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Extra View

Stimulus-Specific Transcriptional Regulation Within the p53 Network

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ABSTRACT

The p53 transcriptional network is composed of hundreds of effector genes involved in varied stress-response pathways, including cell cycle arrest and apoptosis. It is not clear how distinct p53 target genes are differentially activated to trigger stress-specific biological responses. We analyzed the p53 transcriptional program upon activation by two DNA-damaging agents, UVC and doxorubicin, versus the non-genotoxic molecule Nutlin-3. In colorectal cancer cells, UVC triggers apoptosis, doxorubicin induces transient cell cycle arrest followed by apoptosis, and Nutlin-3 leads to cell cycle arrest with no significant apoptosis. Quantitative gene expression analysis allowed us to group p53 target genes into three main classes according to their activation profiles in each scenario. The CDK-inhibitor p21 was classified as a Class I gene, being significantly activated under cell cycle arrest conditions (i.e., doxorubicin and Nutlin-3) but not during UVC-induced apoptosis. Chromatin immunoprecipitation analysis of the p21 locus indicates that the level of p53-dependent transcription is determined by the effects of stimulus-specific transcriptional coregulators acting downstream of p53 binding and histone acetylation. In particular, our analysis indicates that the subunits of the CDK-module of the human Mediator complex function as stimulus-specific positive coregulators of p21 transcription.

ABBREVIATIONS

APAF1, apoptotic peptidase activating factor 1; CDK8, cyclin dependent kinase 8; GADD45, growth arrest and DNA-damage-inducible; GTF, general transcription factor; HDM2, human homolog of mouse double minute 2; Q-RT-PCR, quantitative-reverse transcription-polymerase chain reaction; TBP, TATA-binding protein; TAF1, TBP associated factor 1; TFIIA, RNA polymerase II transcription factor II A; TFIIB, RNA polymerase II transcription factor II B; UVC, ultraviolet light C

INTRODUCTION

The p53 transcription factor functions as a signaling hub of pivotal importance for cellular stability and transformation. Multiple upstream signaling pathways lead to p53 activation, including those induced by potentially oncogenic events, such as DNA damage, oncogene hyperactivation, telomere erosion, nutrient deprivation and hypoxia. Activated p53 participates in the orchestration of varied cellular responses to these stressful stimuli, such as cell cycle arrest, apoptosis or senescence, which in turn generates a strong selective pressure to inactivate this protein during cancerous growth. Accordingly, p53 is mutated in about half of all human cancers and is considered to be the most commonly mutated tumor suppressor gene.[1-3]

The p53 protein functions primarily as a transcriptional regulator. Recent analysis of p53 binding to chromatin on a genome-wide scale revealed that the total number of p53 target genes may reside in the several hundreds, including protein-coding genes as well as non-coding RNAs.[4,5] Seminal microarray experiments clearly defined the existence of stimulus-specific p53-dependent transcriptional programs. Zhao et al. demonstrated that, depending on the way p53 was activated (e.g., DNA damage caused by ionizing versus non-ionizing radiation), different subsets of p53 target genes were activated.[6] In these experiments, the set of p53 target genes activated in all signaling scenarios tested represented a very small fraction of the total p53 transcriptional program.

These observations reiterate one of the most challenging questions in the p53 field: What are the mechanisms driving differential regulation of p53 target genes? In the past few years, several models have been put forward to explain this phenomenon. One model proposes that post-translational modifications of p53 play a critical role in this process. For example, phosphorylation and/or acetylation of specific residues in the p53 protein have been shown to enhance its ability to activate pro-apoptotic genes in certain experimental conditions.[7,8] However, it is not known how specific p53 post-translational modifications may affect its transactivation potential in a gene-specific manner. Another model proposes that the ability of p53 to recognize response elements on specific genes is modulated by p53 'cofactors' such as the p53-binding protein ASPP1 or the p53-related proteins, p63 and p73.[9,10] Other views would suggest that the quality of the p53-dependent transcriptional program is affected by other transcriptional regulators acting in parallel pathways and modulating subsets of p53 target genes. For example, the oncogenic transcription factor c-myc promotes the apoptotic response by specifically repressing transcription of the *p21* gene, a key mediator of p53-dependent cell cycle arrest.[11] A modest review of the literature suggests that not one single model can reconcile all the observations generated so far (for an excellent review of these issues see ref. 12). It is likely that multiple mechanisms, both known and undiscovered, contribute to define the ultimate p53-dependent transcriptional profile provoked by a given p53-activating stimulus. In this report, we summarize recent findings from our group as well as present new data that generate further insight into the mechanisms of stimulus-specific regulation of p53-dependent transcription.[12-14] We found that the ultimate activation status of p53 target genes can be defined at steps downstream of p53 binding to chromatin and p53-mediated recruitment of histone-modifiying activities. Our studies reveal the existence of stimulus- and gene-specific transcriptional coregulators acting within the p53 network, including subunits of the Mediator complex, as well as specific general transcription factors (GTFs) and elongation factors.

MATERIALS AND METHODS

Cell culture, FACS Analysis of Cell Cycle Profile, Q-RT-PCR, Western Blots and ChIP assays were performed as described in refs. 13 and 14. Primer sequences for *HDM2* Q-RT-PCR are described in ref. 14. Primer sequences for Q-RT-PCR analysis of *APAF1* and *GADD45* are: *APAF1* sense: 5'-CCTAGGCGCAAAGGCTTG-3'; *APAF1* antisense: 5'-GATCTTTCTCTCTCTGAGCTGTCAAC-3'; *GADD45* sense: 5'- ATTCTCGGCTGGAGAGCAGA-3'; *GADD45* Antisense: 5'-GCATCCCCCACCTTATCCAT-3'.

RESULTS

Stimulus-specific regulation of p53 target genes. In order to study the mechanisms driving stimulus-specific regulation of p53 target genes, we set out to investigate the p53 transcriptional response in a single cell type subjected to distinct p53-activating stimuli. We chose to employ the well-characterized human colorectal carcinoma cell line HCT116 and three well-known p53-activating agents: Ultra Violet Light C (UVC), the chemotherapeutic agent doxorubicin, and Nutlin-3, the small molecule inhibitor of the p53 repressor HDM2.[15] When treated with 25 J/m^2 of UVC, 0.5 μM doxorubicin or 10 μM Nutlin-3, HCT116 cells display identical accumulation of p53 protein levels, both in terms of overall fold induction and kinetics of activation.[12,14] However, only UVC and doxorubicin induce DNA-damage-mediated post-translational modifications of p53, such as serine phosphorylation, which are not observed in Nutlin 3-treated cells.[12,14,16] Interestingly, these three p53-activating agents induce different cellular responses in this cell line (Fig. 1A). UVC induces apoptosis, as evidenced by a significant increase in the subG1 population at 48 h post-irradiation. UVC-induced apoptosis is partially p53-dependent, as apoptosis is decreased, but not absent, in the HCT116 p53$^{-/-}$ isogenic cell line.[14] Doxorubicin induces a well-characterized cell cycle arrest response, with most cells stalling at the G_2/M boundary.[13,17] At later time points (48 h and beyond), doxorubicin-treated cultures show clear signs of apoptosis (Fig. 1A).[17] In this scenario, p53 plays an anti-apoptotic role, as the lack of p53 results in less cell cycle arrest and increased apoptosis.[17] Finally, Nutlin-3 induces a cell cycle arrest response with cells accumulating in both G_1 and G_2/M. This response is fully dependent on p53, as it is not observed in HCT116 p53$^{-/-}$ cells, and is also completely reversible, as cells resume proliferation once Nutlin-3 is removed from the cell culture media.[14,16,18] Importantly, there is no significant apoptosis in HCT116 cells upon Nutlin-3 treatment (Fig. 1A). In summary, UVC, doxorubicin and Nutlin-3 induce different cellular responses involving p53 activation.

Next, we aimed to identify differences in the p53-dependent transcriptional program evoked by UVC, doxorubicin and Nutlin-3. Toward this end, we employed Q-RT-PCR assays to measure induction of known p53 target genes in each scenario. Analysis of more than twenty p53 target genes allowed us to identify three different groups of genes based on their distinct expression profiles (Fig. 1B). One group, represented here by *HDM2* and referred hereto as Class I, was maximally activated in response to Nutlin-3, significantly activated by doxorubicin and very modestly activated by UVC. The *p21* gene, a key contributor to p53-dependent cell cycle arrest, also belongs to this class and shows an expression profile identical to that of HDM2 (data not shown, see refs. 13 and 14). Class II genes, represented here by *APAF1*, were maximally induced by doxorubicin, modestly induced by Nutlin-3 and not induced at all by UVC. Finally, Class III genes were activated to a similar extent by all three agents, as illustrated here by *GADD45*. These results confirmed the flexibility of the p53 transcriptional program and allowed us to begin more mechanistic studies. We decided to focus on the Class I gene *p21*. Importantly, *p21* protein levels correlated tightly with the cell fate choice adopted in our paradigm, displaying significant accumulation in arrested cells (i.e., upon treatment with doxorubicin or Nutlin-3) and no expression in cells undergoing UVC-induced apoptosis (Fig. 1C).

Stimulus-specific transcriptional complexes acting on the p21 locus. We hypothesized that differences in *p21* mRNA accumulation were due to stimulus-specific transcriptional events at the *p21* locus. To test this, we performed high-resolution, quantitative Chromatin Immunoprecipitation assays (ChIP) using antibodies against p53, histone modifications, RNAP II, GTFs and Mediator. Table 1 shows a summary of our ChIP analysis. Some of the primary data for this Table can be found in refs. 12–14.

ChIP assays with p53 antibodies showed that p53 binding to the response elements found in the *p21* locus increases significantly and to similar extent upon all three stimuli, mirroring total p53 accumulation by Western blot. Furthermore, p53-mediated acetylation of histone H3 lysine 9 and histone H4 lysines 5, 8, 12 and 16 are also equivalent

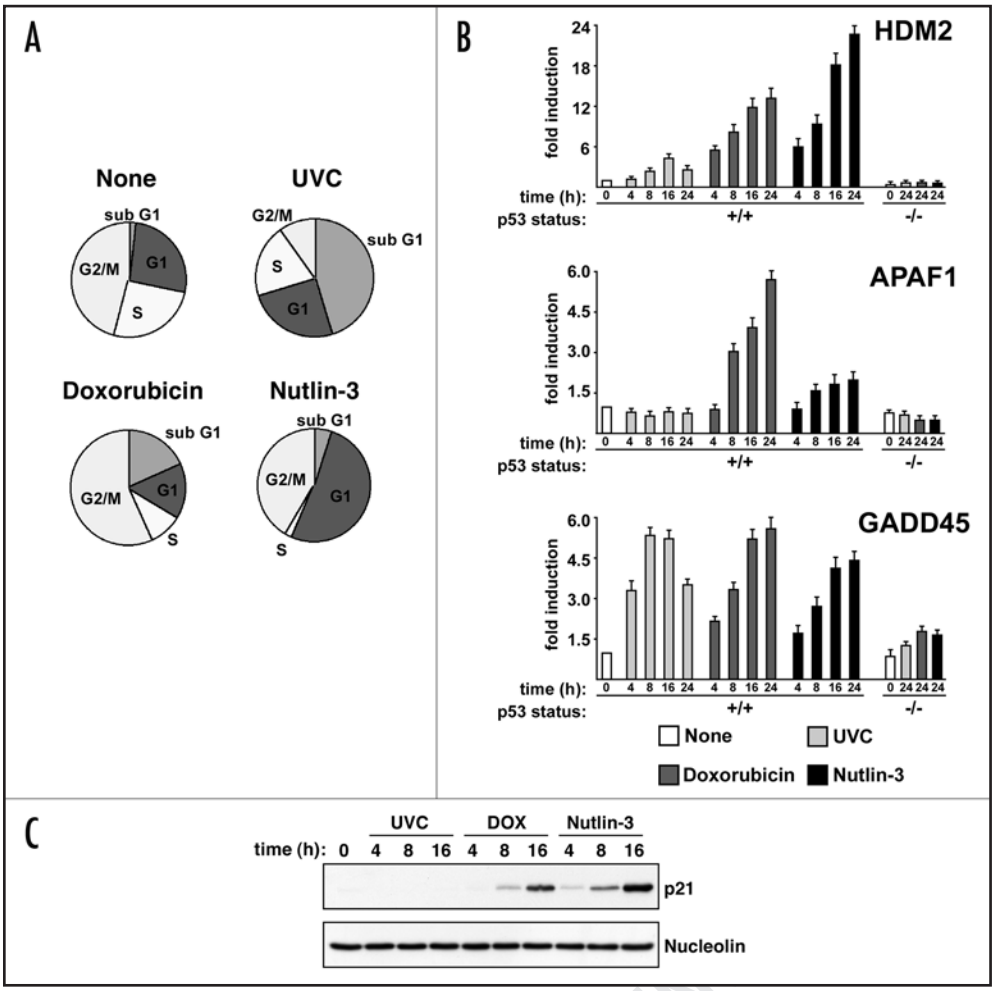

Figure 1. Stimulus-specific regulation of p53 target genes. (A) Distinct p53-activating stimuli induce different cellular responses. Subconfluent cultures of HCT116 $p53^{+/+}$ cells were left untreated (None) or treated with 25 J/m^2 of UVC, 0.5 μM doxorubicin or 10 μM Nutlin-3 and harvested 48 h post-treatment for analysis of cell cycle profile as described in ref. 14. Cells were fixed and their DNA content was determined by staining with propidium iodide and FACS analysis. Pie charts display the percentage of cells in different phases of the cell cycle or undergoing apoptosis (subG1). (B) Stimulus-specific induction of p53 target genes. Cells were treated as in (A) and harvested at 4, 8, 16 and 24 h post-treatment for RNA isolation and Q-RT-PCR analysis with primers specific to human *HDM2*, *APAF1* and *GADD45* mRNAs. HCT116 $p53^{-/-}$ cells were used as controls. Values were normalized to those of 18S rRNA and expressed as fold induction over untreated $p53^{+/+}$ cells. Data represents the average of three independent experiments. Error bars denote standard deviation. (C) Differential expression of p21 correlates with cell fate choice. Cells were treated as in (A) and harvested for preparation of protein extracts at the indicated time points before Western blot analysis of p21 expression. Nucleolin serves as a loading control.

in each scenario. In contrast, RNAP II activation is clearly distinct in UVC- versus doxorubicin- and Nutlin-3-treated cells. Significant levels of paused RNAP II are found at the *p21* proximal promoter before stimulation, indicating that this gene is regulated at post-recruitment steps.[12,13] After UVC irradiation, pre-loaded RNAP II is lost after a transient wave of transcription.[12] In contrast, activation of p53 by doxorubicin or Nutlin-3 leads to a sustained increase of RNAP II occupancy in the *p21* intragenic region, which is indicative of active elongation and productive transcription. When using antibodies recognizing the Ser5-phosphorylated form of the RNAP II C-terminal domain repeats (S5P-CTD), a significant increase in the low basal signals is observed only after doxorubicin and Nutlin-3. Ser5 phosphorylation peaks at the 5' end of the gene and is thought to mediate promoter escape by RNAP II.[19] Differential activation of RNAP II at the *p21* locus is also evident in ChIP assays using antibodies against the Ser2-phosphorylated form of the RNAP II CTD (S2P-CTD). S2P-CTD phosphorylation accumulates toward the 3' end of genes and is a marker of active elongation.[19] Significant increases in S2P-CTD signals at the *p21* locus are observed after treatment with doxorubicin or Nutlin-3, but not upon UVC irradiation. Overall, these results indicate that the ultimate activation status of the *p21* locus is not determined by p53 binding to chromatin or recruitment of histone acetyl-transferases, and that it may instead be defined at other regulatory steps. Additional results from our group indicate that this conclusion is not specific to the *p21* locus and it applies to many p53 target genes analyzed so far. To generate further insight into this issue, we analyzed the association of transcriptional coregulators acting at the *p21* proximal promoter, including several GTFs and Mediator. These studies revealed the assembly of stimulus-specific transcriptional complexes at the *p21* locus. The results from this analysis and their interpretation can be itemized as follows:

(1) Overall, GTFs were easily detectable at the *p21* proximal promoter before p53 stimulation, consistent with the observed pre-loading of RNAP II discussed above.[12-14] In contrast, binding of Mediator subunits was less evident before p53 activation. The fact that significant amounts of RNAP II were present at the *p21* promoter before association of the Mediator complex suggests that the latter may regulate post-initiation steps at this locus.

(2) TFIIA seems to sense activation signals arising from p53 activation in every scenario, as it displays enhanced recruitment to the proximal promoter of *p21* after all three stimuli. The lack of correlation between recruitment of TFIIA and transcriptional activity suggests it is not a key determinant of the ultimate activation status of *p21*.

(3) The TATA-binding protein (TBP), a subunit of the TFIID complex, is present at the *p21* proximal promoter before p53 activation, maintains its association following UVC and doxorubicin treatment and increases only upon Nutlin-3 treatment. Increased TBP binding may allow for the maximum activation observed during Nutlin-3 treatment.

(4) Enhanced recruitment of the TBP-associated factor 1 (TAF1) is observed only after UVC irradiation. The distinct behaviors of TBP and TAF1 reveal a degree of plasticity in the assembly of the TFIID complex. The fact that TAF1 is the only protein identified

Table 1 **ChIP analysis reveals the assembly of stimulus-specific transcriptional complexes on the *p21* locus**

Epitope	Location	None	UVC	Doxorubicin	Nutlin-3
p53	E	-/+	+++	+++	+++
S15P-p53	E	-	+++	+++	-
Ac-H3	E	-/+	+++	+++	+++
Ac-H4	E	-/+	+++	+++	+++
Total RNAP II	P	++	-	+++	+++
Total RNAP II	G	-	-	+++	+++
S5P-CTD	P	+	-	+++	+++
S2P-CTD	G	-	-	+++	+++
TFIIA	P	+	+++	+++	+++
TBP (TFIID)	P	+	+	+	+++
TAF1 (TFIID)	P	+	+++	+	+
TFIIB	P	+	-	+++	++
TFIIF	P	+	+	+++	+++
MED1	E	-	+++	+++	+++
CDK8	P	-/+	-/+	++	+++
Cyclin C	P	-/+	-/+	ND	+++
MED12	P	-/+	-/+	ND	+++

Subconfluent cultures of HCT116 $p53^{+/+}$ cells were left untreated (None) or treated with 25 J/m2 of UVC, 0.5 μM doxorubicin or 10 μM Nutlin-3 for 8 hours before harvest. ChIP assays were performed with antibodies against the indicated epitopes and ChIP-enriched DNA was analyzed by Q-PCR using amplicons covering different regions of the p21 locus. E, Enhancer, amplicon covering the high affinity p53 binding site and referred as to - 2283 in refs. 13 and 14. P, Promoter, amplicon covering the transcription start site and core promoter elements and referred to as -20 in refs. 13 and 14. G, Gene, amplicon located at the 3′ end of the p21 intragenic region and referred as to +8566 in refs. 13 and 14. Maximum signals observed for each epitope are marked by (+++).

so far that is recruited to the *p21* promoter preferentially upon UVC irradiation leads us to hypothesize that it may function as a transcriptional repressor in this scenario. Current efforts in our lab aim to test this notion.

(5) TFIIB binding increases significantly with doxorubicin, more modestly with Nutlin-3 and actually decreases with UVC. TFIIB behavior suggests that it is a key positive regulator of *p21* transcription. Its decreased binding following UVC may explain the lack of sustained transcription in this scenario. However, the fact that TFIIB binding is maximal upon treatment with doxorubicin rather than Nutlin-3 indicates that it is not the ultimate indicator of *p21* transcriptional activity.

(6) TFIIF recruitment increases with doxorubicin and Nutlin-3, but not with UVC. This behavior makes TFIIF a better predictor of the transcriptional status of the *p21* locus than TFIIA or TFIIB. Of note is the observation that while the association of other GTFs is restricted to the proximal promoter, TFIIF is also present several kilobases into the 5' end of the intragenic region, perhaps indicative of its role in elongation control.[13,20]

(7) MED1, a 'core' Mediator subunit known to interact directly with p53, is recruited upon treatment with all three stimuli.[21] The recruitment profile of MED1 at the *p21* locus is interesting in that the increased association of MED1 occurs at both the p53 enhancer elements and at the proximal promoter, and to a lesser extent the 5' end of the intragenic region.[14] This occupancy profile supports the idea that Mediator acts as a molecular bridge to connect sequence-specific DNA binding proteins, often acting from distal elements, with the basal transcription machinery acting at core promoters. However, it is important to take note that the recruitment of the core Mediator complex does not determine the ultimate activation status of the *p21* locus.

(8) Unlike MED1, subunits of the CDK-Module of Mediator (CDK8, Cyclin C and MED12) are recruited to the *p21* locus only during conditions of activation.[14] The CDK-module is preferentially recruited to the proximal promoter of *p21*. In fact, of all of the transcriptional coregulators analyzed, the level of recruitment of CDK8 most closely correlates with the level of *p21* transcriptional activation. This suggested that CDK8, a protein often associated with transcriptional repression at other human loci, functions instead as a positive coregulator in this scenario.[22,23] We tested this hypothesis using anti-CDK8 siRNAs and reporter assays and confirmed that CDK8 acts as a positive coregulator of *p21* and *HDM2* expression.[14]

DISCUSSION

Our systematic analysis of transcriptional complexes acting on the *p21* locus under different signaling scenarios has revealed an unexpected role for transcriptional coregulators previously considered to act in a 'generic' fashion. Instead, many of them clearly act in a stimulus-specific manner. Our current efforts are aimed to: (1) identify other stimulus-specific coregulators acting on the *p21* locus, (2) elucidate which stimulus-specific factors are determinants of RNAP II activity at the *p21* locus versus those whose behavior is epiphenomenal to RNAP II activity and (3) analyze other Class I genes, as well as Class II and Class III genes, and identify common and/or differential regulatory patterns. Our analysis of p53 target genes beyond *p21* has begun to generate some interesting observations. For example, the pattern of recruitment of transcriptional coregulators to the promoter of *HDM2*, another Class I gene, is very similar to that observed for *p21*. Specifically, stimulus-specific recruitment of TFIIB, TAF1 and CDK8 is identical at both loci. Particularly interesting are Class II genes (e.g., *APAF1*),

which are not maximally induced by Nutlin-3 treatment, suggesting that their full activation may require additional events beyond p53 stabilization, such as p53 phosphorylation or the action of other stress-induced transactivators. On the other hand, only Class III genes are effectively induced upon UVC irradiation as compared to Nutlin-3 and doxorubicin treatment. We hypothesize that UVC-mediated DNA damage generates repressive signals that block transactivation of Class I and Class II genes, but not of Class III genes. Of note, doxorubicin and UVC produce different types of DNA damage thus leading to activation of distinct downstream signaling pathways. Doxorubicin is a drug thought to act mostly trough inhibition of topoisomerase II activity, thus producing accumulation of double-stranded breaks and activation of the ATM signaling pathway.[24-26] In contrast, UVC irradiation produces pyrimidine dimers, which activate the ATR signaling pathway.[26] It is unclear how ATM- and/or ATR-dependent signaling pathways may affect the activity of specific transcriptional coregulators acting within the p53 network. It would be of great interest to determine if DNA damage-induced signaling modulates the activity of Mediator or GTF subunits.

The fact that p53 target genes show distinct stimulus-specific patterns of activation suggests the existence of gene-specific regulatory events within the network. Comparative analysis of the transcriptional complexes acting on different p53 target genes may lead to the identification of gene-specific coregulators. In this regard, we recently discovered that CDK9, a positive elongation factor once considered to be essential for all transcription, is required for transcriptional activation of some, but not all, p53 target genes. Current efforts in our lab aim to elucidate how the combinatorial use of the so-called 'transcriptional CDKs' (i.e., CDK7, CDK8, CDK9, CDC2L6 and CDK11) may provide flexibility and specificity to the p53 transcriptional program. In summary, we propose that combinatorial use of both stimulus-specific and gene-specific coregulators within the p53 network generates the diversity required to fine tune the p53 transcriptional response to the changing needs of the cell. Deciphering this complex regulatory web will allow the scientific community to envision novel therapeutic strategies aimed to evoke a desired p53-dependent biological response.

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