

# Disparate chromatin landscapes and kinetics of inactivation impact differential regulation of p53 target genes

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**Key words:** transcription, PUMA, p21, FAS, CTCF, chromatin boundary, core promoter, non-coding RNA

**Abbreviations:** 3C, chromosome conformation capture; CDK8, cyclin dependent kinase 8; CTCF, zinc finger CCCTC-binding factor; CPE, core promoter element; GTF, general transcription factor; HZF, hematopoietic zinc-finger; MDM2, human homolog of murine double minute 2; p53AIP1, p53-regulated apoptosis-inducing protein; p53REs, p53 response elements; PIG3, p53 inducible gene 3; PUMA, p53 upregulated modulator of apoptosis; P-TEFb, positive transcription elongation factor b; PUMA-TUF, PUMA transcript of unknown function; Q-PCR, quantitative PCR; RNAPII, RNA polymerase II; S5P and S2P-CTD, serine 5 and 2 phosphorylation of the RNAPII C-terminal domain; TBP, TATA-binding protein; TFIIB and F, transcription factor II B and F; USF, upstream transcription factor

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The p53 transcription factor regulates the expression of genes involved in cellular responses to stress, including cell cycle arrest and apoptosis. The p53 transcriptional program is extremely malleable, with target gene expression varying in a stress- and cell type-specific fashion. The molecular mechanisms underlying differential p53 target gene expression remain elusive. Here we provide evidence for gene-specific mechanisms affecting expression of three important p53 target genes. First we show that transcription of the apoptotic gene *PUMA* is regulated through intragenic chromatin boundaries, as revealed by distinct histone modification territories that correlate with binding of the insulator factors CTCF, Cohesins and USF1/2. Interestingly, this mode of regulation produces an evolutionary conserved long non-coding RNA of unknown function. Second, we demonstrate that the kinetics of transcriptional competence of the cell cycle arrest gene *p21* and the apoptotic gene *FAS* are markedly different in vivo, as predicted by recent biochemical dissection of their core promoter elements in vitro. After a pulse of p53 activity in cells, assembly of the transcriptional apparatus on *p21* is rapidly reversed, while *FAS* transcriptional activation is more sustained. Collectively these data add to a growing list of p53-autonomous mechanisms that impact differential regulation of p53 target genes.

## Introduction

The importance of the p53 network in cancer biology is undisputed. *TRP53* is

the most commonly mutated tumor suppressor gene, with inactivating mutations occurring in about half of human cancers.<sup>1</sup> Importantly, it is estimated that 11 million patients worldwide carry tumors with wild-type p53 that could be activated to induce tumor regression, thus making research into p53-based therapies a top priority in modern medicine.<sup>2</sup> However, the development of these therapies is hampered by the fact that p53, which acts as a signaling node within a vast gene network, is a highly pleiotropic factor. Cells can adopt starkly different responses upon p53 activation, such as reversible cell cycle arrest versus apoptosis. The effects of this pleiotropy are manifested in the clinic, where activation of p53 by genotoxic stress leads to cancer cell death and tumor regression only in a fraction of cases.<sup>3-9</sup> Conversely, systemic activation of p53 causes many of the undesirable side effects of genotoxic therapies by triggering apoptosis in healthy tissues.<sup>10</sup> Therefore, understanding the mechanisms defining cell fate choice in response to p53 activation is a prerequisite for the design of therapeutic tools that selectively drive cancer cells into p53-dependent apoptosis while sparing normal tissues.

First and foremost, p53 is a transcription factor.<sup>11-13</sup> Although some transcription-independent functions have been ascribed to this tumor suppressor,<sup>14-19</sup> its role as a transcriptional regulator accounts for most of its biological activity.<sup>20-25</sup> p53 induces cell cycle arrest via transcriptional activation of genes such as the CDK-inhibitor *p21* (*CDKN1A*)<sup>26</sup> and the inhibitor of the G<sub>2</sub>/M transition *14-3-3 $\sigma$*  (*SFN*).<sup>27</sup> Conversely, p53-dependent

apoptosis is mediated by transactivation of genes involved in the intrinsic mitochondrial apoptotic pathway (e.g., *PUMA/BBC3*, *NOXA*),<sup>28-30</sup> and the extrinsic death receptor pathway (e.g., *FAS*, *DR5*).<sup>31,32</sup> Much of the pleiotropy associated with p53 is due to the flexible nature of the p53 transcriptional program. Distinct subsets of p53 target genes are activated in different cell types and in response to diverse stimuli, which reveals the action of factors modulating p53 transactivation potential in a gene-specific manner.<sup>33-45</sup> A major goal in the p53 field is to identify these gene-specific co-regulators and to elucidate how they work. Eventually, this knowledge will enable strategies to manipulate the activity of these factors toward more efficient p53-based therapies.

A prevalent hypothesis in the field is that cell fate choice is defined by selective binding of p53 to the response elements found at target genes. This “p53-centric” form of regulation could be achieved through p53 post-translational modifications and/or p53 interacting partners. For example, phosphorylation of Ser46 of p53 was shown to induce preferential binding and transactivation of *p53AIP1*, and purportedly increasing p53-dependent apoptosis.<sup>46</sup> However recent studies demonstrated that over a panel of cell lines there is no correlation with Ser46 phosphorylation and the induction of apoptosis or the expression of *p53AIP1*.<sup>47</sup> Furthermore, Nutlin-3, the small molecule inhibitor of MDM2, induces p53 activation without phosphorylation and stimulates apoptosis in some cell types.<sup>48,49</sup> Similarly, interaction of p53 with the ASPP proteins has been suggested to lead to preferential binding of p53 to the apoptotic genes *PIG3* and *BAX* but not to cell cycle arrest genes such as *p21*.<sup>50</sup> Conversely, interaction of p53 with the HZF protein seems to facilitate p53 association with the cell cycle arrest genes *p21* and *SFN*, while preventing interaction with the pro-apoptotic genes *BAX* and *NOXA*, thus promoting cell survival.<sup>51</sup> While intriguing, these studies stand in contrast to several others that demonstrate that p53 binding to its cognate p53REs is invariant under a variety of stress stimuli. For example, under conditions of UV-irradiation and  $\gamma$ -irradiation, which lead to different p53 target gene

expression patterns,<sup>33</sup> p53 binding to its canonical targets *p21*, *PUMA*, *BAX*, *p53AIP1* and *PIG3* is indistinguishable.<sup>52</sup> Furthermore, recent genome-wide studies of p53 chromatin binding demonstrated that under conditions where p53 promotes apoptosis, it binds nonetheless to target genes in multiple functional categories, rather than to a distinct apoptotic subset.<sup>53</sup>

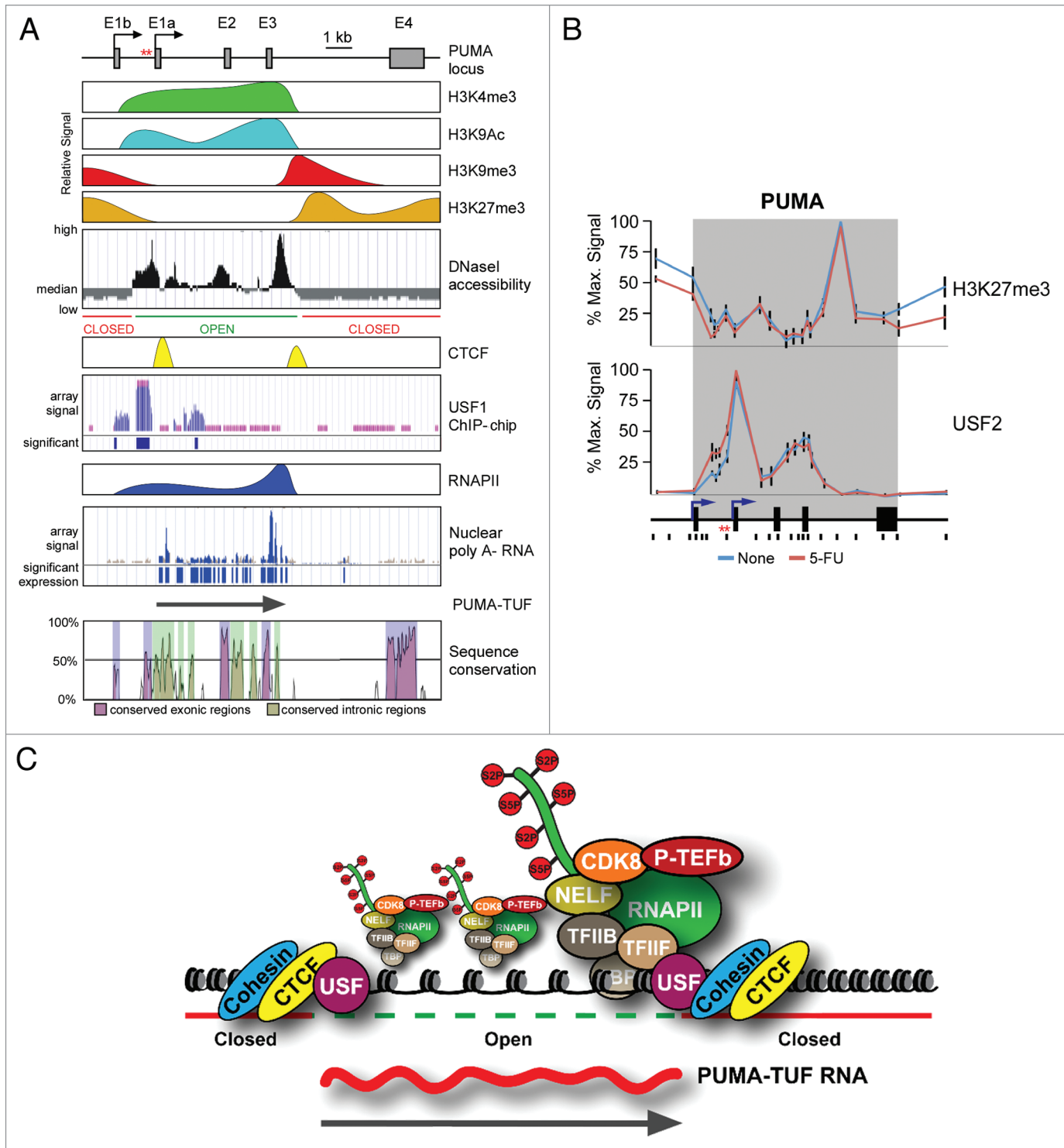
As the hypothesis that differential p53 binding as a determinant of cell fate choice has come into question, it has become increasingly clear that the context in which p53 functions at its individual target genes is exceedingly important. Several “p53-autonomous” mechanisms could influence gene expression independently of p53 modification, p53-DNA association or p53 interacting partners. Recently several instances of these context-dependent regulatory mechanisms have been described. For example, the hCAS/CSE1L protein associates with a distinct subset of p53 target genes (*PIG3*, *p53AIP1* and *p53R2*) and loss of hCAS/CSE1L results in an imbalance in the p53 transcriptional program leading to an attenuated apoptotic response.<sup>55</sup> Importantly, hCAS does not affect p53 binding to DNA but rather acts by somehow reducing the levels of repressive histone marks at select p53 target loci. More recently, it was demonstrated that core promoter elements found at the p53 target genes *p21*, *FAS* and *APAF1* determine the rate of transcriptional apparatus assembly and the duration of transcription re-initiation, all in a p53-independent manner.<sup>54</sup> The *p21* promoter harbors elements that facilitate rapid but brief rounds of transcription, while elements found in *FAS* and *APAF1* promoters dictate slow but sustained rounds of transcription.<sup>54</sup> Collectively these results suggest that p53 target genes exist within unique regulatory landscapes, as defined by chromatin environments and promoter sequences, which play a significant and previously underappreciated role in determining eventual gene expression in response to stress. Here we provide further evidence for the existence of p53-autonomous mechanisms that impact differential regulation of p53 target genes. We first expand upon our recent findings that expression of the apoptotic gene *PUMA* is regulated by non-canonical

intragenic chromatin boundaries.<sup>55</sup> We then shift gears and provide in vivo evidence confirming the finding that “hard-wired” core promoter elements regulate the kinetics of p53 target gene expression.

## Results

**The PUMA locus displays a curious chromatin landscape that reveals an unusual mode of regulation.** PUMA is a BH3-only domain protein that antagonizes the function of pro-survival members of the Bcl-2 family and facilitates BAX translocation to the mitochondria.<sup>56,57</sup> PUMA is a direct transcriptional target of p53 and a key mediator of p53-induced apoptosis.<sup>15,21,28,29</sup> The *PUMA* gene is comprised of an ~12 kb locus, which harbors two alternative promoters, with two adjacent p53REs residing just upstream of Exon 1a (Fig. 1A).

Recently we demonstrated that *PUMA* transcription is regulated by non-canonical mechanisms involving intragenic chromatin boundaries.<sup>55</sup> The first half (~6 kb) of the *PUMA* locus constitutively harbors histone marks of transcriptional activation. For example, histone H3 tri-methylation of lysine 4 (H3K4me3), which is typically observed ~500 bp downstream of poised and active promoters,<sup>58</sup> is found throughout the first 6 kb of the *PUMA* locus, with consistent levels detectable from Exon 1b until a precipitous drop off after Exon 3 (Fig. 1A). An additional mark of activation, histone H3 lysine 9 acetylation (H3K9Ac), normally associates with enhancers and core promoters of transcriptionally active genes.<sup>59</sup> On the *PUMA* locus H3K9Ac is again constitutively found throughout the first half of gene, with major peaks around the promoters and Exon 3 (Fig. 1A). Interestingly these histone marks of active transcription are flanked by histone marks associated with transcriptional silencing. Histone H3 lysine 9 tri-methylation (H3K9me3) is typically associated with a repressed transcriptional state and the presence of heterochromatin.<sup>60</sup> We observed the clear presence of H3K9me3 upstream of the transcriptional start sites, and interestingly just downstream of the precipitous drop in H3K9Ac levels within intron 3 (Fig. 1A). Tri-methylation of histone 3



**Figure 1.** Non-canonical transcriptional regulation at the *PUMA* locus. (A) A linear scale model of the *PUMA* locus indicating the exon structure and dual transcription start sites followed by a schematic summary of ChIP data, DNase I accessibility data and RNA analysis. Primary ChIP data for H3K4me3, H3K9Ac, H3K9me3, CTCF and RNAPII was previously published in Gomes and Espinosa.<sup>55</sup> Primary data for H3K27me3 and USF2 is shown in (B). Data on DNase I accessibility, USF1 occupancy and poly A- nuclear RNA was obtained from the UCSC genome browser after analysis of publicly available genome wide datasets.<sup>62,66,67</sup> Several lines of experimental evidence supporting the existence of PUMA-TUF were published in Gomes and Espinosa.<sup>55</sup> *PUMA* locus conservation plot is an adapted view of VISTA plot data (<http://genome.lbl.gov/vista/index.shtml>) comparing human and murine genomic sequences. (B) ChIP assays were performed with whole-cell extracts from control and 5-FU-treated (8 h) HCT116 p53<sup>+/+</sup> cells with antibodies recognizing H3K27me3 and USF2. The locus maps are a condensed scale model of those seen in (A). The location of 20 Real-Time PCR amplicons used in ChIP assays is also shown; red asterisks represent the p53REs. The gray band represents the annotated transcribed region. (C) A model summarizing the transcriptional state of the *PUMA* locus prior to p53 activation.

Lysine 27 (H3K27me3) is another mark of associated with transcriptional silencing.<sup>61</sup> On *PUMA* H3K27me3 is found upstream of the transcriptional start sites and again interestingly just downstream of Exon 3, following the precipitous drop in H3K4me3 and H3K9Ac levels (Fig. 1A). Interestingly, H3K27me3 levels do not change following transcriptional activation upon p53 activation by 5-fluorouracil (5-FU) (Fig. 1B). Collectively these data suggest that the *PUMA* locus harbors an unusual chromatin environment, with the first 6 kb being defined by marks of active transcription and an 'open' chromatin state, while the flanking regions being defined by marks of transcriptional repression and a 'closed' chromatin state. This model is clearly supported by DNaseI accessibility assays, which show a long stretch of 'open' chromatin starting upstream of Exon 1a and extending downstream of Exon 3 (Fig. 1A).<sup>62</sup> What factors might be mediating this unusual intragenic chromatin domain?

The CTCF zinc finger protein is involved in definition of chromatin boundaries, enhancer blocking and formation of chromatin loops.<sup>63,64</sup> We observed that CTCF occupies two intragenic sites within *PUMA*, one around the core promoters and a second site just downstream of Exon 3, both of which overlap with the boundaries of the 'open' and 'closed' chromatin domains (Fig. 1A). We further demonstrated that CTCF knockdown leads to loss of H3K9me3 within *PUMA* and elevated basal *PUMA* mRNA expression.<sup>55</sup> Collectively these data suggest that CTCF helps initiate/maintain the chromatin boundaries within *PUMA* and functions to repress the basal transcription of this potent apoptotic gene. However, as several chromatin signatures were still preserved upon CTCF knockdown,<sup>55</sup> we hypothesized that additional factors must play a role in maintaining the observed chromatin boundaries. Accordingly, we observed that USF2 is constitutively associated within the *PUMA* locus in a manner overlapping with the previously defined chromatin boundaries (Fig. 1B). In addition to being associated with direct transcriptional activation, the USF factors have also been demonstrated to function as chromatin boundary elements that

prevent the spreading of heterochromatin at  $\beta$ -globin genes.<sup>65</sup> It is therefore possible that USF factors are also responsible for initiating/maintaining the peculiar intragenic chromatin architecture observed on *PUMA*. Of note, genome-wide studies of USF1 occupancy detected binding around the upstream boundary within *PUMA* (Fig. 1A).<sup>66</sup>

What is the consequence of this unusual chromatin architecture on the transcriptional competence of *PUMA*? Correlating with the 'open' nature of the first half of *PUMA* and the association of histone marks indicative of active transcription, we demonstrated that RNA polymerase II (RNAPII) is also constitutively associated with this region (Fig. 1A).<sup>55</sup> Along with RNAPII a variety of general transcription factors (GTFs) (including TBP, TFIIB and TFIIF), Mediator (CDK8) and elongation factors (P-TEFb) are also constitutively associated with the first half of *PUMA*.<sup>55</sup> We further demonstrated that these transcriptional complexes are active and give rise to a large sense-strand RNA of unknown coding capacity representing regions of the *PUMA* locus starting close to Exon 1a and terminating 100–200 bp after the end of Exon 3.<sup>55</sup> We have dubbed this transcript *PUMA*-TUF, for *PUMA* transcript of unknown function (Fig. 1A). Genome-wide studies of nuclear, non-polyadenylated RNAs supports our observations that the first half of *PUMA* is constitutively transcribed (Fig. 1A).<sup>67</sup> Interestingly this portion of the *PUMA* locus shows unusually high sequence conservation within intronic regions (Fig. 1A), which raises the intriguing possibility that *PUMA*-TUF may have a conserved cellular function and not simply be a consequence of promiscuous transcription from 'open' chromatin.

**Do CTCF and cohesin complexes induce gene looping at the *PUMA* locus?** In addition to CTCF and USF1-2, we observed that the CTCF-associated Cohesins SMC1 and Rad21 also associate with the boundaries in the *PUMA* intragenic region.<sup>55</sup> Genome-wide studies revealed that most CTCF binding sites occur within intergenic regions of the genome,<sup>64</sup> which agrees with CTCF's described roles in enhancer blocking and the prevention of heterochromatin

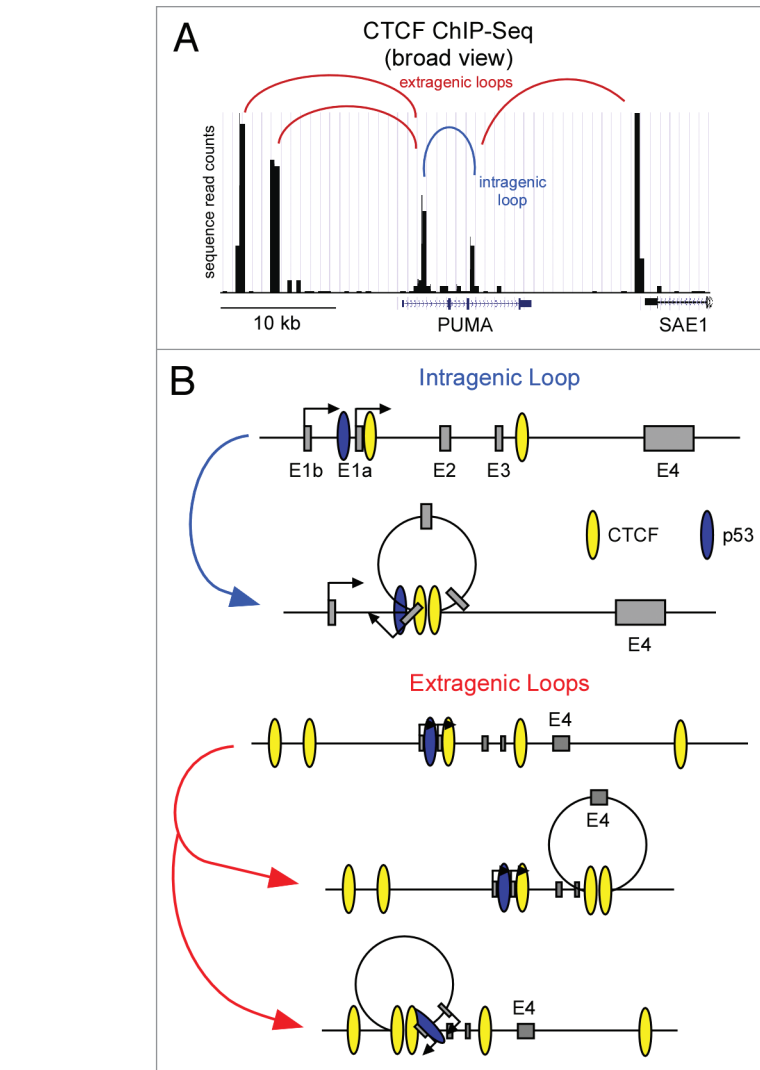
spreading into actively transcribed genes.<sup>68</sup> It has been proposed that these CTCF functions involve the formation of 'chromatin loops', in which two distant sites of CTCF binding are brought into close proximity in the three-dimensional milieu of the nucleus.<sup>69</sup> These loops, which can be detected by 3C technology, may be facilitated by Cohesins and seem to be regulated in a cell type- and signaling-specific manner.<sup>69</sup> These observations create the intriguing possibility that the sites of CTCF-Cohesin binding within *PUMA* could mediate the formation of chromatin loops (Fig. 2). One possibility is that the dual sites of intragenic CTCF-Cohesin occupancy create an intragenic loop (Fig. 2B). Such a hypothesis fits well with our current understanding of the transcriptional regulation of *PUMA*. Recall that the first half of *PUMA* is constitutively transcribed and harbors an 'open' chromatin state. This intragenic region could be kept in isolation from the rest of the gene via an intragenic loop, and p53 activation could 'break' this loop, allowing for RNAPII travel into the 3' end of the gene, with ensuing expression of *PUMA* mRNA (Fig. 2B). Consequently, by reducing the cellular pools of CTCF we might be undoing chromosomal loops, which then allows for more RNAPII to travel into the 3' end of *PUMA*. Alternatively, these CTCF-Cohesin complexes could be forming 'extragenic loops' with CTCF binding sites flanking the *PUMA* locus observed in genome-wide studies (Fig. 2A).<sup>64</sup> These extragenic interactions could bring important regulatory elements to the *PUMA* proximal promoters to allow proper regulation of the locus. The possibility that gene looping may be occurring on the *PUMA* locus is highly interesting and 3C studies are underway to determine if any loops exist and if they have a functional consequence on the expression of *PUMA*.

**p53-dependent transcriptional activation of *p21* is rapidly reversed in vivo.** Recent biochemical analyses by the Emerson lab revealed an important role for core promoter elements in differential regulation of p53 target genes.<sup>54</sup> The core promoter of a gene is defined as the minimal DNA sequence required for accurate RNAPII recruitment and transcription



initiation, and can be comprised of a myriad of core promoter elements (CPEs) including the TATA box, the Downstream core Promoter Elements (DPE), GC-rich islands and Initiator motifs (Inr).<sup>70</sup> These CPEs are responsible for the recruitment of GTFs to the core promoter, which in turn are responsible for the recruitment of RNAPII and formation of the pre-initiation complex (PIC). Additionally, CPEs can determine the sensitivity of a given promoter to distal enhancer elements, thus providing specificity in response to activator-induced gene expression.<sup>71</sup> Core promoters found at p53 target genes harbor a diverse collection of CPEs, but it is unclear how these different architectures may influence their mode of expression in response to stress.<sup>72</sup>

Using elegant *in vitro* transcription assays, Morachis et al.<sup>54</sup> showed that the CPEs found at the *p21* and *FAS* promoters determine both the kinetics of PIC assembly and the re-initiation capacity of the transcriptional apparatus.<sup>54</sup> They observed that the *p21* promoter undergoes rapid PIC assembly and transcription initiation, but re-initiates poorly, thus being transcriptionally competent for a short period of time. In contrast, the *FAS* promoter undergoes slow PIC formation and transcriptional initiation, but was competent for numerous rounds of effective re-initiation. Importantly, the assays employed did not include p53REs or p53 protein. These data suggest that, at least *in vitro*, the core promoter architecture of p53 target genes can influence their expression kinetics independently of p53. A key prediction of these studies is that after a pulse of p53 activity in cells, different p53 target genes will not only engage in productive transcription at different velocities, but would also be inactivated with different kinetics. Early microarray experiments documented extensively the differential kinetics of p53 target gene activation, with *p21* being classified as an early response gene.<sup>33</sup> However, the kinetics of p53 target gene inactivation has not been analyzed. To investigate this issue we took advantage of the small molecule activator of p53, Nutlin-3. Nutlin-3 binds to and antagonizes the function of the p53 repressor MDM2, leading to activation of p53 in the absence of DNA damage,

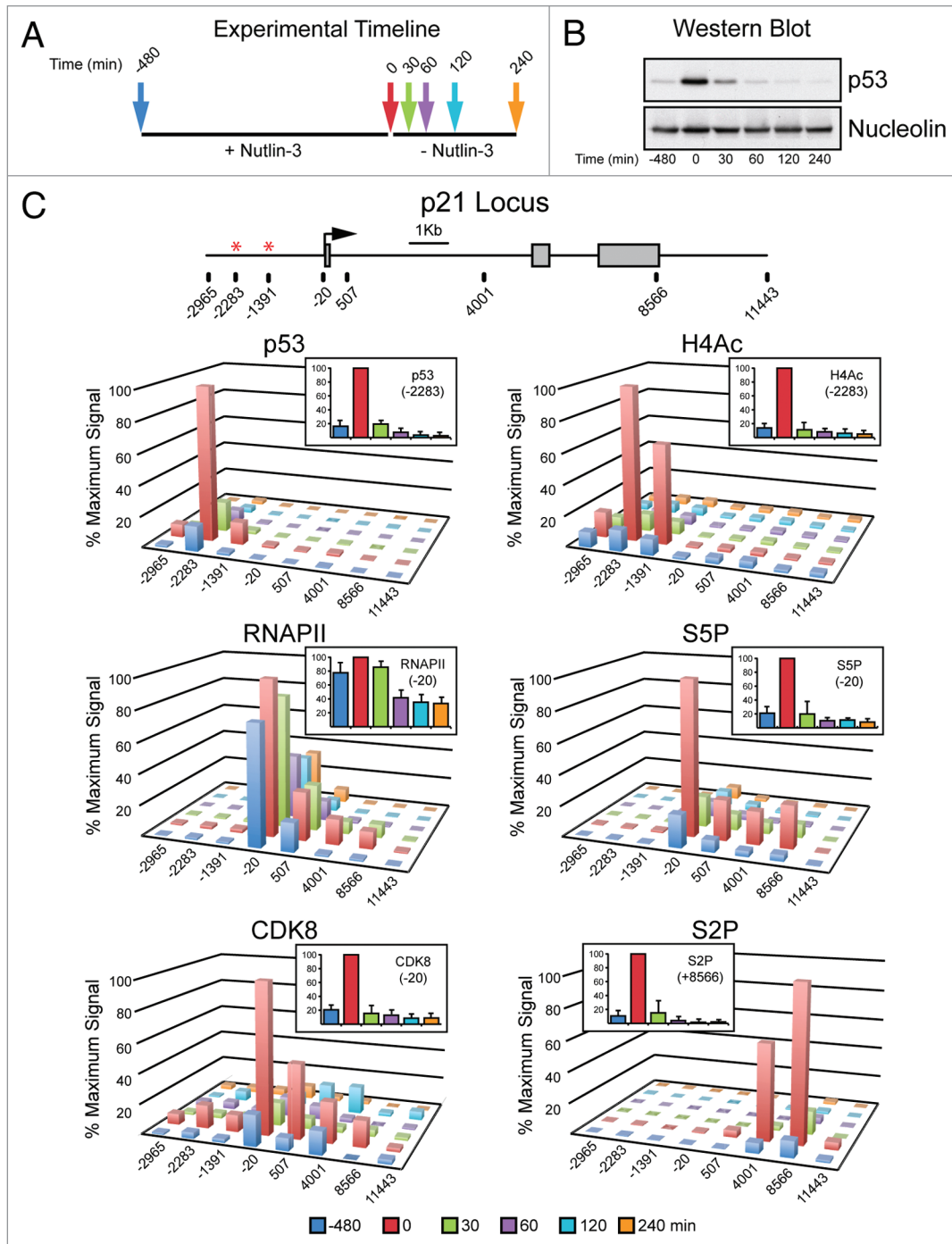


**Figure 2.** Models of potential CTCF-induced gene looping on the *PUMA* locus. (A) An adapted UCSC genome browser view of *PUMA* and surrounding genomic sequence displaying CTCF binding data from CD4<sup>+</sup> T cells<sup>90</sup> denoting potential CTCF looping sites. (B) A general schematic of potential *PUMA* locus intragenic and extragenic CTCF-mediated loops.

and importantly its effects are completely reversible upon removal from cell culture.<sup>49</sup> Treatment of HCT116 cells with Nutlin-3 for 8 h leads to the accumulation of cellular p53 (Fig. 3B) and induces the transcriptional activation of p53 target genes, including *p21* and *FAS*.<sup>49,73,74</sup> Following removal of Nutlin-3 from cell cultures, p53 levels are drastically reduced within 30 min and drop below basal levels after 60 min (Fig. 3B). If the *in vitro* observation that *p21* is rapidly activated and shut off, while *FAS* is slowly activated but sustained, this should be reflected in the reversal of the transcriptional events taking place at these individual loci. We began to test this prediction by performing

first a detailed ChIP analysis of the *p21* locus post-Nutlin-3 removal (Fig. 3C).

We have previously demonstrated that activation of the *p21* locus correlates with p53-dependent increases in histone acetylation, recruitment of the Mediator complex and conversion of paused RNAPII into an elongation competent form.<sup>40,42,43</sup> Following 8 h of Nutlin treatment, a clear increase in chromatin-bound p53 is observed at the p53REs on the *p21* locus along with enhanced histone acetylation (H4Ac) (Fig. 3C). The CDK8 subunit of the Mediator complex is also recruited to the *p21* promoter, consistent with its demonstrated role as a positive-regulator of transcription on select p53 target genes.<sup>40</sup> Following Nutlin-3



**Figure 3.** Rapid inactivation of *p21* transcription following transient p53 activation in cells. (A) Experimental timeline. HCT116 cells were treated with Nutlin-3 for 8 h, drug was washed out and cell extracts were harvested at 0, 30, 60, 120 and 240 min following Nutlin-3 removal. (B) Western blot analysis demonstrating p53 accumulation in response to Nutlin-3 treatment and rapid degradation following drug removal. Nucleolin serves as a loading control. (C) A linear scale model of the *p21* locus indicating exon structure, transcription start site and red asterisks representing the p53REs. The location of 8 PCR amplicons used in ChIP assays is also shown. ChIP assays were performed with antibodies recognizing total p53, H4Ac, CDK8, total RNAPII, S5P-CTD and S2P-CTD.

treatment, transcriptional activation is evidenced by increased levels of RNAPII within the body of the gene (Fig. 3C). It is widely accepted that phosphorylation of the heptad repeats (amino acid sequence

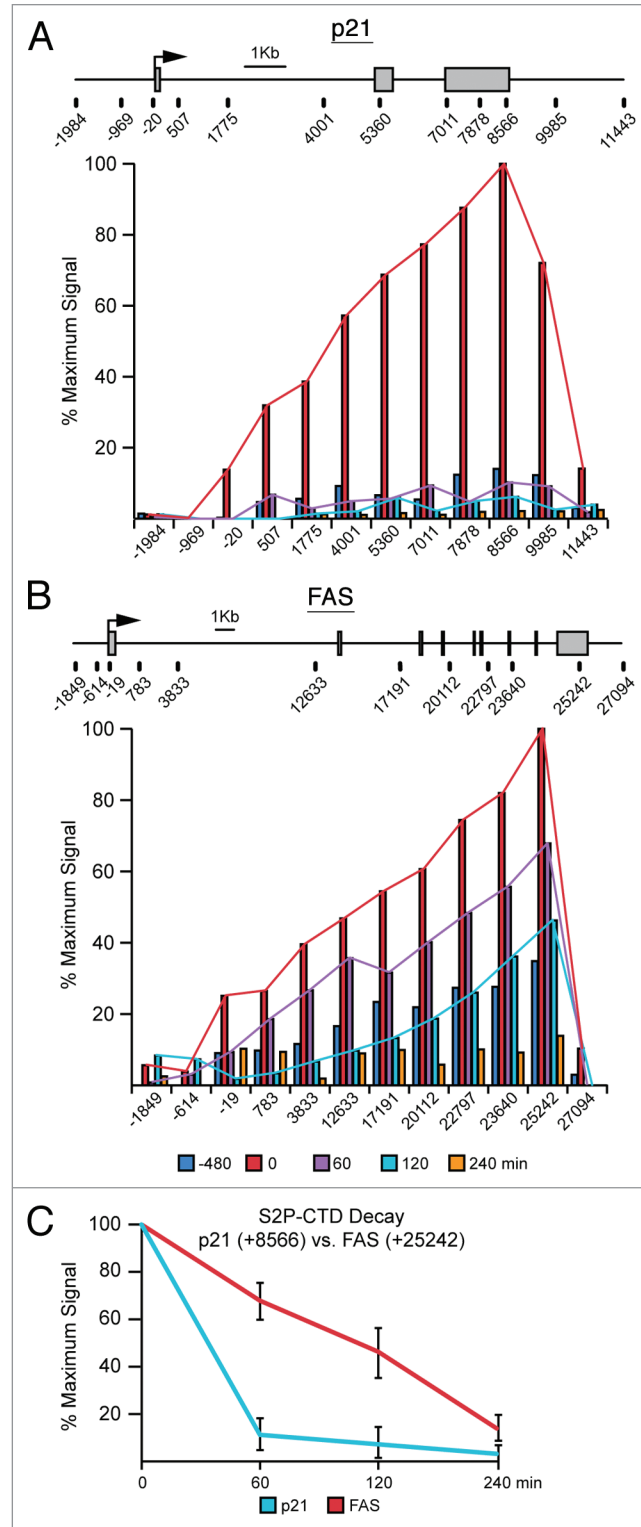
YSPTSPS) in the C-terminal domain (CTD) of the largest subunit of RNAPII is indicative of transcriptional state. Whereas phosphorylation of serine 5 (S5P-CTD) is associated with promoter escape, serine 2

phosphorylation (S2P-CTD) accumulates towards the 3' end of genes and is indicative of effective elongation.<sup>75</sup> Accordingly, levels of both S5P-CTD and S2P-CTD increase drastically at the *p21* locus upon Nutlin-3

treatment with their characteristic 5' and 3' polarities, respectively (Fig. 3C).

Surprisingly, all marks of transcriptional activation at the *p21* locus are reduced to near or below basal levels after only 30 min of Nutlin-3 removal (Fig. 3C). The dramatic loss of p53 is perhaps not too surprising as MDM2 inhibition is immediately relieved after Nutlin-3 removal. However, the rapid loss of H4Ac suggests that histone deacetylases (HDACs) may be constitutively associated with the *p21* locus and that, while their function is overcome during transcriptional activation, they quickly reverse the hyperacetylated state induced by p53.<sup>76,77</sup> The rapid, concomitant loss of CDK8 and active RNAPII from the gene is indicative of an immediate disruption of the enhancer-promoter dialog that leads to stimulation of RNAPII elongation. How is this rapid inactivation achieved? A simple hypothesis is that the Mediator complex, which is recruited directly by p53, arbitrates this communication, and that as soon as the levels of chromatin-bound p53 are reduced, so is Mediator association and stimulation of RNAPII activity at post-recruitment steps. While the reductions in S5P-CTD and S2P-CTD levels correlate with reduced RNAPII levels, it is also possible that CTD phosphatases function on this locus to facilitate immediate transcriptional silencing upon loss of the activator.<sup>78,79</sup> Taken together these data provide strong evidence that some p53 target genes, such as *p21*, can be rapidly silenced upon reversal of p53 activation, which could be dictated by a core promoter architecture that is non-permissive to sustained transcriptional re-initiation, as first evidenced by the in vitro transcription assays performed by Morachis et al.<sup>54</sup>

*p21* and *FAS* display different kinetics of transcriptional inactivation after a pulse of p53 activity in cells. Next, we employed ChIP assays with antibodies recognizing actively elongating RNAPII (S2P-CTD) to compare the timing of transcriptional silencing of *p21* versus *FAS*. As expected, after 8 h of Nutlin-3 treatment S2P-CTD levels are dramatically increased over basal and progressively increase towards the 3' end at both the *p21* and *FAS* loci (Fig. 4A and B). Interestingly, the decay in S2P-CTD



**Figure 4.** *FAS* displays extended transcriptional competence after p53 shut down. (A and B) Linear scale models of the *p21* and *FAS* loci indicating exon structures and transcription start sites. The location of 12 PCR amplicons used in ChIP assays for each locus is also shown. ChIP assays were performed with antibodies recognizing S2P-CTD. (C) Summary of ChIP data from the 3' end of *p21* (+8,566) and *FAS* (+25,242) over the time course following Nutlin-3 treatment and removal.

signal is much slower on the *FAS* locus, only falling below basal levels 240 min after removal of the drug (Fig. 4). Focusing in on the 3' end of each of these genes following Nutlin-3 removal demonstrates an immediate drop of S2P-CTD levels on *p21*, whereas elongating RNAPII persists for a longer time on *FAS* (Fig. 4C). Collectively these data demonstrate that the *p21* and *FAS* loci clearly have different kinetics of inactivation, with *p21* being quickly silenced following loss of active p53 while *FAS* inactivation is clearly delayed. Furthermore these data correlate well with in vitro observations for these loci and suggest that the core promoter architecture of p53 target genes plays an important role in determining their eventual pattern of expression.

## Materials and Methods

**Chromatin immunoprecipitation (ChIP) assays.** All ChIP assays were performed as finely detailed in Gomes et al. 2006.<sup>42</sup> Briefly for *PUMA* ChIPs, subconfluent HCT116 cultures were treated with 5-FU (375  $\mu$ M) for 8 h, fixed with 1% formaldehyde and then whole-cell lysates were prepared. Protein lysates (1 mg) were subject to ChIP with the indicated antibodies, followed by DNA purification and Q-PCR with the indicated primer sets, detailed in Gomes and Espinosa 2010.<sup>55</sup> For Nutlin reversal experiments, HCT116 cells were treated with Nutlin-3 (10  $\mu$ M) for 8 h, cells were washed twice with 1X PBS, replaced with Nutlin-free media and harvested at 0, 30, 60, 120 and 240 minute intervals. Primers for *FAS* are detailed in Supplemental Table 1.

**Protein immunoblot analysis.** 10  $\mu$ g of total protein extract harvested as described above were loaded onto 10% SDS-PAGE and transferred to PVDF membranes. Blots were probed with primary antibodies: p53 (DO-1, Oncogene) and nucleolin (sc-8031, Santa Cruz), developed with peroxidase-conjugated secondary antibodies (Santa Cruz) and ECL detection reagents (GE Healthcare).

## Concluding Remarks

Here we have described two gene-specific mechanisms that mediate p53 target

gene expression in a manner independent of p53 function. First, we demonstrated that unique intragenic chromatin domain environments ultimately determine functional expression from the apoptotic *PUMA* gene locus. Second, we provided in vivo data supporting the in vitro results indicating that the core promoter architecture of p53 target genes determine the duration of *p21* and *FAS* transcriptional competence after RNAPII initiation.

These observations raise several intriguing possibilities in regards to the regulation of p53 target gene expression in response to cellular stress. First, functional *PUMA* mRNA expression may be achieved in the absence of stress and p53 activation simply by modulating the chromatin architecture of the locus, perhaps via modulation of chromatin boundary factors such as CTCF, Cohesins and USF1-2. Can this endogenous mechanism be exploited to facilitate preferential expression of apoptotic *PUMA* in the treatment of cancer? Second, the identification of *PUMA-TUF*, which appears to be highly conserved, suggests this RNA may have a cellular function. Is that function involved in the regulation of *PUMA* expression or other cellular processes? Third, select groups of p53 target genes (e.g. cell cycle arrest vs. apoptosis) seem to harbor CPEs dictating rapid but brief as opposed to slow but sustained rounds of transcription. Can these intrinsic characteristics be exploited to facilitate the sustained expression of apoptotic genes upon therapeutic activation of p53?

Collectively these findings provide important evidence that differential gene expression following p53 activation, and ultimately cell fate choice in response to cellular stress, cannot simply be defined as a consequence of differential p53 binding to DNA. Rather, the individual 'personalities' of each p53-target gene are likely to strongly influence their ultimate expression pattern. The illumination and deciphering of these mechanisms brings us closer to the ultimate goal of developing targeted therapies allowing for the tipping of the cell fate choice balance, thereby forcing cancer cells into apoptosis in response to p53 activation.

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## Note

Supplementary materials can be found at: [www.landesbioscience.com/supplement/GomesCC9-17-sup.pdf](http://www.landesbioscience.com/supplement/GomesCC9-17-sup.pdf)

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