

Tumor suppressor p53 down-regulates expression of human leukocyte marker CD43 in non-hematopoietic tumor cells

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Abstract. CD43 (leukosialin, sialophorin), a cell surface protein on most hematopoietic cells, is an important regulator of immune cell function and is involved in regulation of cell adhesion and proliferation. Aberrant expression of CD43 is a common event observed in human tumors of non-hematopoietic origin suggesting a role in tumor development. We have previously shown that overexpression of CD43 causes activation of the ARF-p53 tumor-suppressor pathway and results in cell death. In a non-functional ARF-p53 background, the cells overexpressing CD43 display an increased cell growth rate due to higher survival. Here we show that p53 specifically downregulates the expression of CD43 at the protein and mRNA level. Transactivating properties of p53 are necessary to affect the expression of exogenous CD43. The downregulation of CD43 mRNA is caused by p53-dependent transrepression, at least in part, via a histone deacetylation mechanism. These studies establish that under certain conditions there exists a negative feedback loop between p53 and CD43: CD43-dependent signaling activates p53, which in turn downregulates the expression of CD43.

Introduction

p53, a well-studied tumor suppressor protein, is a transcription factor that is activated in response to different types of cellular stress e.g. DNA damage and oncogenic activation (1). Activated p53 induces cell cycle arrest and/or apoptosis through activation and repression of downstream target genes. p53 activation by oncogenes is mediated by ARF tumor suppressor protein that stabilizes p53 protein by interfering with Mdm2-mediated p53 degradation (2). Besides transactivation of target genes, p53 inhibits directly or indirectly the expression of several genes related to cell survival, such as *c-myc* (3), *cyclin D1* (4,5) and *survivin* (6). There are different ways of transrepression by p53. It can repress promoters that lack a p53-binding site or acts in

sequence-specific manner, either through interference with the functions of basal transcriptional machinery or specific transcriptional activators. Alternatively, p53-dependent repression may occur through recruitment of histone deacetylases and chromatin remodeling (7). Data from several studies suggest that p53-mediated transrepression is mostly required for apoptosis induction. The elimination of damaged and abnormally proliferating cells by p53 is considered to be crucial for tumor prevention, especially because the p53 gene is the most commonly inactivated gene in human cancers.

Leukocyte marker CD43 is a heavily glycosylated transmembrane protein, which is involved in regulation of cell adhesion and cell proliferation in hematopoietic cells and is an important contributor to immune homeostasis. CD43 has multiple, even opposite functions: it plays a role both in pro-adhesion and anti-adhesion, locomotion, apoptosis modulation and differentiation (8). The cytoplasmic domain of CD43 mediates its role as a signal transducer (8). In hematopoietic cells, CD43 expression is tightly regulated at the transcriptional level (9,10) as well as at the protein level e.g. by increasing or decreasing its surface expression (11).

CD43 expression has been detected in different tumors of non-hematopoietic origin, including lung and colon, but not in normal tissue (12-14). Its expression has also been described in several cancer cell lines (15,16). CD43 expression has so far been considered to be specific exclusively for immune cells, and therefore the role of CD43 protein and regulation of its expression have been studied mainly in the hematopoietic system. In non-hematopoietic cancer cells CD43 protein is located primarily intracellularly and even nuclearly. In contrast, in blood cells CD43 is expressed on the cell membrane (17). The cytoplasmic domain of CD43 contains a nuclear localization signal, which offers an explanation to its presence in the nucleus. Moreover, the cytoplasmic domain of CD43 interacts with β -catenin, a known proto-oncogene, and causes the upregulation of β -catenin downstream target genes *c-Myc* and *cyclin D1*. These data suggest that CD43 can be involved in cell proliferation via the β -catenin signaling pathway (18).

Our previous studies have shown that CD43 overexpression in non-hematopoietic cells activates the ARF-p53 tumor suppressor pathway and leads to apoptosis of these cells (19). It is well known that loss of p53 or ARF function allows cell survival and tumor progression. We have found that in cancer cells with defective ARF-p53 signaling, elevated CD43 expression promotes cell growth by increasing cell survival due to

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helping the cells to escape from Fas-mediated apoptosis (20). Our results support a suggestion that CD43 may have a role in non-hematopoietic tumor development due to its stimulating effect on cell proliferation.

Based on the above observations, we hypothesized that potential crosstalk exists between CD43 and p53-mediated signaling. The aim of the present study was to further characterize the interplay between p53- and CD43 signaling pathways. We found that p53 downregulates CD43 expression at both the transcriptional and posttranscriptional levels. p53 specifically reduces the levels of both endogenous and exogenous CD43 protein and transcriptional activity of p53 is required for this downregulation. We show that p53 represses CD43 transcription, at least in part, through a mechanism that involves histone deacetylation. Our results suggest that also other events are likely to contribute to p53-mediated repression of CD43 expression. Together, these results demonstrate that p53 transcriptionally downregulates CD43 expression providing a negative regulatory loop between p53 and CD43.

Materials and methods

Cell culture, expression vectors and transfections. The H1299 (p53-null human non-small cell lung carcinoma), Colo205 (mutant p53 expressing colon adenocarcinoma) and HCT116 (human wild-type p53 expressing colon carcinoma) cells (obtained from the American Type Culture Collection) were cultured in 5% CO₂ and at 37°C in IMDM (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Gibco Life Technologies).

Transfections were performed using the electroporation method as described earlier (21) or the ExGen reagent according to the manufacturer's instructions (Fermentas). Human wild-type and mutant p53 in pCG vector, p14ARF in pCG vector, and CD43 in pcDNA vector have been previously described (19,21). The pEGFP-F vector containing farnesylated EGFP was purchased from Clontech. The sequence of the p21^{CIP1/WAF1} promoter was cloned into the promoterless luciferase reporter plasmid pGL-3-basic (Promega). The reporter vector containing cyclin G promoter was kindly provided by Dr M. Oren. The TOPFLASH reporter plasmid containing TCF-binding sites and Renilla luciferase control vector were purchased from Upstate and Promega, respectively.

Western blot analysis. Western blot analysis was performed as described previously (19). The primary antibodies used were anti-CD43 4D2 (17), anti-p53 DO-1 (Eurogenetics), anti-p14ARF (Abcam Ltd.), anti-EGFP (Abcam Ltd.) and anti-β-actin (Abcam Ltd.). Secondary antibodies used were AP-conjugated (alkaline phosphatase) or HRP (horseradish peroxidase)-conjugated (LabAS Ltd., Estonia). Signals were detected using the color reaction or ECL Western blotting detection kit (Millipore).

mRNA analysis. Total RNA from cells was extracted using the Qiagen RNeasy kit according to the manufacturer's protocol. Northern blot analysis was performed as described previously (19). Quantitative RT-PCR was performed with gene-specific primers using the ABsolute™ QPCR SYBR-Green ROX mix (Abgene) and the ABI Prism 7900HT real-time PCR system

(Applied Biosystems) according to the manufacturer's recommendations. Data were normalized against GAPDH signals. PCR primers (all from Sigma-Proligo) for qRT-PCR were as follows: 5'-AGTGCTGCGTCCTTATCAGC-3' and 5'-GGCTC GCTAGTAGAGACCAA-3' for CD43, 5'-AGTCTGGCTTA TATCCAACACTTCG-3' and 5'-GACTTTGCTTTCCTTGG TCAGG-3' for HPRT, 5'-tgtctttcagcaaggactgg-3' and 5'-gatg ctgcttacatgtctcg-3' for B2M, 5'-CTGGAAGATGGTGATGG GATT-3' and 5'-ATGTTCCAATATGATTCCACCCA-3' for GAPDH.

Chromatin immunoprecipitation (ChIP) assay. Cells (6x10⁶) were cross-linked with 1% formaldehyde for 15 min at room temperature. The cells were then washed, lysed in FA lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate and protease inhibitors) and sonicated to shear DNA to an average fragment size of 500 bp. Antibodies against K14-acetylated histone H3 and total histone H3 (Upstate), linked to protein A-Sepharose beads (Amersham), were used for immunoprecipitation. After overnight incubation at 4°C, the immune complexes were washed three times and extracted with elution buffer (100 mM Tris, pH 7.8; 10 mM EDTA; 1 % SDS; 400 mM NaCl), and heated at 65°C for 4 h to reverse crosslinks. DNA was purified and analyzed by quantitative real-time PCR using an ABI Prism 7900HT real-time PCR system under standard conditions (95°C for 15 sec and 60°C for 1 min for 40 cycles) with Absolute QPCR SYBR-Green Mix (Abgene). The primer pairs were used to amplify the flanking region of the CD43 promoter (5'-ATCCCATTCTCAGCCAAGA-3' and 5'-ACCATCACCT GCTCCTTCA-3') and encompass the promoter region of p21 (5'-CTGTGGCTCTGATTGGCTTT-3' and 5'-AGGTCTCTG TCTCTACCA-3), and for GAPDH (5'-CTGGAAGATGGT GATGGGATT-3' and 5'-ATGTTCCAATATGATTCCAC CCA-3') to normalize the data.

Reporter assay. Transfected cells were prepared for reporter assays 24 h after transfection and reporter activity was determined with the Dual-Luciferase Reporter Assay System (Promega). Results were expressed as relative luciferase activity or folds of induction obtained by the ratio of the Firefly/Renilla luminescence.

Results

p53 influences the expression of CD43 protein. To investigate the possible effect of p53 on CD43 we analyzed the influence of exogenous p53 on ectopically expressed CD43 protein levels in p53-null human non-small cell lung carcinoma H1299 cells. Coexpression of exogenous p53 and CD43 resulted in a significant decrease in CD43 protein levels in these cells compared to p53-null cells (Fig. 1A). Under the same conditions p53 does not reduce the levels of farnesylated EGFP (EGFP-F), which is expressed on the cell membrane similarly to CD43. This also rules out a possible effect of p53 on the CMV promoter function. Our previous studies showed that overexpression of full length CD43 activates p53 and leads to programmed cell death (19). The current result suggests the existence of a negative feedback loop between p53 and CD43, where CD43 overexpression induces p53 and results in the downregulation

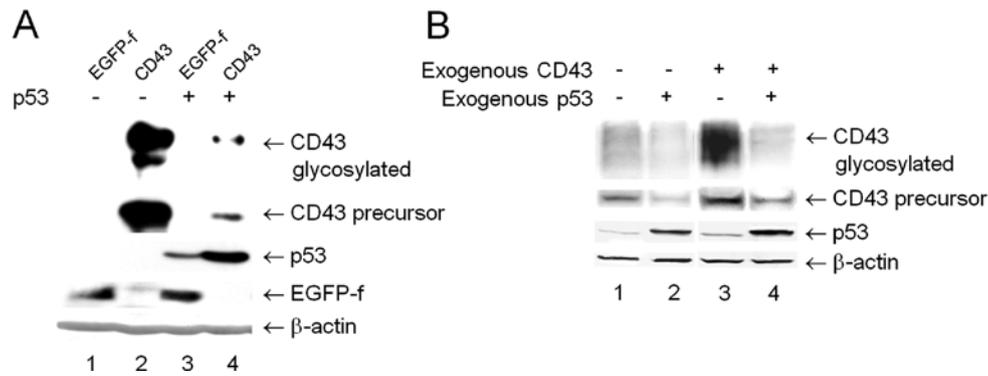


Figure 1. p53 reduces both endogenous and exogenous CD43 protein levels. H1299 (A) and Colo205 (B) cells were cotransfected with indicated expression plasmids. Proteins were extracted 24 h after transfection, and analyzed by Western blot using specific antibodies.

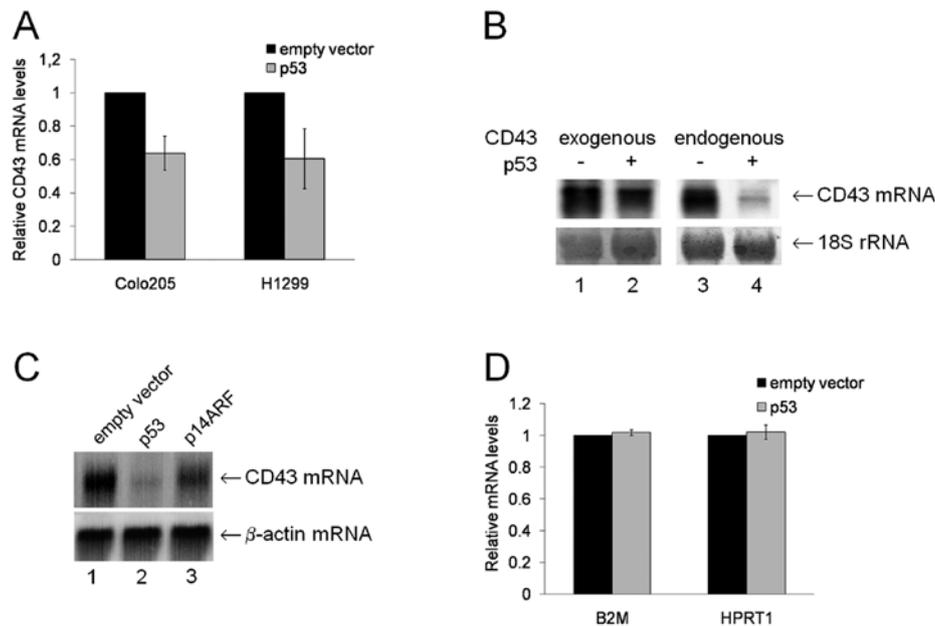


Figure 2. wt p53 specifically reduces the endogenous CD43 mRNA level. (A) CD43 mRNA levels in H1299 and Colo205 cells analyzed by quantitative RT-PCR. (B) Exogenous and endogenous CD43 mRNA levels in H1299 cells analyzed by Northern blot. (C) Endogenous CD43 mRNA levels in H1299 cells analyzed by Northern blot. (D) B2M and HPRT1 mRNA levels in H1299 cells analyzed by quantitative RT-PCR. The cells were mock-transfected or transfected with expression vectors as indicated on graphs and blots. The mRNA level was determined 24 h posttransfection with either specific primers by qRT-PCR or with specific probes by Northern blotting. In the case of qRT-PCR, after normalization to GAPDH, the mRNA levels of interest are expressed as an average of three independent experiments, and Northern blots shown are representative of three independent experiments.

of CD43 expression. Having established that p53 influences the expression of exogenous CD43, we next examined whether introduction of wt p53 into Colo205 colon carcinoma cells expressing mutant p53 affects the levels of endogenous CD43. The endogenous CD43 levels were affected in a similar way in human colon carcinoma cells (Fig. 1B). Expression of wt p53 resulted in the reduction of the CD43 protein levels of both exogenous and endogenous CD43 in these cells. Wt p53 specifically reduces the levels of both precursor and fully glycosylated CD43 (Fig. 1A and B). We also confirmed that the lower levels of CD43 protein observed in p53-expressing cells were not caused by apoptosis at the time point of 24 h (data not shown), as p53 and CD43 coexpression might induce apoptosis of the cells (19). Elevated levels of CD43 induce p53 activity via the ARF tumor suppressor protein (19). We tested the possibility that ARF alone can affect CD43 levels, because ARF has both

p53-dependent and p53-independent tumor-suppressive activities (22). ARF alone did not affect CD43 protein levels (data not shown). Taken together, these results indicate that p53 has specific impact on CD43 expression and this repression occurs, at least in part, at the posttranscriptional level.

p53 reduces CD43 mRNA levels in H1299 and Colo205 cells. p53 reduced the endogenous levels of CD43 protein in Colo205 cells. To determine whether this p53-dependent decrease in endogenous CD43 protein levels was due to the reduction in the amount of CD43 mRNA, we used H1299 and Colo205 cells. The cells were transfected with the p53 expression vector or an empty control vector and harvested 24 h posttransfection followed by RNA purification and analysis by qRT-PCR. We observed a significant reduction in CD43 mRNA levels in cells overexpressing wt p53 (Fig. 2A). Northern blot analysis

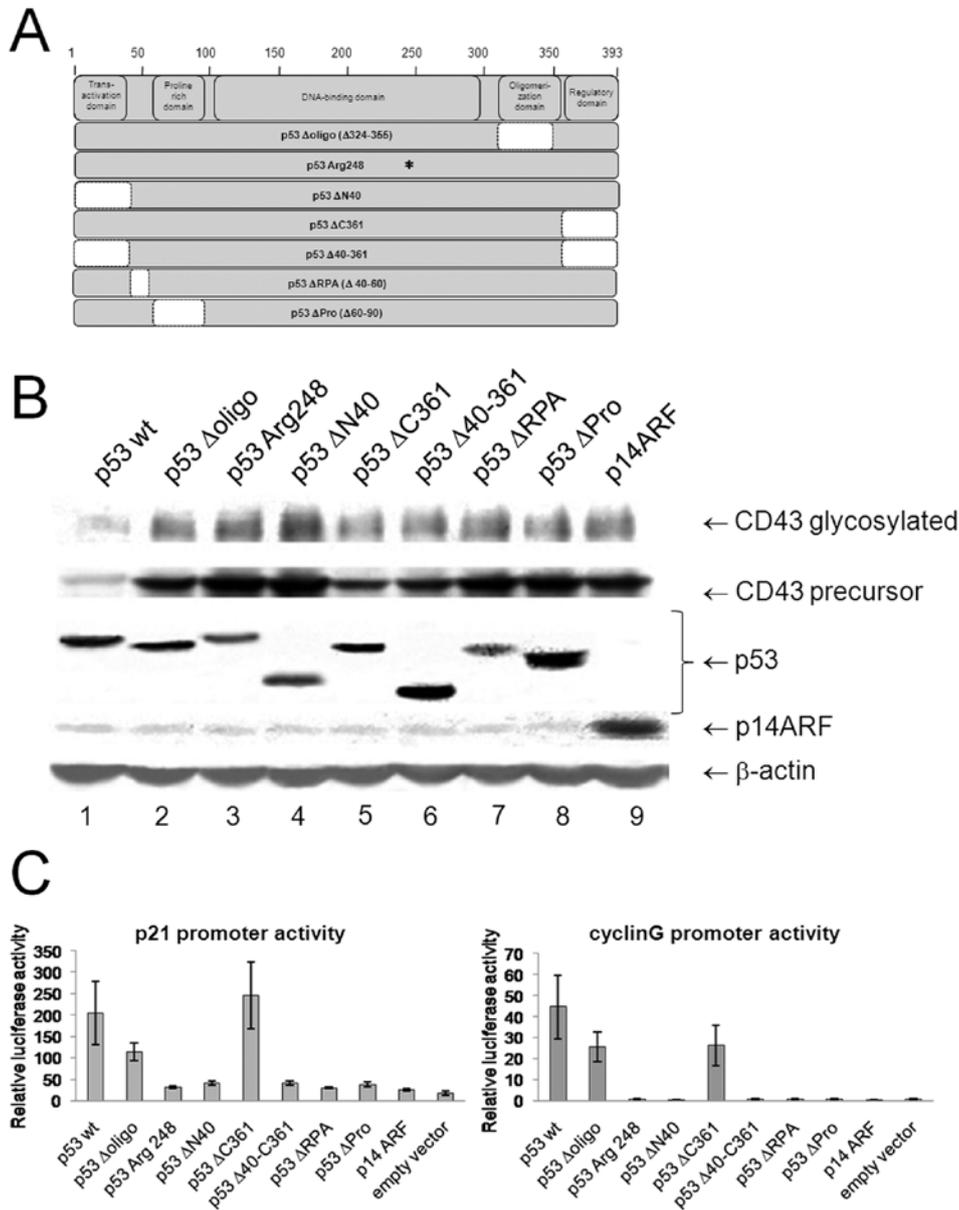


Figure 3. Transactivation activity is required for p53-dependent downregulation of CD43 protein level. (A) Schematic representation of the mutant p53 proteins used in the study. Functional domains of the wt p53 protein are shown on the top. The p53 mutants are listed below. White squares indicate deleted regions in the protein. The asterisk indicates the location of a point mutation (Arg to Trp). Δ Pro marks for deletion of amino acids 60-90, corresponding to the proline rich domain and Δ RPA for deletion of amino acids 40-60, corresponding to the replication protein A-binding domain. (B) Ability of different p53 mutants to reduce ectopic CD43 protein levels analyzed by Western blot with specific antibodies. In the p53-negative H1299 cell line CD43 is coexpressed with wt p53 or p53 mutant proteins or p14ARF as a control. (C) Transactivating properties of p53 mutants from p21 and cyclin G promoters. A reporter plasmid is cotransfected with indicated expression vectors into H1299 cells. Twenty-four hours posttransfection, cell extracts were subjected to luciferase assay. Luciferase activity is presented as fold-induction relative to the cells transfected with a reporter plasmid and an empty vector. Results presented are the average of three independent experiments.

showed that p53 downregulates the expression of endogenous but not exogenous CD43 mRNA levels in H1299 cells (Fig. 2B), demonstrating that p53 does not affect the stability of CD43 mRNA, and the observed effect is indeed due to reduced CD43 gene expression. Our results also indicated that CD43 mRNA level is reduced when the cells overexpress p53, but this level is not considerably affected by tumor suppressor ARF expression alone (Fig. 2C). We used the expression of ARF tumor suppressor protein as an additional control to ensure that the effect is p53-specific.

To rule out a possible non-specific effect of wt p53, we assessed the mRNA levels from two non-relevant genes, β 2-microglobulin (B2M) and hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) by qRT-PCR, using similar experimental conditions in H1299 cells. B2M is a component of MHC class I molecules. HPRT1 gene encodes hypoxanthine guanine phosphoribosyltransferase, an enzyme involved in purine metabolism. p53 did not affect the expression of these two genes (Fig. 2D). Our results confirmed that CD43 gene expression is regulated by the tumor suppressor p53 and that

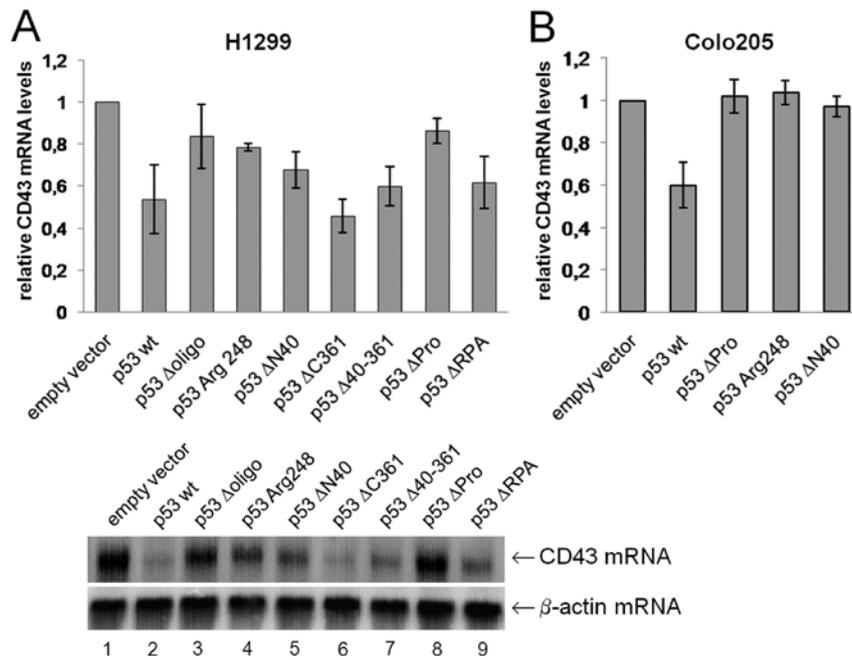


Figure 4. Influence of different p53 proteins on CD43 mRNA expression level analyzed by Northern blot in H1299 (A) and qPCR in Colo205 (B) cells. Cells are transfected with an empty vector control, with expression vectors of wt p53 and its mutants. The results from (A) were quantified by Phosphor Imaging (n=3). The bar graphs show relative CD43 mRNA levels to empty vector control. All values have been normalized against corresponding β -actin or GAPDH level, and represent the averaged results for three independent experiments.

p53 affects CD43 expression at both transcriptional and protein levels.

The transcriptional activation ability of p53 is required for regulation of CD43 protein level. To find out the regions and activities of p53 which are necessary to control the expression of CD43, we expressed different p53 deletion mutants and tumor-derived mutant Arg248 (Arg \rightarrow Trp) p53 protein (presented in Fig. 3A) in H1299 cells and assessed their ability to affect exogenous CD43 protein level by Western blot analysis. Again, we used the expression of ARF tumor suppressor protein as an additional control. Fig. 3B shows that the cells overexpressing wt p53 have lower CD43 protein levels than in the ones overexpressing ARF (lanes 1 and 9, respectively). None of the p53 mutants used, except the one with C-terminal deletion (Δ 361) was able to considerably affect CD43 protein levels.

In parallel, we monitored the transactivation activity of different p53 proteins by luciferase reporter assay using reporter construct containing the p53-binding motif from cyclin G1 and p21 genes (23,24) (Fig. 3C). The ability of different p53 mutants to reduce CD43 protein expression levels is clearly correlated with the ability of these mutants to activate transcription, since p53 mutants with absent transactivation domain or deficient in DNA binding are not capable of reducing CD43 levels (Fig. 3B). Although the levels of mutant p53 proteins vary, as seen in Fig. 3B, it does not correlate with their transactivation ability or reduced levels of CD43 protein. The results indicate that the DNA binding and transactivating properties of p53 are required to influence CD43 protein expression levels.

The proline-rich region of the p53 protein is required for p53-mediated repression of CD43 mRNA. We next determined which regions of p53 are required for the downregulation of CD43 mRNA. We performed Northern blot analysis or qRT-PCR of H1299 and Colo205 cells transfected with expression vectors encoding wt p53 or different mutant proteins (Fig. 4A and B). As shown in Fig. 4A, p53 mutant proteins have distinct effect on CD43 expression levels, but there is no clear correlation between p53-dependent transcriptional activity and CD43 mRNA levels, as was the case for the levels of CD43 protein. N-terminal transactivation domain, oligomerization domain and DNA-binding ability of p53 are, at least partly, responsible for the modulation of CD43 mRNA level in H1299 cells (Fig. 4A), but not in Colo205 cells (Fig. 4B). It has been shown that tetrameric p53 binds DNA more effectively than monomer, and amino acids 339-346 in the oligomerization domain are required for the transcriptional repression of p53 (25,26). p53 mediates transcriptional repression through various mechanisms. The ability of p53 to repress CD43 mRNA level may occur both via p53-dependent transactivation of other factors and transrepression of the CD43 gene itself. Our results show that p53 protein without proline rich region failed to down-regulate CD43 mRNA level both in H1299 and Colo205 cells (Fig. 4A, lane 8 and Fig. 4B, respectively). The proline-rich domain of p53 is important for transcriptional repression involving recruitment of histone deacetylases to target promoters (7). Our finding suggests that p53 downregulates CD43 mRNA level by the mechanism of transrepression.

p53 negatively regulates CD43 gene expression through histone H3 K14 deacetylation of CD43 promoter region. Based on our previous results, we hypothesized that histone deacety-

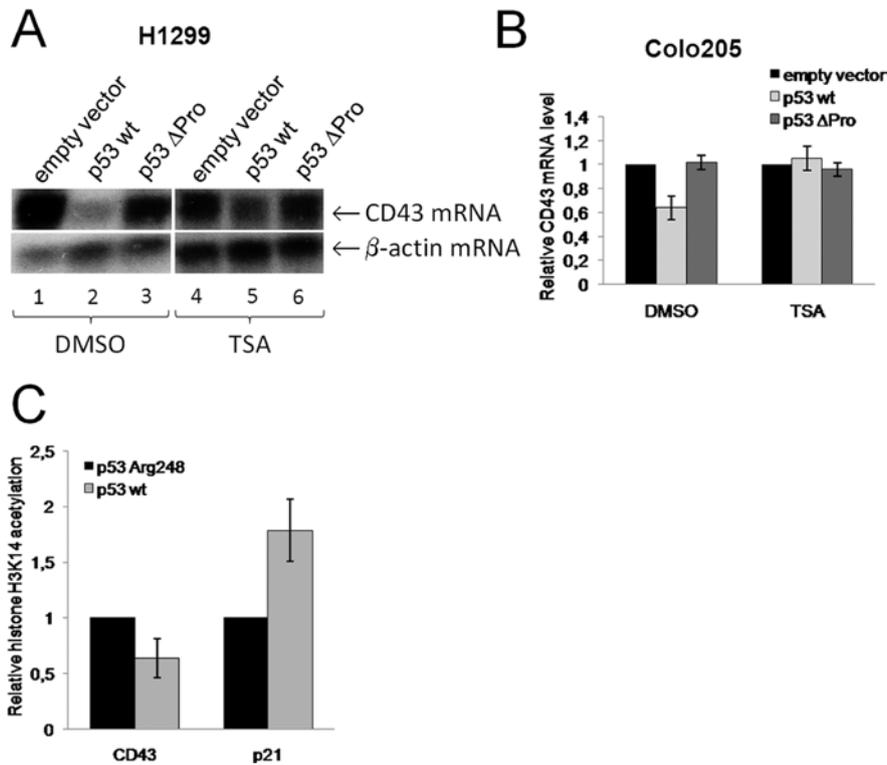


Figure 5. Transcriptional repression of CD43 by p53 is inhibited by the HDAC inhibitor TSA. The effect of trichostatin A on p53-mediated decrease in the level of CD43 mRNA analyzed by Northern blot in H1299 cells (A) and by qRT-PCR in Colo205 cells (B). ChIP for H3 acetylation in CD43 gene promoter region in H1299 cells (C). Wt p53 or p53 Arg248 proteins are expressed in H1299 cells and the chromatin is precipitated with an antibody which is specific for H3 K14 acetylation and an acetylation-insensitive anti-H3 antibody. p21 gene is used as a control. The bar graph shows relative acetylation.

lation could be involved in p53-mediated transrepression of the CD43 gene. To test this hypothesis, we used trichostatin A (TSA), an inhibitor of histone deacetylases (HDAC). H1299 and Colo205 cells (Fig. 5A and B, respectively) were transfected with wt p53 or p53ΔPro expression vectors and then treated with 100 ng/ml of TSA for 5 h. Expression of wt p53, but not of p53ΔPro, resulted in significant reduction of CD43 mRNA. In the presence of TSA, however, this repression was inhibited. Obviously, trichostatin A disrupts the ability of wt p53 to repress CD43 expression, elucidating the molecular mechanism underlying p53-mediated repression of CD43 transcription.

p53-dependent acetylation of histone H3 contributes to the promoter regulation of several genes. To test the possibility that p53 decreases the histone acetylation in the CD43 gene, wt p53 or p53Arg248 proteins were expressed in H1299 cells. The amount of histone acetylation was measured by chromatin immunoprecipitation (ChIP) assay using H3- and H3K14-specific antibodies. The result of the ChIP assay indicates that repression of CD43 expression is indeed accompanied by reduced acetylation at histone H3 K14. At the same time, increased acetylation at p21/Waf1 promoter was observed in wt p53 expressing cells (Fig. 5C). It is already known that p53-mediated transactivation of p21 transcription correlates with increased histone acetylation (27,28). These results prove that p53 initiates the deacetylation of histone H3 near the CD43 promoter.

The CD43 promoter region contains a p53 response element and the promoter activity is repressed by p53. The data shown so far indicate that p53 directly or indirectly represses CD43 expression. p53 can downregulate CD43 expression via sequence-specific transactivation of other genes or directly repress the activity of CD43 promoter. The consensus DNA-binding site for p53-mediated transactivation contains two copies of a 10-bp motif 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3', separated by a 0-13-bp spacer region (29). In addition to the p53 consensus sequence, binding sites required for p53-dependent repression have been described (6,30), but p53 is also capable of transcriptional repression from the consensus binding site (31,32).

Although trans-repression by p53 does not always require direct DNA binding, we decided to search for the possible p53 binding sites in the CD43 gene. We allowed up to 2 mismatches for the p53 consensus sequence and no mismatches for the trans-repression sequence. As shown in Fig. 6A, three putative p53 response elements were identified. Two sites with similarity to the consensus sequence lie ~1.5 kb upstream and 1.7 kb downstream of the transcription start site, and one sequence for trans-repression is adjacent to the minimal promoter region of the CD43 gene. These findings suggest a direct effect of p53 on CD43 promoter activity.

We therefore examined the p53-mediated transcriptional repression of CD43 by performing CD43 promoter-luciferase reporter gene assay. The CD43 genomic sequence from -91 to +90 exhibits strong promoter activity as found by Kudo and Fukuda (33). Fragments of 0,5 kb (-91 to +439) and 1,5 kb (-1029 to +439) containing the potential p53-dependent

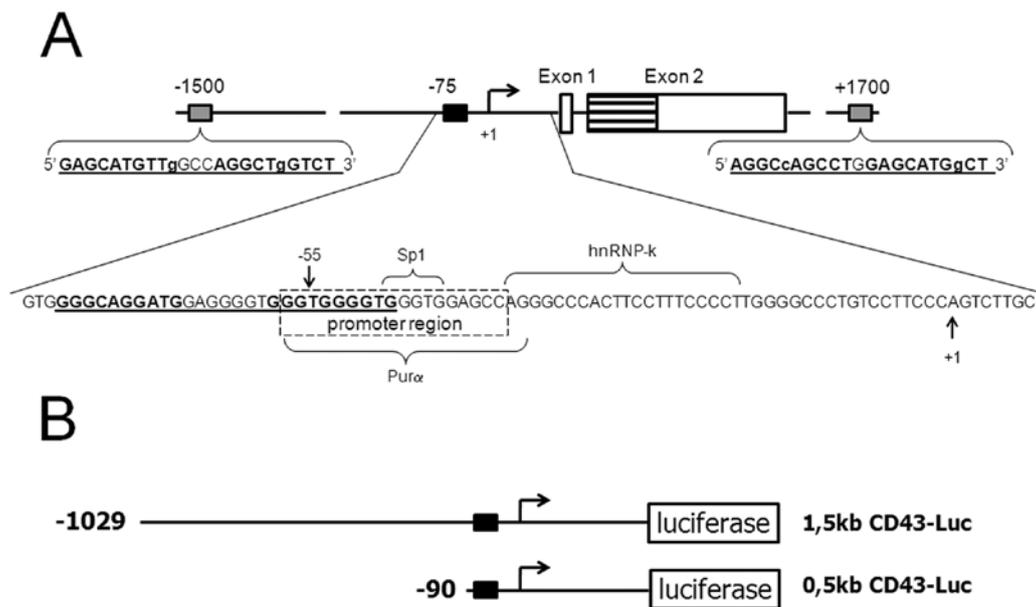


Figure 6. (A) Schematic representation of the CD43 gene. The promoter region and exons of the CD43 gene are shown. The striped box within the exon 2 box corresponds to the translated region. Three potential binding sites for p53 are indicated schematically and by underlined sequences. The black box represents the p53 binding site known to be necessary for p53-mediated transrepression. p53 consensus sites are indicated with grey boxes. Mismatches to the consensus sequence are shown by lowercase letters. Locations of the p53-binding sequences are given relative to the second transcription start site (+1 and bent arrow). The first transcription start site is marked as -55. The locations of the binding sites for known regulatory factors are indicated using braces. (B) Structure of CD43 promoter reporter constructs containing putative p53 binding site for transrepression (black boxes).

repression sequence, depicted in Fig. 6A and B, were cloned into a luciferase reporter vector. The resultant constructs were named 0,5 kb CD43-luc and 1,5 kb CD43-luc, respectively. An insert-free reporter vector served as a negative control. The reporter constructs, along with the vector controls and p53 expression plasmid, were transfected into H1299 cells (p53^{-/-}). As shown in Fig. 7A, p53 expression causes ~4-fold repression of luciferase activity in the constructs driven by the CD43 promoter sequences. Repression of the TCF-reporter vector (TOPFLASH) activity by p53 has been reported by others (34) and we used it as an additional control. p53-dependent luciferase repression was also seen in HCT116 colon carcinoma cells, a wild-type p53 cell line, when coexpressed with p14ARF to activate p53 (Fig. 7B). Thus, the promoter of CD43 is subject to direct regulation by p53 and the sequence from -91 to +439 is sufficient for p53-mediated repression on CD43 promoter activity. We also identified the p53 domains that mediate the p53 effect on CD43 promoter. The result in Fig. 7C shows that the same regions of p53 modulate the activity of CD43 promoter and the level of its mRNA.

Discussion

There is a growing body of evidence showing aberrant expression of CD43 in human solid tumor cells (12-14,35,36). CD43 expression has been observed already at early stages of colorectal tumors, but was not detectable in normal colonic epithelium (12,17). Among multiple functions attributed to CD43, its ability to impair apoptotic cell response has been reported and accounts for its potential role in tumorigenesis (20,37). Tumor suppressor protein p53 becomes activated in response to various detrimental stimuli, including inappropriate cell proliferation, followed by elimination of such cells

(1), p53-dependent downregulation of cell survival-related genes is an important mechanism in apoptosis regulation (7).

We present evidence to suggest that p53 could be an important factor that negatively regulates CD43 expression. We show that p53 down-regulates CD43 expression both at the levels of mRNA and protein. Lower levels of CD43 protein correlate with the transactivation ability of p53, suggesting that p53-dependent downregulation of CD43 protein requires transactivation of a subset of target genes. Using various p53 deletion mutants we identified the regions responsible for p53-mediated downregulation of CD43 mRNA: N-terminal transactivation domain, oligomerization domain, proline rich domain and DNA-binding domain of p53. A number of studies have described the domains of p53 required for its trans-repression activity. Among these were the N-terminus, the proline-rich domain and the C-terminus (7). It has been shown that tetrameric p53 binds DNA more efficiently than monomeric (26). Residues 339-346 in the oligomerization domain have been reported to be required for p53 trans-repression activity (25). In our study, the p53 proline-rich domain was crucial for the downregulation of CD43 mRNA levels. The p53 proline-rich domain is necessary for p53-mediated transcriptional repression, while it interacts with corepressor protein mSin3a (38-40). mSin3a tethers p53 in a repressor complex with histone deacetylases (HDAC)(38). The results of the present study showed that the histone deacetylation mechanism is involved in p53-mediated transcriptional repression of CD43, suggesting that CD43 expression is inhibited by the HDAC-mSin3a-p53 complex.

The CD43 gene has an unusual genomic organization consisting of only two exons, of which the second exon encodes the entire translation product (Fig. 6A). The CD43 genomic sequence from nucleotides -53 to -40 is an essential promoter region for the expression of human CD43 and it contains a

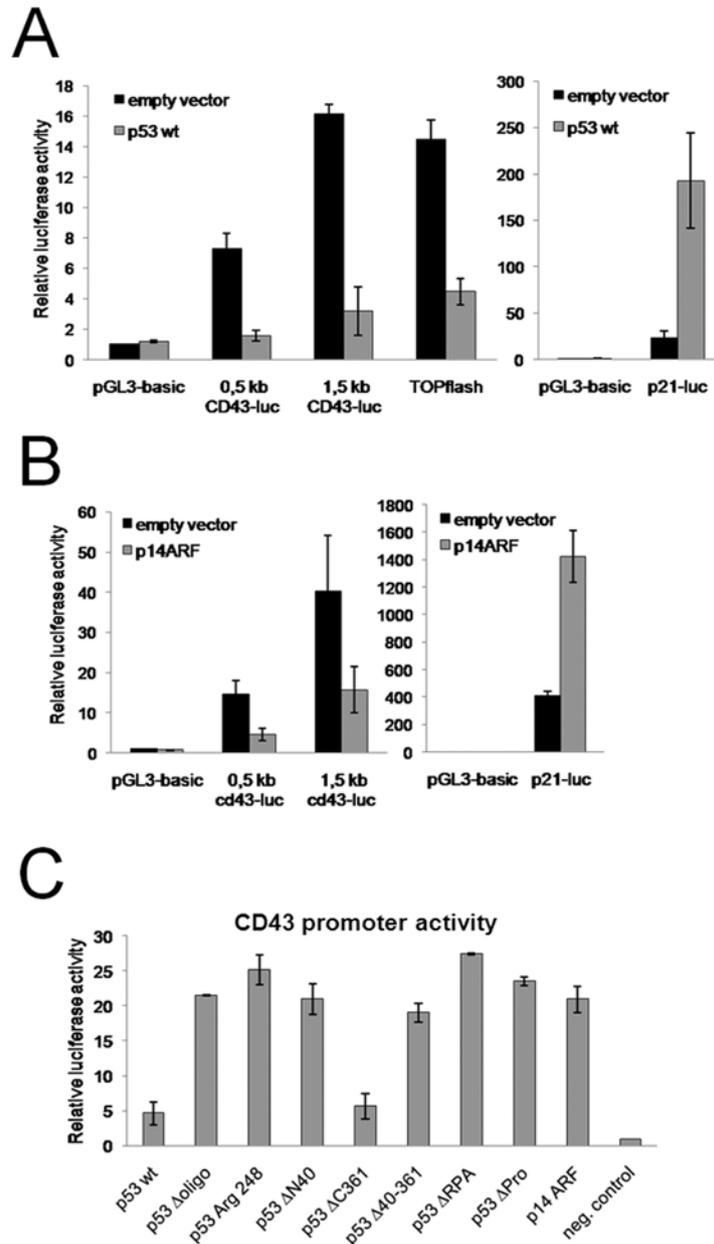


Figure 7. Activity of the CD43 promoter in H1299 (A) and HCT116 cells (B). The effect of different p53 proteins on CD43 promoter activity in H1299 cells (C). Luciferase activity is presented as fold-induction relative to the cells transfected with a promoterless luciferase pGL-3 reporter plasmid and an empty vector. Results represented are the average of three independent experiments.

binding site for the Sp1 transcription factor which is critical for the activation of CD43 expression (33,41). Sp1 transactivating properties are repressed by the methylation-specific repressor MeCP2 (methyl CpG binding protein 2), probably by interfering with Sp1 binding to the methylated CD43 promoter (10). DNA methylation can induce a decrease in histone acetylation level (42). Transcriptional repressor MeCP2 bound to methylated DNA recruits the Sin3 corepressor and histone deacetylases to repress transcription (43,44). One can speculate that p53 is involved in this process, as p53 has been shown to participate in DNA methylation to silence gene expression.

We identified a potential p53-binding sequence for trans-repression described by others (30), spanning -75 to -48 bp within the CD43 promoter region. Interestingly, it partially overlaps with the Sp1- and Pura α (-57 to -37)-binding sequences

and lies almost adjacent to hnRNP-K binding site (-38 to -17). The transcription factor Pura α and the heterogeneous nuclear ribonucleoprotein K (hnRNP-K) have been shown to repress transcription from the CD43 promoter (9,45). A transient reporter gene assay revealed that sequence from -91 to +439 was sufficient for p53-mediated repression of CD43 promoter activity. These observations suggest molecular mechanisms for p53-mediated regulation of CD43 expression besides recruitment of histone deacetylases. One plausible explanation is that p53 physically displaces Sp1 from its binding site as provided by repression of the human DNA polymerase δ catalytic subunit gene (POLD1) (46). Alternatively, p53 could interact with Sp1 that is bound to the promoter and the resulting complex is transcriptionally inactive. This mechanism has been described in the case of p53-dependent repression of the cyclin B1

promoter (47). Our reporter assay also revealed that in addition to the transactivation and proline-rich domains, the sequence-specific DNA-binding and oligomerization domains of p53 are required for exogenous CD43 promoter repression. C-terminal amino acids 321-363, which include the oligomerization domain of p53 protein, are required for the physical interactions with Sp1 (48). These data support the idea that p53 might interact with Sp1 to suppress CD43 promoter activity. Interestingly, the C-terminal regulatory domain was completely dispensable for inhibition of CD43 transcription. The C-terminal domain of p53 has been shown to bind DNA without sequence specificity, but truncation of this entire basic domain activates p53 binding to its consensus DNA (49). These observations suggest that p53 directly binds to the CD43 promoter. However, in some cases the promoters of downregulated target genes contain one or more potential p53 response elements which do not participate in p53-mediated regulation, but functional DNA-binding domain of p53 is still required for the inhibition (50).

In conclusion, we demonstrate that CD43 expression is downregulated following induction of wt p53 in human tumor cells and that p53 directly inhibits CD43 transcription at least partly by initiating the deacetylation of histones near CD43 promoter. Given that aberrant CD43 expression potentially contributes to both non-hematopoietic and hematopoietic tumor development, this insight into the regulation of CD43 expression may help to evaluate the role of p53-mediated tumor suppression. Accordingly, our data should contribute to a better understanding of not only regulation of CD43 expression but also its potential role in tumorigenesis.

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