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Research article

STRESS-INDUCED EXPRESSION OF p53 TARGET GENES IS INSENSITIVE TO SNW1/SKIP DOWNREGULATION

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Abstract: Pharmacological inhibition of protein kinases that are responsible for the phosphorylation of the carboxy-terminal domain (CTD) of RNA Pol II during transcription by 5,6-dichloro-1-beta-D-ribofuranosyl-benzimidazole (DRB) leads to severe inhibition of mRNA synthesis and activates p53. Transcription of the p53 effectors that are induced under these conditions, such as p21 or PUMA, must bypass the requirement for CTD phosphorylation by the positive elongation factor P-TEFb. Here, we have downregulated SNW1/SKIP, a splicing factor and a transcriptional co-regulator, which was found to interact with P-TEFb and synergistically affect Tat-dependent transcription elongation of HIV 1. Using the colon cancer derived cell line HCT116, we have found that both doxorubicin- and DRB-induced expression of p21 or PUMA is insensitive to SNW1 downregulation by siRNA. This suggests that transcription of stress response genes, unlike, e.g., the SNW1-sensitive mitosis-specific genes, can proceed uncoupled from regulators that normally function under physiological conditions.

Key words: P-TEFb, SNW1, p21, p53, Transcriptional elongation, Genotoxic stress

INTRODUCTION

The ability of a cell to respond to stress depends on its capacity to induce the expression of the proper set of genes. Both the regulators and the effectors of the stress response have to emerge at appropriate times and in the required qualities

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Abbreviations used: BrdU – bromodeoxyuridine; CTD – carboxy-terminal domain; Doxo – doxorubicin; DRB – 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole; P-TEFb – positive transcription elongation factor b; RNA Pol II – RNA polymerase II

and quantities. p53 is a transcription factor that activates target genes leading to cell cycle block or apoptosis in response to DNA damage, hypoxia, signals of misregulated oncoproteins, and related forms of stress [1]. The cell cycle arrest program involves the induction of p21, the cyclin-dependent kinase inhibitor of the CIP/KIP family, or 14-3-3σ, the inhibitor of G2/M transition [2]. The apoptotic program induces both the components of the mitochondrial pathway, such as PUMA, or the death receptor pathway, such as FAS/APO1 [3, 4, 5]. The stress-induced activities of p53 depend on the type of stress as well as the type and status of the cell so that it can either repair the damage or subject itself to apoptotic destruction [6].

Timely induction of gene products can be achieved at various steps of the gene expression process. Inducible expression strategies of primary versus secondary response genes differ. Typically, the primary response genes are regulated at post-initiation steps [7]. Their promoters often contain CpG islands leading to less stable nucleosomes, maintain high levels of H3K4me and H4K5/8/12Ac modifications, and have RNA Pol II pausing close to or at the transcription start site in the uninduced state [7, 8, 9]. Among the target genes of p53, the cell cycle arrest genes, such as p21, harbor RNA Pol II with S5-phosphorylated carboxy-terminal domain (CTD) and negative elongation factors within their core promoters and are regulated at the post-initiation stage of the transcriptional cycle [10, 11]. In contrast, the proapoptotic genes, which are induced later relative to p21, contain low levels of promoter-bound RNA Pol II; they are likely regulated at the level of preinitiation complex assembly and their expression usually lasts longer [12]. Recently, the p21 core promoter was found to assemble preinitiation complex (PIC) efficiently but had limited capacity for reinitiation. In contrast, the FAS/APO1 core promoter was inefficient in PIC assembly but supported multiple rounds of transcription [12]. In addition, most cell cycle arrest genes have few start sites and typical core elements (focused promoters), whereas most of the proapoptotic promoters have several start sites and few core elements (dispersed promoters) [12, 13]. In the proapoptotic PUMA gene, the dispersed association of general transcription and elongation factors was found to be constitutive, leading to basal transcription within ~6 kb of the 5' part of the gene. p53 induced the production of full length mRNA of this gene by levying intragenic boundaries of repressor proteins CTCF and cohesin [14, 15].

Induction of expression at the post-initiation stage requires regulation of the interplay between negative and positive elongation factors. Positive transcription elongation factor b (P-TEFb) is recruited to the p21 locus after p53 stimulation [11]. P-TEFb is a heterodimer formed from Cdk9 kinase and C-type cyclin (CycT1, CycT2A, CycT2b or CycK) [16]. Activity of Cdk9 leads to serine phosphorylation of RNA Pol II (at position 2 of its CTD) and of the Spt5 subunit of the negative elongation factor DSIF. These and other events trigger the elongation phase of transcription [17]. Remarkably, inhibition of the P-TEFb kinase Cdk9 by 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) leads to

extensive inhibition of mRNA synthesis, but leaves the p53 response mostly unimpaired [18]. Gomes and coworkers showed that while the expression of the housekeeping genes decreases after DRB treatment, both p21 and PUMA were induced. ChIP, RNA, and apoptosis analyses confirmed that neither RNA Pol II S2 phosphorylation nor FACT recruitment were required for proper mRNA maturation and protein induction of these p53 targets [11].

P-TEFb was shown to cooperate with a number of other factors to support productive transcription elongation; at the same time, it was found to be dispensable for at least some intron-containing stress-induced genes, such as p21, or intronless genes, such as histone H2B [19]. Partial specific inhibition of Cdk9 activity by the overexpression of dominant negative Cdk9 protein led to both repression and upregulation of only a limited subset of genes in human cells [20]. It is intriguing to ask whether any of the P-TEFb partners can substitute its function in cases of stress-induced Cdk9/CycT-independent gene expression. One such factor is SNW1, which was found to bind P-TEFb in nuclear extracts and Tat:P-TEFb on the HIV-1 promoter [21]. SNW1 was classified as a transcriptional co-regulator in a number of instances including the pathways of nuclear hormone receptors, Notch/CBF1, TGFβ/Smad2/3, MyoD, or pRb [22]. At the same time, it was shown to be part of catalytically competent spliceosomes [23] and to affect the splicing of both HIV-1 and endogenous transcripts [21, 24].

In this report, we have asked whether SNW1 is required for the transcription of stress-induced genes of the p53 transcriptional program. We show that DRB- and doxorubicin-induced accumulation of several p53 target proteins in HCT116 cells is refractory to SNW1 downregulation. This extends for endogenous genes the observation that genotoxic stress induced the transcription of HIV independently of both P-TEFb and SNW1 [25].

MATERIALS AND METHODS

Cell culture and transfections

HCT116 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum. Transfection with siRNA and subsequent incubations with inhibitors were performed in Accell medium (Dharmacon) supplemented with 1% fetal bovine serum. All cultures were grown at 37°C in a humidified atmosphere containing 5% CO_2. For transfections, cell cultures were grown to 50%–60% confluency. Transfections were performed according to the manufacturer's protocol using 1 μM concentration of SNW1 targeting Accell siRNA mix (Dharmacon). Doxorubicin (0.5 μM; Sigma-Aldrich) or DRB (50 μM; Sigma-Aldrich) was added 3 days after transfection.

Real-time RT-PCR analyses

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription (RT) reactions were

performed using RevertAid First Strand cDNA Synthesis Kit (Fermentas) using Oligo(dT)$_{18}$ Primers. Real-time PCR reactions were done with 12.5 µl SYBR Green PCR Master Mix (BioRad) on a BioRad iCycler with the following primers (published in [11]) – 14-3-3σ: forward 5′- GCCGAACGCTATGAGGACAT, reverse 5′- CTTCTCCACGGCGCCTT; PUMA: forward 5′- ACGACCTCAACG CACAGTACG, reverse 5′- TCCCATGATGAGATTGTACAGGAC; HPRT: forward 5′- TGACACTGGCAAAACAATGCA, reverse 5′- GGTCCTTTT CACCAGCAAGCT; 18S rRNA (published in [26]): forward 5′- GCTTAATTTGA CTCAACACGGGA, reverse 5′- AGCTATCAATCTGTCAATCCTGTC. Values were normalized to those of 18S rRNA using the ddC$_T$ method [27].

Protein immunoblot analyses

Typically, 10 µg of total protein extract was loaded onto 7.5, 10, or 12% SDS-PAGE gel and transferred to nitrocellulose membrane (Bio-Rad). Blots were probed with primary antibodies against SNW1 (H-300, sc-30139; Santa Cruz), p53 (DO-1, sc-126; Santa Cruz), p21 (12D1, 2947; Cell Signaling), RNA Pol II (H-224, sc-9001, 8WG16, sc-56767; Santa Cruz), 14-3-3σ (C-18, sc-7683; Santa Cruz), or PUMA (N-19, sc-19187; Santa Cruz) and developed with the appropriate peroxidase-conjugated secondary antibodies (Santa Cruz).

Immunofluorescence

For indirect immunofluorescence, cells were washed twice in PBS, fixed with 4% paraformaldehyde in PBS with 10 mM $MgCl_2$ for 10 min at room temperature, and subsequently permeabilized with 0.5% Triton X-100 in Mg-PBS for 15 min at room temperature. The cells were then rinsed in Mg-PBS, blocked in 3% BSA in Mg-PBS, incubated for 2 h with primary antibodies diluted in blocking solution, washed in PBS, and incubated for 60 min with the appropriate secondary antibodies (Alexa Fluor; Molecular Probes). We used antibodies directed against SNW1, RNA Pol II (see above), α-tubulin (B-7, sc-5286; Santa Cruz), or BrdU (PRB-1, A21300; Molecular Probes); DNA was labeled with DAPI (Lonza). Images were acquired by a Leica TCS SP2 microscope system using Leica 63×/1.45 and 20×/0.7 oil objectives.

Run-on transcription assay

Cells were grown on cover slips to approx. 50% confluency, washed with glycerol buffer (20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EGTA, and 25% glycerol), and incubated at room temperature for 3 min each in glycerol buffer and in glycerol buffer containing 0.05% Triton X-100 and 10 U/ml RNasin. Transcription reactions were run for 20 min at room temperature in Run-on buffer (50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM EGTA, 100 mM KCl, 25% glycerol, and 20 U/ml RNasin) containing 0.5 mM each of ATP, CTP, GTP, and BrUTP (B7166; Sigma). Cells were then washed three times in TBS buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, and 0.05% Triton X-100) and fixed in 4% paraformaldehyde. Immunofluorescence staining was performed as described above.

RESULTS AND DISCUSSION

SNW1 associates with chromosomes after mitosis

SNW1 is an essential gene [28] present in one copy per genome throughout eukaryotes [29]. The protein was found as part of interchromatin granule clusters in interphase nuclei [30] and its nuclear distribution paralleled the speckled pattern of the SR protein SC-35 [31]. Since SNW1 was shown to be associated with both ongoing transcription, through its association with P-TEFb [21], and splicing, through its participation in spliceosomal complexes [23], we were interested in obtaining a more detailed view of its localization in relation to sites of active transcription. Using confocal microscopy, we analyzed HeLa cell nuclei double stained for SNW1 and Pol II (Fig. 1A). The signals colocalized in places, but there was no generalized overlap. Line scans through two selected areas document such overlap of the signals, but in most instances the distribution patterns suggest close proximity rather than colocalization. To visualize the sites of active transcription, a run-on assay was performed on permeabilized HeLa cells using BrUTP incorporation. Immunostaining was done on fixed cells using anti-BrdU antibody. Similarly to anti-RNA Pol II, anti-BrdU and anti-SNW1 antibody signals colocalized only in part, confirming that only a fraction of the SNW1 pool directly colocalizes with actively transcribing complexes (Fig. 1B). At the same time, many foci of BrUTP incorporation are devoid of SNW1 presence. SNW1 thus exists in pools which are distinct in part from the sites of transcribing RNA Pol II. Such distribution is similarly seen with other splicing factors, such as the SR-type splicing factor SF2/ASF [32], and is in agreement with the localization of SNW1 to speckles. It has been reported that BrUTP incorporation can be found proximal to speckles, although not within the speckle domain itself [33, 34].

During mitosis, SNW1 showed no tendency of colocalization with the chromosomes or the microtubule organizing center(s) (MTOC), as would be expected for a splicing factor or a protein binding to various transcription factors (Fig. 1C, 2B and data not shown). However, during cytokinesis, before the chromosomes were decondensed in G1, they became enriched in SNW1 (Fig. 1C, last panel). This observation is interesting, because P-TEFb enters chromosomes earlier than other transcription factors and associates with late anaphase and telophase chromosomes through its interaction with the bromodomain protein Brd4 [35].

Mitosis proceeds aberrantly when SNW1 is downregulated by RNA interference. We reduced the protein levels to ~30% transfecting cells with SNW1-directed siRNA oligos in Accell medium (Dharmacon; Fig. 2A). Control oligos showed no effect (data not shown). We analyzed mitoses in HeLa cells and observed the formation of aberrant spindles with multiple MTOCs in a subpopulation of cells (Fig. 2B); we did not observe any decrease of tubulin using either immunofluorescence or Western analyses (data not shown). The

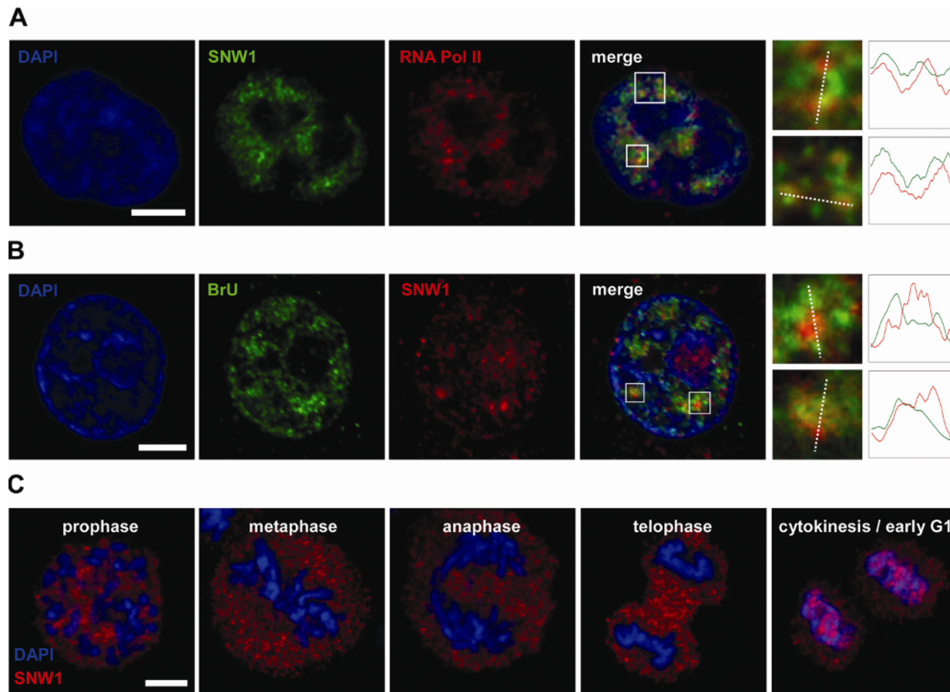

Fig. 1. Nuclear distribution of SNW1. HeLa cells were fixed and labeled with antibodies against SNW1 and RNA Pol II (8WG16); DNA was visualized by DAPI (A). Sites of transcription were marked by the incorporation of BrUTP in permeabilized HeLa cells. The cells were then fixed and labeled by antibodies against SNW1 and BrdU; DNA was stained by DAPI (B). Two areas boxed in the merge panel are zoomed out in the small panels on the right. Dotted lines indicate the x-axis of the line scan of fluorescent intensities (y-axis). The intensity profiles in the red and the green channel are then plotted in the far right panels using corresponding colors (A, B). The localization of SNW1 in mitotic nuclei was marked by SNW1 antibody on fixed HeLa cells; chromosomes were labeled by DAPI (C). Scale bars are 5 μm.

cells which were able to accomplish nuclear division with the aberrant spindles apparently formed aneuploid daughters (Fig. 2B, last panel). We also observed the distribution of Cdk9 and RNA Pol II (8WG16) in siRNA-treated interphase nuclei and found no differences in their distribution (data not shown).

We assume that SNW1 affects splicing or expression of genes involved in spindle formation and chromosome segregation. Similar cell cycle phenotype was observed for the alleles of the splicing factor UAF35 [36]. The role of SNW1 in cell cycle regulation was proposed previously based on similar observations in high-throughput analyses [37, 38]. Nevertheless, nuclear distribution of SNW1 during mitosis does not indicate that this protein has a direct role in chromosome organization or the formation of MTOCs. Cell division requires that multiple transcripts are switched on and off in a concerted way, which is perhaps why this process is primarily affected in mutants of

splicing or transcription regulators. In *Saccharomyces cerevisiae*, mutations of the SNW1 homolog Prp45 lead to cell shape and division defects at permissive temperatures; at restrictive temperatures, when the cells cease to divide, they are stalled without any apparent shape deformations ([39] and unpublished observations).

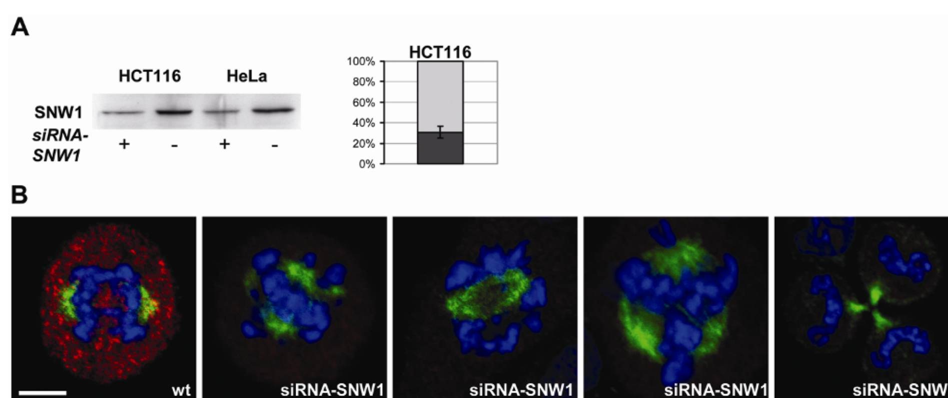

Fig. 2. Downregulation of SNW1 leads to aberrant nuclear division. HCT116 and HeLa cells were transfected with SNW1 targeting siRNA using the Accell protocol (Dharmacon). After 3 days, cells were lysed and the levels of SNW1 protein were compared on a Western blot using anti-SNW antibody (A). The immunoblots from independent HCT116 transfections were quantified densitometrically using Image Quant software and expressed as percent of control (right). SNW1 levels were reduced to 30.9 ± 5.6% (n = 11). Mitotic HeLa cells from control and siRNA-treated cultures were fixed and labeled by antibodies against SNW1 and α-tubulin; DNA was stained by DAPI (B). The panels show merges of all three signals; four different siRNA-treated cells are shown for comparison. SNW1 levels in siRNA-treated cells are substantially reduced. Scale bars are 5 μm.

p53 response genes are induced independently of SNW1

The comparison of the pharmacological inhibition of Cdk9 activity by DRB with the induction of DNA damage by doxorubicin demonstrated that P-TEFb is differentially required for the activation of p53 target genes [11]. DRB inhibits both Cdk7 and Cdk9 and decreases mRNA synthesis by 50% at concentrations above 40 μM. The inhibition of transcription by DRB activates the p53 transcriptional program and eventually leads to apoptosis [40, 18]. In contrast, DNA damage inducing doxorubicin, which does not inhibit CTD phosphorylation, activates a partly different panel of genes and leads to cell cycle block [10]. p53, p21, and PUMA were induced after both DRB and doxorubicin treatment, whereas 14-3-3σ or DR5/Killer accumulated only under doxorubicin [11]. These results suggested that while genes such as 14-3-3σ are dependent on P-TEFb activity for efficient induction, p53 and some of its targets are not. We asked whether SNW1, which is a P-TEFb partner implicated in HIV-1 elongation [21], is likewise dispensable for p53-dependent induction of p21.

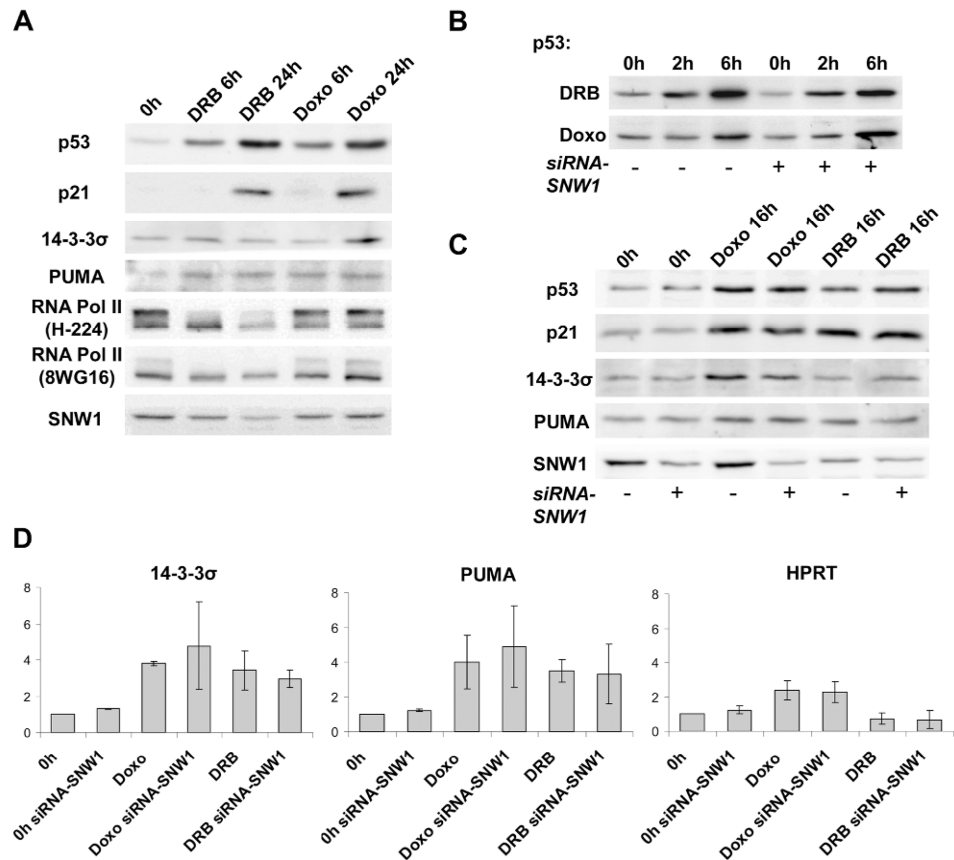

Fig. 3. Stress induction of p53 response genes. HCT116 cells were transfected with SNW1 targeting siRNA using the Accell protocol (Dharmacon). After 3 days, the cells were treated with doxorubicin (0.5 μM) or DRB (50 μM) for the indicated times. Proteins were detected using specific antibodies (Methods). Treatment of cells with DRB or doxorubicin led to the accumulation of p53 and to the induction of p53 effectors p21 and PUMA (A). Phosphorylation status of RNA Pol II was analyzed by two commonly used antibodies, 8WG16 and H224. DRB-treated cells showed hypophosphorylated RNA Pol II, slightly reduced SNW1, and failed to induce 14-3-3σ. Downregulation of SNW1 had no effect on the accumulation of p53 (B) or the p53 effectors after either drug treatment (C). The changes of mRNA levels of PUMA and 14-3-3σ were quantified by qRT-PCR and compared to the housekeeping gene HPRT (D). The data are from four independent experiments.

We examined the time-dependent accumulation of p53, p21, 14-3-3σ, and PUMA in colon cancer derived cell line HCT116 following DRB or doxorubicin treatment (Fig. 3A). We confirmed the accumulation of p53, p21, PUMA, and 14-3-3σ in response to doxorubicin-induced DNA damage. After DRB treatment, only p53, p21, and, to a lesser extent, PUMA were induced. In addition, phosphorylated RNA Pol II was depleted as expected and we also observed a slight reduction of SNW1 level under DRB. The latter finding

indicates that P-TEFb is required for transcription of the SNW1 gene or that DRB treatment leads to SNW1 degradation.
The siRNA-mediated downregulation of SNW1 did not affect p53 accumulation in either DRB- or doxorubicin-treated cells (Fig. 3B and 3C). Likewise, we reproducibly observed the induction of p21 or PUMA proteins after both treatments irrespective of SNW1 levels. Doxorubicin-induced expression of 14-3-3σ also did not show significant differences between siRNA-treated and control cells (Fig. 3C). Quantitative RT-PCR analysis of 14-3-3σ and PUMA mRNA levels confirmed the Western analyses (Fig. 3D). We included HPRT as a housekeeping gene which is not under p53 control. Values were normalized to 18S rRNA.
Our results on SNW1-downregulated cells imply that SNW1 is dispensable for stress-induced accumulation of p53 and p21 proteins. p21 was shown to be regulated at the level of stalled transcription elongation and proved to be inducible even under DRB, when CTD of RNA Pol II is devoid of S2-phosphorylation and the association of elongation factors (SPT16) is derailed [11]. SNW1 was proposed to act in concert with P-TEFb and in complex with c-Myc and Menin to promote HIV-1 transcription elongation [25, 21]. Recently, it was found that P-TEFb and SNW1 are dispensable for UV stress-induced HIV-1 transcription [25]. Our results extend this observation for the endogenous regulators p53 and p21. For p21, splicing must proceed unhampered in SNW1-downregulated cells for the protein to accumulate. We cannot refute the possibility that the residual level of SNW1 is sufficient to maintain functional splicing of most genes, leading to defects only in some instances of alternative splicing of, e.g., cell cycle related genes. Alternatively, SNW1 may have gene-specific roles in (alternative) splicing.
In response to stress, cells apparently activate transcriptional programs which are uncoupled from the regulatory circuitries that normally operate to ensure tight and timely coordination of transcription and transcript processing [25]. Because P-TEFb is believed to overcome the activities of negative transcription elongation factors, there are two scenarios possible: (i) P-TEFb is substituted by some other, as yet unidentified, factor, or (ii) stress-induced transcription is exempt from the activities of both the negative and positive regulators.

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