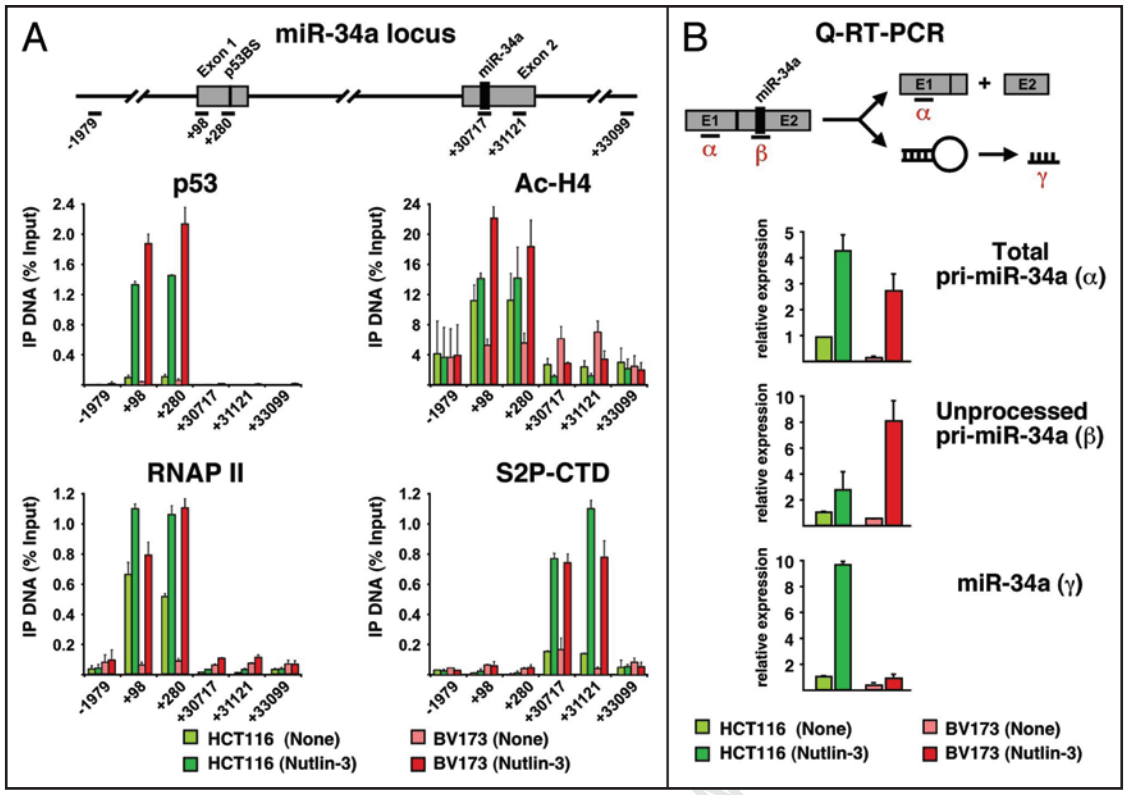

Figure 5. Unequal pri-miR-34a processing among cancer cell types. (A) ChIP analysis demonstrates effective RNAP II activation at the *miR-34a* locus in both HCT116 and BV173 cells. (B) Q-PCR analysis of pri-miR-34a expression reveals its reduced processing in BV173 cells. RNA was extracted before and 16 h after Nutlin-3 treatment and analyzed by standard Q-RT-PCR with amplicons recognizing total pri-miR-34a (α) or unprocessed pri-miR-34a (β). Levels of mature miR-34a were analyzed by Taqman assays (γ). Error bars indicate standard deviations over three independent experiments.

dependent apoptosis in cancer cells for their selective elimination is a top priority in cancer research. However, these efforts are hampered by our ignorance about the mechanisms that determine the cellular response to p53 activation. Most efforts have focused on mechanisms of gene selectivity within the p53 transcriptional program (reviewed in ref. 29). One view proposes that p53 binds selectively to target genes as instructed by p53 post-translational modifications and/or p53-interacting proteins. An alternative model postulates that gene selectivity is achieved at regulatory steps subsequent to p53 binding to chromatin by gene- and/or stimulus-specific transcriptional coregulators. Here, we demonstrate that the p53 transcriptional response can be strongly qualified by yet a different set of p53-independent mechanisms acting at the epigenetic and post-transcriptional levels. As we understand more about this enormous regulatory diversity, we will be able to envision new therapeutic strategies to harness the power of p53 in the clinic.

Materials and Methods

Cell culture, FACS analysis, western blots, ChIP assays and Q-RT-PCR reactions. Cell culture, FACS analysis, Western Blots, ChIP assays and Q-RT-PCR reactions were performed as previously described.[17,30] Information about antibodies and oligonucleotides can be found in Supplementary Materials.

Nuclear run-on assays. Cell cultures were treated with 10 μM Nutlin-3 (Cayman Biochemicals) or left untreated for 8 h before nuclei isolation as described previously.[31] Nuclei were resuspended in 100 μl of Reaction Buffer[31] and incubated at 30°C for 15 min. Labeled RNA was isolated and hybridized to nitrocellulose filters onto which the p21 cDNA had been blotted. Filters were washed as described and analyzed by PhosphoImager.

mRNA half-life studies. Cell cultures were treated with 10 μM Nutlin-3 or left untreated for 8 h followed by the addition of the transcription inhibitor actinomycin D (ActD, Sigma) at 7.5 μg/ml. Total RNA was isolated at several time points after the addition of ActD and subjected to Q-RT-PCR reactions as described.[30]

Methylation-specific PCR. Genomic DNA was isolated and modified by sodium bisulfite treatment as previously described.[32] Methylation-specific PCR analysis was done using alternative primer sets covering CpG dinucleotides in the *14-3-3*σ promoter region (see Suppl. Table S4) and 50 ng of bisulfite-treated gDNA. For gel electrophoresis analysis, PCR reactions were stopped at 37 cycles and run on EtBr-stained agarose gels. For Q-PCR quantification, reactions were run along standard curves with known amounts of PCR products obtained from either HCT116 cells (unmethylated) or LNCaP cells (methylated) and analyzed by SYBR green incorporation.

Analysis of miR-34a expression. Levels of mature *miR-34a* were measured using Taqman MicroRNA Assays (Applied Biosystems). Small RNAs were purified using the miRVana miRNA Isolation Kit (Ambion). Expression was normalized to that of *U6* snRNA. Levels of total and unprocessed *pri-miR-34a* were measured by standard Q-RT-PCR using primer pairs against sequences in Exon 1 and around Drosha cleavage sites in Exon 2, respectively, and normalized to 18s rRNA.

Knock-down of miR-34a activity. miR-34a activity was inhibited in cultures of HCT116 $p21^{-/-}$ $14\text{-}3\text{-}3\sigma^{-/-}$ using a specific Anti-miR™ oligonucleotide (Ambion) targeting mature miR-34a. Control or anti-miR-34a oligonucleotides were transfected with siPORT™ NeoFX™ transfection reagent (Ambion) following manufacturer's instructions. Media was replenished at 24 h post-transfection -/+ 10 μM Nutlin-3 and cells were harvested 96 h later for apoptotic index assays.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/ParisCC7-15-Sup.pdf

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