Gene-specific requirement for P-TEFb activity and RNA polymerase II phosphorylation within the p53 transcriptional program

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Activation of the p53 pathway mediates cellular responses to diverse forms of stress. Here we report that the p53 target gene *p21^{CIP1}* is regulated by stress at post-initiation steps through conversion of paused RNA polymerase II (RNAP II) into an elongating form. High-resolution chromatin immunoprecipitation assays (ChIP) demonstrate that p53-dependent activation of *p21^{CIP1}* transcription after DNA damage occurs concomitantly with changes in RNAP II phosphorylation status and recruitment of the elongation factors DSIF (DRB Sensitivity-Inducing Factor), P-TEFb (Positive Transcription Elongation Factor b), TFIIF, TFIIF, and FACT (Facilitates Chromatin Transcription) to distinct regions of the *p21^{CIP1}* locus. Paradoxically, pharmacological inhibition of P-TEFb leads to global inhibition of mRNA synthesis but activation of the p53 pathway through p53 accumulation, expression of specific p53 target genes, and p53-dependent apoptosis. ChIP analyses of *p21^{CIP1}* activation in the absence of functional P-TEFb reveals the existence of two distinct kinases that phosphorylate Ser5 of the RNAP II C-terminal domain (CTD). Importantly, CTD phosphorylation at Ser2 is not required for *p21^{CIP1}* transcription, mRNA cleavage, or polyadenylation. Furthermore, recruitment of FACT requires CTD kinases, yet FACT is dispensable for *p21^{CIP1}* expression. Thus, select genes within the p53 pathway bypass the requirement for P-TEFb and RNAP II phosphorylation to trigger a cellular response to inhibition of global mRNA synthesis.

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The p53 tumor suppressor acts as a signaling node of critical importance for cellular stability and transformation. p53 enables cells to undergo cell cycle arrest, cell death, or senescence in response to various forms of cellular stress including DNA damage, oncogene hyperactivation, and hypoxia. The ability of p53 to prevent genetic instability and uncontrolled proliferation generates a selective pressure to inactivate this protein during tumor growth, and as a result p53 is the most commonly mutated tumor suppressor gene (Vogelstein et al. 2000; Vousden 2002). p53 is a sequence-specific DNA-binding protein that functions primarily as a transcriptional regulator (Kern et al. 1991; Farmer et al. 1992; Zhao et al. 2000). Induction of cell cycle arrest by p53 is mediated

mostly by transcriptional activation of the *p21/cip1/waf1/cdkn1a* gene (referred here to as *p21*), an inhibitor of cyclin-dependent kinases (el-Deiry et al. 1993). The molecular events that lead to p53-dependent apoptosis are more complex and occur through p53 induction of numerous apoptotic genes that activate the death-receptor pathway (*Fas/APO1*, *DR5/Killer*) or the mitochondrial apoptotic pathway (*PUMA*, *Bax*, *Noxa*) (Vousden and Lu 2002). However, there is also evidence for transcription-independent mitochondrial functions of p53 in apoptosis (Erster and Moll 2004; Chipuk et al. 2005).

Control of gene expression in eukaryotic cells involves regulatory events at multiple transcriptional and post-transcriptional stages. The elongation phase of transcription has gained increasing importance as numerous positive and negative elongation factors have been identified (Sims et al. 2004). Moreover, recent evidence indicates that elongation is tightly linked to post-transcriptional events such as mRNA capping, splicing, polyadenyla-

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tion, and nuclear export (Hirose and Manley 2000; Proudfoot et al. 2002; Bentley 2005). The C-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAP II) plays a critical role in the integration of these events. Several elongation factors, 5' and 3' RNA processing factors, and histone-modifying activities interact with this domain in an orchestrated fashion (Hirose and Manley 2000; Gerber and Shilatifard 2003; Bentley 2005; Kizer et al. 2005; Xiao et al. 2005). The CTD is comprised of multiple heptad repeats (YSPTSPS motifs, 52 in mammals) each containing two main phospho-acceptor sites, Ser2 and Ser5. Phosphorylation of Ser5 occurs primarily at the 5' region of genes, whereas Ser2 phosphorylation accumulates on the elongating polymerase and therefore is more abundant at the 3' region of genes (Komarnitsky et al. 2000). Several CTD kinases have been identified, with CDK7 and CDK9 being the most prominent among them. CDK7 is part of the general transcription factor TFIIF and catalyzes Ser5 phosphorylation (Trigon et al. 1998). CDK9 is the catalytic subunit of the Positive Transcription Elongation Factor b (P-TEFb), and its site specificity is somewhat controversial since it seems to phosphorylate preferentially Ser2 or Ser5 depending on the experimental paradigm used (Garber et al. 2000; Ramanathan et al. 2001; Shim et al. 2002; Ahn et al. 2004; Ni et al. 2004). CTD phosphorylation seems to play a critical role in mRNA synthesis, and its alternate phosphorylation patterns may direct the transitions between consecutive stages of this process. For example, Ser5 phosphorylation accompanies the transition from initiation to elongation and may mediate recruitment and activation of the 5' capping enzyme (Ho and Shuman 1999), whereas Ser2 phosphorylation is implicated in recruitment of 3' RNA processing factors and efficient 3' cleavage and polyadenylation (Ahn et al. 2004; Ni et al. 2004).

Similar to the regulation of transcriptional initiation, it is likely that control of elongation occurs through the combinatorial use of elongation factors and that gene- and signaling-specific elongation factors exist. Here we present evidence that CTD kinase activities and RNAP II phosphorylation are differentially required for expression of distinct p53 target genes. Our studies show that RNAP II is regulated at a post-initiation stage on the *p21* gene. Paused RNAP II assembles on the *p21* promoter before cellular stress and is converted to the elongating form upon stress-induced p53 activation (Espinosa et al. 2003). This process is accompanied by recruitment of several positive elongation factors and changes in CTD phosphorylation. High-resolution quantitative chromatin immunoprecipitation (ChIP) assays reveal important differences in the site of recruitment and distribution of the elongation factors NELF (Negative Elongation Factor), DSIF (DRB Sensitivity-Inducing Factor), P-TEFb, TFIIF, TFIID, and FACT (Facilitates Chromatin Transcription) on the *p21* locus. Pharmacological inhibition of P-TEFb kinase activity by 5,6-di-chloro-1- β -D-ribofuranosyl-benzimidazole (DRB) produced several interesting observations: (1) P-TEFb activity is differentially required for activation of distinct p53 target genes; (2) at

least two distinct Ser5 kinases act consecutively on the *p21* locus based on DRB sensitivity and site of action; (3) CTD kinases are required for recruitment of the elongation factor FACT to the *p21* locus; (4) P-TEFb kinase activity, Ser2 phosphorylation, and FACT recruitment are dispensable for *p21* mRNA transcription, 3' processing, and accumulation in response to stress-induced p53 activation; and (5) global inhibition of transcription triggers a stress response that leads to p53-dependent apoptosis.

Results

Activation of the p53–p21 axis in response to DNA damage

The p53 pathway is activated by a plethora of stimuli including numerous DNA-damaging agents. This process is characterized in human colon carcinoma cells (HCT116) after exposure to the chemotherapeutic agent doxorubicin, which inhibits topoisomerase II, leading to DNA double-stranded breaks. As shown in Figure 1, doxorubicin treatment results in rapid accumulation of p53 protein and delayed appearance of p21 protein (Fig. 1A). Real-time RT-PCR analyses reveal that *p21* mRNA accumulation in HCT116 cells is clearly p53-dependent, since it is not observed in the isogenic cell line not expressing p53 (Fig. 1B; Bunz et al. 1998). A FACS analysis shows that doxorubicin treatment results in efficient cell cycle arrest of HCT116 cells in both the G1 and G2/M phases of the cell cycle (Fig. 1C). It has been clearly demonstrated that HCT116 cells lacking either p53 or p21 escape arrest and undergo apoptosis in response to doxorubicin (Bunz et al. 1998). In order to study the mechanisms of p53-dependent transcriptional activation of the *p21* gene, we established a high-resolution quantitative ChIP assay that enables us to generate detailed maps of protein occupancy on the *p21* locus at different stages of the transactivation process. The gene map in Figure 1D shows the most relevant features of this locus, including the two upstream p53-binding sites (high-affinity BS1 and low-affinity BS2), the transcription start site (+1), the exon–intron organization, the start (ATG) and stop codons (TAA), and the polyadenylation signal (AATAAA). The location of 20 amplicons used in real-time PCR quantification of the ChIP-enriched DNA is also indicated.

Transcriptional activation of p21 involves conversion of RNAP II from a paused to an elongating form concurrent with changes in CTD phosphorylation

ChIP analysis of the *p21* locus before and 8 h after doxorubicin treatment indicates that levels of chromatin-bound p53 mimic those of total cellular p53 (Fig. 2). p53 occupancy peaks at the high-affinity BS1, which binds up to three times more p53 than BS2. Importantly, no p53 is detected at any other region of the *p21* locus, including the proximal promoter (–20 amplicon), indicating that

Figure 1. Kinetics of activation of the p53–p21 axis in HCT116 cells in response to DNA damage. (A) Western blot showing accumulation of p53 and p21 proteins upon doxorubicin treatment (0.4 μ M). Nucleolin was used as a loading control. (B) Real-time RT–PCR analysis of p21 mRNA accumulation. Values were normalized to those obtained for 18S ribosomal RNA, and are expressed as fold induction over untreated HCT116 p53^{+/+} cells. (C) FACS analysis of cell cycle profile. HCT116 p53^{+/+} cells were harvested at indicated times after addition of doxorubicin, and their DNA content was determined by FACS. Pie charts display the percentage of cells in each stage of the cell cycle. (D) Linear up-to-scale map of the p21 locus showing the location of p53-binding sites (p53BS1 and p53BS2), the transcription start site (+1), exons and introns, the start codon (ATG), the stop codon (TAA), and the polyadenylation signal (AATAAA). The location of 20 amplicons used in real-time PCR quantification of ChIP-enriched DNA is also shown. Numbers indicate the relative position of the central base pair of the amplicon relative to the transcription start site. Amplicon sizes range between 50 and 75 bp.

p53 truly acts distally from its enhancer elements. ChIP assays using antibodies that recognize the N terminus of RNAP II reveal that large amounts of total polymerase are found at the p21 core promoter before stress induction (up to 100-fold higher signal than upstream regions) and that only trace amounts of RNAP II transit the gene before DNA damage. During activation, total RNAP II levels increase only modestly at the core promoter (~50%) but rise significantly in the coding region of the gene (10- to 20-fold). Interestingly, RNAP II seems to proceed well beyond the polyadenylation signal (spanning +8573 to +8578, covered by amplicon +8566) and dissociates from chromatin somewhere between 1.5 and 3 kb past the cleavage site. In contrast to other mammalian genes regulated by distal elements, RNAP II cannot be detected at the p53 enhancer elements either before or during activation, suggesting that no chromatin loop structures are formed between the enhancers and the proximal promoter (Hatzis and Talianidis 2002; Wang et al. 2005). Accumulation of RNAP II at the 5' region of the gene both before and after stress induction suggests that a pausing mechanism exists and that regulation of post-initiation steps may be important for p21 transactivation.

Phosphorylation of the RNAP II CTD has been implicated in several post-initiation regulatory steps (Kobor and Greenblatt 2002). ChIP assays with antibodies recognizing the phosphorylated form of Ser5 (S5P-CTD) reveal that Ser5 phosphorylated RNAP II peaks at the core promoter, is present throughout the transcription unit, and decays past the polyadenylation signal. Interestingly, Ser5 phosphorylation at the core promoter in-

creases much more than total RNAP II levels upon stress induction (up to three- to fourfold), suggesting that preloaded RNAP II is not fully phosphorylated on this residue before activation. A very different distribution pattern is observed using antibodies specific to phospho-Ser2 (S2P-CTD). In agreement with studies on yeast genes, Ser2 phosphorylation is not present at the core promoter and accumulates toward the 3' region of the gene (Komarnitsky et al. 2000). Evidently, a drop in phospho-Ser2 signal precedes that of total RNAP II signal (cf. +8566 and +9985 signals for all three RNAP II antibodies), indicating that CTD de-phosphorylation may come before RNAP II dissociation. Importantly, the clearly dissimilar distribution patterns of Ser5 and Ser2 phosphorylation argue strongly against the notion that anti-phospho-Ser2 antibodies cross-react significantly with phospho-Ser5 (cf. values for both antibodies at the –20 amplicon) (Jones et al. 2004), and indicate that these antibodies truly discriminate between distinct RNAP II species in our experimental paradigm. Finally, before activation, phosphorylated RNAP II is more easily detected in the body of the gene than total RNAP II, which could be attributed to the presence of up to 52 epitopes per molecule when using S5P-CTD and S2P-CTD antibodies.

Specific elongation factors are differentially recruited and distributed to unique regions within the p21 locus during stress-induced transcriptional activation

Previously we have demonstrated that, in addition to RNAP II, several components of the preinitiation com-

Figure 2. Distribution of RNAP II and its phospho-isoforms on the *p21* gene locus before and during p53-dependent transcriptional activation. ChIP assays were performed with protein extracts obtained from HCT116 p53^{+/+} cells before or 8 h after doxorubicin treatment with antibodies recognizing the p53 transactivation domain (p53), total RNAP II (RNAP II), or phosphorylated isoforms of Ser5 and Ser2 of the RNAP II CTD (S5P-CTD, S2P-CTD). ChIP-enriched DNA was quantified by real-time PCR using the indicated amplicons. Values are expressed as percentage of input DNA immunoprecipitated. The results shown are the average of at least eight independent PCRs from four separate immunoprecipitations from two independent cell cultures. All standard deviations are <15%. Asterisks indicate the positions of the transcription start site and polyadenylation signal.

plex (PIC) are preloaded onto the *p21* core promoter before activation and that their recruitment is modulated in a stress-specific manner (Espinosa et al. 2003). As shown in Figure 3, levels of TFIIB increase significantly at the *p21* core promoter during activation by doxorubicin treatment, whereas initial levels of the TFIID subunits TAF1 and TBP do not. Importantly, our high-resolution maps indicate that these three components of the PIC localize exclusively to the core promoter, do not accompany RNAP II into the coding region, and are not

detected at the distal p53 enhancer elements. Because expression of the *p21* gene is primarily controlled at the level of elongation, we tested for recruitment of several elongation factors of diverse function to the *p21* locus. First, we used antibodies against the NELF complex, which is known to mediate RNAP II pausing in a variety of experimental systems (Yamaguchi et al. 1999). Interestingly, the NELF-A subunit is detected at the *p21* core promoter before activation, and its levels remain unchanged upon doxorubicin treatment, suggesting that NELF may be involved in RNAP II pausing observed at the *p21* locus. Importantly, NELF distribution is restricted to the 5' region of the gene. Next, we used antibodies against the DSIF complex (SPT4/SPT5), which interacts with NELF to mediate RNAP II pausing (Yamaguchi et al. 1999). ChIP assays with anti-SPT5 antibodies reveal that DSIF has a distribution pattern almost identical to total RNAP II, supporting the notion that SPT5 functions as an adaptor that mediates interaction of different factors with RNAP II (Sims et al. 2004). It has been established that the negative impact of NELF-DSIF on transcriptional elongation can be relieved when SPT5 is phosphorylated by the catalytic subunit of the P-TEFb complex, CDK9 (Wada et al. 1998; Price 2000). Our experiments show that CDK9 is indeed recruited to the *p21* locus upon activation. Interestingly, CDK9 signals consistently peaked at the +182 amplicon, which indicates that P-TEFb is recruited to RNAP II after promoter escape. Importantly, CDK9 seems to accompany RNAP II throughout the transcription unit. Although the CDK9 distribution pattern is dissimilar from that of phospho-Ser2-CTD (Fig. 2), this does not conflict with the notion that CDK9 is a Ser2-CTD kinase, since the continuous presence of low amounts of CDK9 in the 3' region of the gene may catalyze accumulative phosphorylation of the CTD repeats. We then tested for recruitment of other positive elongation factors including TFIIF, TFIIF, and FACT. The levels of these three proteins increase significantly at the *p21* locus upon activation, but they display distinct occupancy profiles. Interestingly, RAP74 (TFIIF) and CDK7 (TFIIF) are recruited to the core promoter region and proceed into the transcription unit for only a few kilobases. Our assays could not detect significant amounts of these factors beyond 4–5 kb downstream of the transcription start site, suggesting that they dissociate from the elongating polymerase well before it reaches the 3' region of the gene. In contrast, SPT16 (FACT) seems to be loaded after promoter escape (note peak at +182 amplicon), and high levels are present throughout the coding region, showing maximum occupancy at the 3' end of the gene (near amplicons +7878 and +8566). This profile agrees with a role for FACT in chromatin remodeling during transcriptional elongation (Orphanides et al. 1999). Interestingly, SPT16 signals decay significantly right after the polyadenylation signal (+9985 amplicon), indicating that FACT dissociates from the chromatin before RNAP II. In summary, we demonstrate that several negative and positive elongation factors are present within the *p21* locus and display significant differences in their sites of recruit-

Figure 3. Recruitment of initiation and elongation factors to the *p21* locus during activation. ChIP assays were performed with protein extracts obtained from HCT116 cells before or 8 h after doxorubicin treatment with antibodies recognizing TFIIB, TAF1, TBP, NELF-A (NELF complex), SPT5 (DSIF complex), CDK9 (P-TEFb complex), RAP74 (TFIIF complex), CDK7 (TFIIH complex), and SPT16 (FACT complex). Quantification and replicates were performed as in Figure 2. Asterisks indicate the positions of the transcription start site and polyadenylation signal.

ment, stress-inducibility, and distribution patterns, all of which may reflect their diverse functions.

The DRB paradox: global inhibition of mRNA synthesis is concomitant with p53 activation and induction of select p53 target genes

In order to define the role of CTD kinases in transcriptional regulation by p53, we used the pharmacological inhibitor DRB, which effectively blocks CDK9 activity and, to a lesser extent, other CDKs (see Discussion). As shown in Figure 4A, exponentially growing HCT116 cells display approximately equal amounts of phosphorylated (IIo) and unphosphorylated (IIa) forms of RNAP II. DNA damage by doxorubicin does not affect total RNAP II phosphorylation, whereas DRB treatment leads to a clear shift toward the unphosphorylated form. Interestingly, global inhibition of transcription by DRB and other reagents triggers activation of the master stress sensor, p53 (Ljungman et al. 1999). As shown in Figure 4A, DRB is as potent an inducer of p53 as DNA damage by doxorubicin. Thus, DRB treatment puts p53 target genes in a conflicting scenario in which their key regulator, p53, is strongly active while a major positive elon-

gation factor in the cell is catalytically inactive. In fact, real-time PCR analysis of RNA expression reveals gene-specific effects of DRB treatment (Fig. 4B). As exemplified by the control genes *HPRT* and *SDHA* and in agreement with previous reports, most of the transcriptome is rapidly down-regulated by DRB treatment (Ljungman et al. 1999; Chao and Price 2001). In stark contrast, some p53 target genes including *p21* and *PUMA* are clearly activated upon DRB treatment in a p53-dependent manner to levels similar to those observed after DNA damage by doxorubicin. Interestingly, other p53 target genes such as *14-3-3σ* and *DR5/Killer* do not respond to DRB-induced p53 activation. Although p21 mRNA accumulation after DRB treatment is slightly diminished and delayed compared with doxorubicin treatment, which may indicate mild defects in elongation, these results suggest that a specific subset of p53 target genes bypass the requirement for CDK9 activity and/or RNAP II phosphorylation.

p21 transcriptional activation upon DRB-induced p53 activation occurs in the absence of RNAP II Ser2 phosphorylation and FACT recruitment

In order to understand how some p53 target genes can be induced even in the absence of active CDK9, we per-

Figure 4. Activation of the p53 pathway by DRB-mediated inhibition of global transcription. (A) Western blot showing the ratio of phosphorylated (IIo) to unphosphorylated (IIa) RNAP II in HCT116 cells treated with doxorubicin (0.4 μ M) or DRB (50 μ M). The same blot was also probed with p53 and actin antibodies. (B) Real-time PCR analysis of gene expression in response to doxorubicin or DRB treatment. HCT116 cells of different p53 status were harvested at the indicated times after treatment with doxorubicin (0.4 μ M) or DRB (50 μ M), total RNA was isolated, and RT-PCR was performed with primers specific to the indicated mRNAs. Values were normalized to those of 18S rRNA and are expressed as fold induction over untreated p53^{+/+} cells. *HPRT* and *SDHA* are housekeeping, non-p53 target genes. Results shown are the average of eight different PCRs from four cDNA preparations of two different RNA extractions.

formed ChIP analysis on the *p21* promoter in DRB-treated HCT116 cells. As shown in Figure 5, DRB treatment resulted in similar patterns of p53, total RNAP II, and CDK9 recruitment to the *p21* locus as compared with doxorubicin-induced DNA damage. Slightly lower p53 and total RNAP II levels bound to the *p21* promoter after DRB treatment correlate with somewhat lower *p21* mRNA expression levels (Fig. 4B). Importantly, CDK9 recruitment is not impaired by DRB treatment, indicating that CDK9 association with the elongation complex does not require CDK9 catalytic activity. Furthermore, these results reveal that CDK9-mediated phosphorylation of SPT5 is not required for RNAP II elongation on the *p21* gene. NELF and SPT5 also showed similar occupancy profiles in DRB- and doxorubicin-treated cells (data not shown). Interestingly, RNAP II phosphorylation patterns are clearly different during DRB-induced *p21* activation. Whereas Ser5-CTD phosphorylation still increases at the 5' region of the gene, the phospho-Ser5

signal drops below basal levels further downstream. These results prompt several observations: (1) At least two different Ser5-CTD kinases seem to function on the *p21* locus: a somewhat DRB-insensitive kinase acting at the 5' region of the gene and a DRB-sensitive kinase acting throughout the transcription unit. (2) Ser5-CTD phosphorylation by the 5' kinase is rapidly dephosphorylated after promoter escape. (3) Ser5-CTD phosphorylation in RNAP II molecules transiting to the 3' region of the gene is dispensable for *p21* mRNA accumulation. Remarkably, our ChIP analyses using phospho-Ser2 antibodies reveal that RNAP II transcribing the *p21* locus upon activation by DRB is completely unphosphorylated on this residue, in striking contrast to elongating RNAP II after doxorubicin induction. This result indicates that the Ser2-CTD kinase functioning on the *p21* gene is DRB sensitive and that Ser2 phosphorylation is also dispensable for *p21* mRNA accumulation. Finally, recruitment of the elongation factor FACT is clearly impaired by DRB treatment as seen by a decrease of SPT16 below basal levels throughout the *p21* coding region. Although it is formally possible that small amounts of SPT16 may still contribute to the passage of RNAP II through nucleosomes, these results suggest that SPT16 function is dispensable for RNAP II transcription through this locus and that FACT recruitment may require CTD kinase activities and/or RNAP II phosphorylation.

3' RNA processing of *p21* mRNA is not affected by the absence of RNAP II Ser2-CTD phosphorylation

RNAP II phosphorylation is implicated in several stages of the multistep mRNA synthesis process. Most recently, Ser2-CTD phosphorylation by CDK9 has been shown to be involved in 3' RNA processing in yeast, *Drosophila*, and *Xenopus* oocytes (Ahn et al. 2004; Bird et al. 2004; Ni et al. 2004). In agreement with our results, these studies demonstrated that CDK9 activity and Ser2-CTD phosphorylation are dispensable for RNAP II elongation. However, the immature mRNAs synthesized showed defects in 3' cleavage and polyadenylation, resulting in rapid turnover and an overall decrease in mRNA levels. Based on these findings, we investigated the status of *p21* mRNA processing in our experimental paradigm. As shown in Figure 6A, DRB treatment leads to increased levels of *p21* protein in HCT116 cells, indicating that the mRNA produced can be efficiently translated. The somewhat lower levels of *p21* protein produced in response to DRB compared with doxorubicin treatment may reflect differences in *p21* mRNA accumulation between the two treatments (Fig. 4B). To investigate *p21* mRNA 3' cleavage, we designed a PCR-based analysis of the *p21* mRNA 3' region. The PCR results shown in Figure 6B indicate that the *p21* mRNA produced in untreated and doxorubicin- or DRB-treated cells is cleaved somewhere between the sequences of reverse primers 2 and 3. This is exactly where the polyadenylation signal (AATAAA) and cleavage site (asterisk) are located as predicted by cDNA cloning (GenBank accession no. NM_000389). As a control, PCR analysis of

Figure 5. Altered patterns of RNAP II phosphorylation and elongation factor recruitment on the *p21* locus upon activation by DRB treatment. ChIP assays were performed with protein extracts obtained from HCT116 cells before or 8 h after treatment with doxorubicin (0.4 μ M) or DRB (50 μ M) with antibodies recognizing p53, total RNAP II (RNAP II), CDK9, phospho-Ser-5 CTD (S5P-CTD), phospho-Ser2-CTD (S2P-CTD), and SPT16 (FACT complex). Quantification and replicates were performed as in Figure 2.

genomic DNA generates all amplification products. Even though the PCR conditions are well beyond the linear range of amplification, no traces of extended transcripts are detected when using cDNA from DRB-treated cells. Finally, we tested for enrichment of *p21* mRNA in the poly(A)⁺ RNA population (Fig. 6C). Real-time PCR quantification reveals that *p21* mRNA is enriched ~20-fold in the poly(A)⁺ preparation over the total RNA fraction and that such enrichment does not change significantly in DRB-treated cells. On the other hand, polyadenylation of HPRT mRNA is strongly affected by DRB treatment, showing a decrease of approximately fivefold. In summary, these results demonstrate that 3' processing of *p21* mRNA does not require phosphorylation of RNAP II at Ser2-CTD.

Inhibition of global mRNA synthesis by DRB leads to p53-dependent apoptosis

Our results suggest that select p53 target genes, including *p21* and *PUMA*, can bypass the requirement for CTD kinase activities and RNAP II phosphorylation at Ser2 in order to be functionally expressed in response to p53 activation. This ability may allow cells to mount a transcriptional response to certain types of stress even when global mRNA synthesis is inhibited. In fact, activation of some p53 target genes has also been observed in response to global inhibition of transcription by ultraviolet light C (UVC) irradiation or actinomycin D treatment (Ljungman et al. 1999). Since DRB treatment triggers a different p53 transcriptional program than DNA damage by doxorubicin, we analyzed the response of HCT116 cells to p53 activation in this scenario. As shown in Figure 7A,

HCT116 cells treated with DRB undergo rapid and efficient p53-dependent apoptosis. Untreated HCT116 cells with or without p53 show a typical cell cycle profile with cells distributed equally in G1, S, and G2/M phases of the cell cycle. Within 24 h post-DRB treatment, HCT116 p53^{+/+} cell cultures show a significant increase in the sub-G1 population, which is characteristic of apoptosis. In Figure 7B, the apoptotic fate of the cell culture is confirmed by an observed increase in staining with annexin V, a marker for cell membrane rearrangements occurring during apoptosis. On the other hand, DRB-induced apoptosis is very much attenuated in HCT116 p53^{-/-} cells. These results suggest that p53-dependent transcriptional activation of certain apoptotic genes, such as *PUMA*, can trigger an apoptotic response even upon global inhibition of mRNA synthesis.

Discussion

Activation of the p53 pathway enables cells to respond to a plethora of stressful stimuli. In mammals, p53 is dispensable for normal growth and development, and p53 function would probably go unnoticed in a utopian stress-free biological system. As a first responder in the defense to stress, the p53 transcription factor is called to action when cells are subjected to scenarios that negatively affect their normal homeostasis. Some of these situations provide challenges for the normal action of canonical transcription factors. For example, p53 must regulate subsets of genes within its complex transcriptional program when cells are dividing out of control, when DNA is damaged, or when RNA synthesis is inhibited. Thus, it is likely that p53 uses unorthodox

Figure 6. Analysis of 3' RNA processing of p21 mRNA. (A) Western blot showing p21 protein accumulation in HCT116 cells in response to doxorubicin (0.4 μ M) or DRB treatment (50 μ M). (B) PCR analysis of p21 mRNA 3' cleavage. Sequence of the 3'-end region of the *p21* locus showing the location of the primers used as well as the polyadenylation signal (AATAAA) and the cleavage site (asterisk) as predicted by cDNA cloning (GenBank accession no. NM_000389). PCR was performed using total genomic DNA (gDNA) or cDNA from HCT116 p53^{+/+} cells before or 16 h after treatment with doxorubicin or DRB. (M) Molecular weight marker. (C) Real-time PCR analysis of p21 mRNA polyadenylation. Total and poly(A)⁺ RNA were prepared from HCT116 p53^{+/+} cells before and 16 h after DRB treatment. Equal mass of either preparation was used as template in RT-PCR reactions with primers specific to p21 or HPRT mRNAs. Relative quantification was achieved using standard curves of known amounts of total cDNA. The results shown are the average of six PCRs from two different RNA extractions.

mechanisms to perform under such compromised physiological conditions. Here we provide evidence that p53 is able to activate select target genes and trigger an apoptotic program in response to inhibition of CTD kinases, RNAP II phosphorylation, and mRNA synthesis. In this paradoxical scenario, global inhibition of mRNA synthesis leads to transcription of specific stress-response genes through p53 action.

Transcription elongation has long been thought of as merely the addition of ribonucleotides to the growing mRNA chain, with the most critical aspects of regulation occurring at the preinitiation and initiation steps. It is now evident that elongation is a multi-event process that is subject to numerous levels of control (Sims et al. 2004). Several elongation factors have been identified, but their requirement for gene expression *in vivo* is largely unknown. Here we provide evidence that *p21*, a key p53 target gene, is regulated at post-initiation steps. Before DNA damage, the *p21* promoter is preloaded with significant amounts of several components of the PIC, including RNAP II itself (Espinosa et al. 2003). Upon activation of p53 by DNA damage, paused RNAP II is converted to an elongating form, and this transition occurs concomitantly with recruitment of several elongation factors and changes in CTD phosphorylation. Quantitative high-resolution ChIP analysis of the *p21* locus has generated new insights into the function of known elongation factors. Their distinct occupancy profiles suggest that these factors act at different steps in the elongation process. NELF, a negative elongation factor, is re-

stricted to the 5' region of the gene, and its occupancy profile mimics that of paused RNAP II before *p21* activation, suggesting that it may, indeed, control RNAP II pausing at this promoter. During transcription of the *p21* gene, NELF levels do not decrease and accumulation of RNAP II in the promoter region is still evident, implying that RNAP II pausing is overcome but not absent during activation. SPT5, a component of the DSIF complex, displays occupancy profiles identical to total RNAP II, confirming the notion that DSIF is constitutively bound to RNAP II and acts as a docking module that allows interaction of other factors such as NELF. It has been demonstrated that release of NELF from the elongating complex requires SPT5 phosphorylation by CDK9 (Yamaguchi et al. 1999). Although CDK9 is recruited to the *p21* locus upon activation, it is unlikely that SPT5 phosphorylation by CDK9 is required to relieve RNAP II pausing at the *p21* promoter since inhibition of CDK9 by DRB does not prevent RNAP II from transcribing this gene. A recent study demonstrated that RNAP II can escape pausing from the *Drosophila* heat shock genes in the absence of CDK9 activity, confirming that SPT5 phosphorylation by CDK9 is not universally required for transcriptional elongation (Ni et al. 2004). Interestingly, CDK9 recruitment to the *p21* locus peaks at ~200 base pairs (bp) downstream of the transcription start site. The CDK9 occupancy profile suggests that it is loaded onto RNAP II after promoter escape and that it accompanies the elongation complex throughout the transcription unit. SPT16, a subunit of the elongation complex FACT,

Figure 7. p53-dependent apoptosis in response to DRB treatment. (A) FACS analysis of cell cycle profile. HCT116 p53^{+/+} cells were harvested at different times after addition of DRB (50 μ M), fixed, and stained with propidium iodide; DNA content was determined by FACS. Pie charts display the percentage of cells in each stage of the cell cycle. (B) Apoptotic index assay. Cells were treated and harvested as in A, stained without fixation with annexin V and propidium iodide (PI), and analyzed by FACS. Numbers indicate the percentage of cells in each quadrant.

also seems to be loaded after promoter escape, but unlike CDK9, its levels rise steadily toward the 3' region of the gene. On the other hand, the general transcription factors TFIIF and TFIIH are recruited to the transcription start site and proceed with the elongating RNAP II for a few kilobases. Our assays failed to detect significant amounts of these two factors in the 3' half of the transcription unit. This suggests that they function at earlier stages of the elongation process and dissociate from the elongation complex before it reaches the 3' region of the gene. Although both TFIIF and TFIIH have been shown to possess post-initiation functions, it is unclear how long they remain associated with the elongation complex. Previous insightful studies using a reconstituted basal transcription system and the Adenovirus Major Late promoter concluded that the general transcription factors TFIIB and TFIID remain bound to the core promoter, whereas TFIIF and TFIIH dissociate from the template later but before transcripts reach a length of 68 nucleotides (nt) (Zawel et al. 1995). Our ChIP analysis confirms the differential association of TFIIB/TFIID versus TFIIF/TFIIH, but suggest that the latter proteins remain bound to the elongation complex much longer. Overall, our detailed protein occupancy maps support the existence of diverse modes of action for these critical elongation factors.

P-TEFb is undoubtedly one of the most widely used elongation factors in eukaryotic cells. In addition to

DSIF, the current model for P-TEFb action identifies the RNAP II CTD as a key target for CDK9 kinase activity (Marshall et al. 1996; Zhu et al. 1997). CTD phosphorylation by CDK9 represents a typical example of healthy scientific debate. The widely held view postulates that CDK9 phosphorylates Ser2-CTD exclusively. This view is supported by RNA interference (RNAi) experiments in *Caenorhabditis elegans*, pharmacological inhibition of CDK9 in flies, and analysis of CTK1 (a CDK9 homolog) mutants in yeast, among others (Shim et al. 2002; Ahn et al. 2004; Ni et al. 2004). However, CDK9 has been also shown to phosphorylate Ser5-CTD in certain scenarios. For example, recruitment of CDK9 to the HIV1 LTR by the Tat viral protein leads to CTD phosphorylation mainly at Ser5 (Garber et al. 2000). CDK9 is inhibited by DRB, and this property led to the identification of P-TEFb (Marshall et al. 1996; Zhu et al. 1997). Pharmacological inhibition of CDK9 by DRB or flavopiridol shuts down most mRNA synthesis in mammalian cells (Chao and Price 2001). However, the contribution of other kinases to this effect remains to be elucidated since both drugs have been shown to inhibit other CDKs with less efficiency (Rickert et al. 1999; Dai and Grant 2003). Flavopiridol is a more potent inhibitor of CDK9 in in vitro kinase assays (IC₅₀ < 10 nM), yet cell culture studies usually use concentrations that could inhibit other kinases (≥ 300 nM) (Chao and Price 2001; Dai and Grant 2003; Ni et al. 2004). In this report we have used DRB (50 μ M) to block CDK9 activity in HCT116 colon carcinoma cells. This concentration of DRB produces a virtually indistinguishable pattern of global changes in gene expression compared with flavopiridol, suggesting that both drugs inhibit the same processes (Lam et al. 2001). Our studies reveal that CDK9 activity is dispensable for activation of select p53 target genes. Furthermore, our ChIP analyses show that DRB-sensitive kinases in HCT116 cells are required to phosphorylate both Ser2 and Ser5 of RNAP II when transiting the *p21* locus, but the precise contribution of CDK9 to this effect cannot be determined at this point. Intriguingly, a DRB-insensitive kinase activity mediates Ser5-CTD phosphorylation at the 5' region of the *p21* gene. The occupancy pattern of DRB-insensitive Ser5 phosphorylation matches that of CDK7, the kinase subunit of TFIIF that is known to be less sensitive to DRB (Marshall et al. 1996). This observation generates two novel conclusions: (1) Two distinct Ser5 kinases function consecutively on the elongating polymerase; and (2) a CTD-phosphatase acts in the 5' region of the gene to remove the phosphate groups added by the 5' kinase. Our analyses reveal a second RNAP II dephosphorylation event in the 3' region of the gene as seen by a decrease in Ser5- and, most prominently, in Ser2-CTD phosphorylation before a drop in total RNAP II signals. This indicates that passage of RNAP II through the polyadenylation signal must trigger the recruitment of a phosphatase that removes these phosphate groups prior to RNAP II dissociation from the chromatin. A similar 3'-dephosphorylation event has been observed in certain yeast genes (Kim et al. 2004). In this regard, several CTD-phosphatases have already been identified.

FCP1 is a phosphatase conserved from yeast to humans that preferentially dephosphorylates Ser2 over Ser5 *in vivo* (Cho et al. 2001). On the other hand, a family of small CTD phosphatases (SCPs) and the Ssu72 phosphatase catalyze selective dephosphorylation of Ser5 (Yeo et al. 2003; Krishnamurthy et al. 2004). Further experiments will be necessary to determine the role of these phosphatases in the dephosphorylation events observed on the *p21* locus. SPT16 also seems to dissociate from the elongation complex immediately after passage through the 3' end of the gene. Interestingly, DRB treatment greatly diminishes SPT16 recruitment. Further studies will be required to identify a possible role for RNAP II phosphorylation in SPT16 recruitment. Studies in yeast also revealed an "unloading" of certain elongation factors after RNAP II passed the polyadenylation signals (Kim et al. 2004). However, yeast cells lacking the CDK9 homolog CTK1 display intact Ser5 phosphorylation and compromised Ser2 phosphorylation, yet show no defects in recruitment of ySPT16 (Ahn et al. 2004). Finally, our studies demonstrate that unlike some yeast and *Drosophila* genes, CDK9 activity and Ser2-CTD phosphorylation are dispensable for efficient 3' processing of *p21* mRNA. Defects in 3' cleavage and polyadenylation in yeast cells lacking the CDK9 homolog CTK1 have been observed (Ahn et al. 2004), and similar conclusions were reached when examining *Drosophila* heat shock genes (Ni et al. 2004). It is not clear at this point how widespread is the requirement for P-TEFb activity and Ser2-CTD phosphorylation in RNA processing. Intriguingly, *ctk1Δ* yeast cells are viable, which indicates that alternative modes of 3' RNA processing must exist, including transcription-uncoupled (Bird et al. 2004) and/or cytoplasmic polyadenylation (Richter 1999). Furthermore, recent studies using *in vitro* assays for coupled transcription–RNA processing showed that inhibition of P-TEFb had no significant effects on 3' RNA processing of a chimeric gene carrying a viral poly(A) site (Adamson et al. 2005). Interestingly, gene-specific requirements for P-TEFb function have been recently described among NFκB target genes (Luecke and Yamamoto 2005).

Taken together, our studies establish that specific *p53* target genes are readily activated in the absence of CDK9 activity, incomplete RNAP II phosphorylation, and defective recruitment of specific elongation factors. It is likely that compensatory mechanisms are used on these target genes to allow transcription and RNA processing to occur under these conditions. It is clear that such alternative processes provide cells with a way to react to stress stimuli that compromise mRNA synthesis. Future studies will be required to elucidate the nature and prevalence of such mechanisms.

Materials and methods

ChIP assays

HCT116 cultures were grown to 50%–60% confluency and treated with doxorubicin (0.4 μM; Sigma-Aldrich) or DRB (50 μM; Sigma-Aldrich). After PBS wash, cells were cross-linked

with a 1% formaldehyde/PBS solution for 15 min at room temperature. Cross-linking was stopped by addition of glycine to 125 mM final concentration. Cells were washed twice with cold PBS and harvested in RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl at pH 8, 5 mM EDTA, 20 mM NaF, 0.2 mM sodium orthovanadate, 5 μM trichostatin A, 5 mM sodium butyrate, and protease inhibitors). Samples were sonicated to generate DNA fragments <500 bp. For immunoprecipitation, 1 mg of protein extract was pre-cleared for 2 h with 30 μL of 50% G protein-Sepharose slurry before addition of indicated antibodies. Antibody sources are described in Supplementary Table 1. Two micrograms to 5 μg of each antibody was added to the samples and incubated overnight at 4°C in the presence of 30 μL of protein G-beads pre-blocked with 1 mg/mL BSA and 0.3 mg/mL salmon sperm DNA. When antibodies against phosphorylated CTD were used, immunocomplexes were recovered using anti-mouse IgM/protein G-Sepharose beads (Sigma-Aldrich). Beads were washed twice with RIPA buffer, four times with ChIP Wash Buffer (100 mM Tris-HCl at pH 8.5, 500 mM LiCl, 1% [v/v] Nonidet P-40, 1% [w/v] deoxycholic acid), twice again with RIPA buffer, and twice with 1× TE. Immunocomplexes were eluted for 10 min at 65°C with 1% SDS, and cross-linking was reversed by adjusting to 200 mM NaCl and incubating 5 h at 65°C. DNA was purified, and a fraction was used as template in real-time PCR reactions. Primers against multiple regions of the *p21* locus were designed with Primer Express 2.0 (Applied Biosystems; see Supplementary Table 2 for primer sequences). PCR products range in size between 50 and 75 bp. Ten-microliter PCR reactions containing 1× SYBR Green Mix (Applied Biosystems), 1/100 fraction of the ChIP-enriched DNA, and 100 nM primers were set up in 384-well plates using an Eppendorf epMotion 5070 robot. Standard curves from 1–200 ng of sonicated genomic DNA were run alongside ChIP samples for each individual primer, and plates were read in an Applied Biosystems 7900HT Real-time PCR instrument (Absolute Quantification Method). Normal serum and input DNA values were used to subtract/normalize the values from ChIP samples.

Real-time RT–PCR reactions

Total RNA was prepared with the Qiagen RNeasy Kit following the manufacturer's instructions. RT reactions were performed with SuperScriptase III (Invitrogen) using the random hexamer protocol following the manufacturer's instructions. Primers were designed against sequences of the indicated mRNAs using Primer Express 2.0 (Applied Biosystems; see Supplementary Table 3 for sequences). PCR reactions containing 1× SYBR Green Mix (Applied Biosystems), 0.1 ng of cDNA, and 500 nM primers were set up and read as described above. Standard curves using 0.001–10 ng of cDNA pools were used. Values were normalized to those of 18S rRNA. For Figure 6B, 50 ng of genomic DNA or 2.5 ng of cDNA was used in standard PCR reactions for 38 cycles (past saturation point for the cDNA samples) using the indicated primers, and PCR products were resolved in 2% agarose gels. For Figure 6C, poly(A)⁺ RNA was prepared with Oligotex resins (Qiagen) following the manufacturer's instructions. Equal amounts of total or poly(A)⁺ cDNAs were used in PCR reactions alongside standard curves of total cDNA to determine fold enrichment.

Protein immunoblot analyses

Typically, 10 μg of total protein extract was loaded onto 10% SDS-PAGE and transferred to PVDF membranes. Blots were probed with primary antibodies: *p53* (DO-1, Oncogene), *p21*,

nucleolin, RNAP II, and actin (Santa Cruz sc-817, sc-8031, sc-9001, and sc-1616, respectively), and developed with peroxidase-conjugated secondary antibodies and ECL detection reagents (Amersham).

Flow cytometry analyses of cell cycle and apoptosis

For cell cycle distribution profiles, cells were harvested by trypsinization, washed twice with cold PBS, and resuspended in PBS/2 mM EDTA. Cells were then fixed overnight in 50% ethanol. Before cell sorting, cells were washed in PBS/2 mM EDTA, treated with RNase A, and stained with 50 µg/mL propidium iodide. For apoptotic index assays, cells were harvested by trypsinization, washed with PBS, and resuspended in Annexin V Binding Buffer (10 mM HEPES at pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Cells were then stained with Annexin V-fluorescein conjugate (Molecular Probes) and propidium iodide, following the manufacturer's instructions. Cells were analyzed on a Beckton-Dickinson FACSscan instrument.

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