

Priority Report

# Differential Regulation of microRNAs by p53 Revealed by Massively Parallel Sequencing

*miR-34a* is a p53 Target That Induces Apoptosis and G<sub>1</sub>-arrest

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## KEY WORDS

p53, microRNA, miRNA, miR-34a, tumor suppression, massively parallel sequencing, cell cycle, transcription factor

## ABBREVIATIONS

DOX	doxycycline
bp	base-pair
pri-miRNA	primary microRNA transcript
miRNA	microRNA
RT-qPCR	real-time quantitative PCR

## ACKNOWLEDGEMENTS

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## NOTE

Supplemental information can be found at: [www.landesbioscience.com/supplement/tarasovCC6-13-sup.pdf](http://www.landesbioscience.com/supplement/tarasovCC6-13-sup.pdf)

## ABSTRACT

In a genome-wide screen for microRNAs regulated by the transcription factor encoded by the p53 tumor suppressor gene we found that after p53-activation the abundance of thirty-four miRNAs was significantly increased, whereas sixteen miRNAs were suppressed. The induction of *miR-34a* was most pronounced among all differential regulations. Also expression of the primary *miR-34a* transcript was induced after p53 activation and by DNA damage in a p53-dependent manner. p53 occupied an evolutionarily conserved binding site proximal to the first non-coding exon of *miR-34a*. Ectopic *miR-34a* induced apoptosis and a cell cycle arrest in the G<sub>1</sub>-phase, thereby suppressing tumor cell proliferation. Other p53-induced miRNAs identified here may also have tumor suppressive potential as they are known to suppress the anti-apoptotic factor Bcl2 (*miR-15a/16*) and the oncogenes RAS and HMGA2 (*let-7a*). Our results for the first time directly integrate the regulation of miRNA expression into the transcriptional network regulated by p53. siRNAs corresponding to p53-induced miRNAs may have potential as cancer therapeutic agents as RNA interference based therapies are currently emerging.

## INTRODUCTION

The p53 tumor suppressor gene encodes a transcription factor that is translationally and post-translationally activated following DNA damage and oncogene activation.<sup>1,2</sup> Because p53 is mutated in a large fraction of cancers of diverse types, it is thought that the tumorigenic process may be intimately related to the deregulation of p53-mediated transcription. Numerous genes that encode proteins which mediate effects of p53 on cell cycle progression, apoptosis and other processes, which presumably contribute to tumor suppression by p53, have been identified.<sup>1,3,4</sup> Although genes encoding miRNAs appear as interesting candidate-mediators of tumor suppression by p53 they have not been described as direct p53 targets so far.

MiRNAs form a class of endogenously expressed small non-coding RNAs that play key roles in the regulation of gene expression. MiRNAs are transcribed by RNA polymerases II or III as primary transcripts (pri-miRNAs), which are further processed by the nuclear RNase III enzyme Drosha to stem-loop-structured miRNA precursor molecules (pre-miRNAs). The pre-miRNAs are subsequently transported to the cytoplasm where the RNase III enzyme Dicer cleaves off the double stranded (ds) portion of the hairpin and generates a short-lived dsRNA of about 20–25 nucleotides (nt) in size. The duplex is subsequently unwound and only one strand gives rise to the mature miRNA, which is incorporated into miRNA-protein complexes (miRNPs) (reviewed in refs. 5–8). MiRNAs guide miRNPs to partially complementary binding sites located in the 3' untranslated region (UTR) of target mRNAs and inhibit their expression either by interfering with translation or by destabilizing the target mRNA.<sup>5,9</sup> MiRNA function has been linked to different forms of cancer. MiRNAs can function as oncogenes as shown for miR-372 and miR-373 in testicular germ cell tumors<sup>10</sup> or the miR-17-92 cluster in B-cell lymphomas.<sup>11</sup> Interestingly, specific miRNAs have also tumor suppressor activity, most likely by repressing the expression of oncogenes. For example the miRNA let-7a inhibits the expression of the oncogenes KRAS, NRAS and HMGA2.<sup>12,13</sup> In chronic lymphocytic leukemias (CLL) the expression of miR-15 and miR-16 is frequently decreased and it has been suggested that miR-15 and miR-16 act as tumor suppressors as well.<sup>14</sup>

Here we set out to characterize the regulation of miRNA expression by p53 in an unbiased manner by using massively parallel sequencing, which is a combination of emulsion PCR and pyrosequencing.<sup>15</sup> In comparison to microarray analyses this approach is

not limited to previously identified miRNAs and is expected to have a superior sensitivity at high sequencing depth.

## MATERIAL AND METHODS

**Cell lines/culture and reagents.** The cell lines H1299 (lung cancer), MCF-7 (breast cancer) and U-2OS (osteosarcoma) were maintained in Dulbecco's modified eagles medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS). HCT116 cells were maintained in McCoy's 5A (Invitrogen) containing 10% FBS. All cell lines were cultivated in presence of 100 units/ml penicillin and 0.1 mg/ml streptomycin. Etoposide (Sigma) was dissolved in DMSO (40 mM stock solution) and used at a final concentration of 20  $\mu$ M. DOX (doxycycline) was dissolved in water (1 mg/ml stock solution) and used at a final concentration of 100 ng/ml.

**Conditional expression.** The human *p53* open reading frame (ORF) was inserted into the pUC19-Sfi shuttle vector<sup>16,17</sup> via an *EcoR* V site. A *Sfi* I fragment was isolated and inserted into pRTS1-eGFP.<sup>16</sup> Flanking *Sfi* I sites were added to *pri-miR-34a* by PCR and allowed direct insertion into pEMI.<sup>17</sup> H1299 or U-2OS cells were transfected with the respective vectors and selected for two weeks in the presence of 150  $\mu$ g/ml hygromycin. Resistant cell pools showed homogenous expression of GFP after addition of DOX as determined by fluorescence microscopy (data not shown).

**Western blot analysis.** Cells were lysed in RIPA lysis buffer (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 1% NP40, 0.5% (w/v) sodium deoxycholate, 0.1% sodium dodecylsulfate, complete mini protease inhibitors (Roche)). Lysates were sonicated and centrifuged at 16,060 g for 15 min at 4°C. Per lane 40  $\mu$ g of whole cell lysate was separated using 12% SDS-acrylamide gels, and transferred on Immobilon PVDF membranes (Millipore Corporation, Massachusetts). For immunodetection membranes were incubated with antibodies specific for p53 (DO-1, Santa Cruz), p21<sup>CIP1</sup> (Ab-11, NeoMarkers), and  $\beta$ -actin (A-2066, Sigma-Aldrich). Signals from HRP (horse-radish-peroxidase)-coupled secondary antibodies were generated by enhanced chemiluminescence (Perkin Elmer Life Sciences) and recorded with a CCD camera (440CF imaging system, Eastman Kodak Co.).

**Real-time quantitative PCR (RT-qPCR).** Total RNA was isolated using the Total RNA Isolation System (Promega, Madison, USA). cDNA was generated from 1  $\mu$ g total RNA per sample using anchored oligo-dT primers (Reverse-iT First Strand Synthesis; ABgene). RT-qPCR was performed by using the LightCycler and the FastStart DNA Master SYBR Green 1 kit (Roche Applied Sciences) as described previously.<sup>18</sup> The oligonucleotides used as PCR primers were: *p21*<sup>CIP1</sup> (221 bp): 5'-GGCGGCAGACCAGCATGACAGATT-3', 5'-GCAGGGGGCGGCCAGGGTAT-3';  $\beta$ -actin (213 bp): 5'-TGACATTAAGGAGAAGCTGTGCTAC-3', 5'-GAGTTGAAGGTAGTTTCGTGGATG-3'; *miR-34a* (128 bp): 5'-CGTCACCTCTTAGGCTTGGGA-3', 5'-CATTGGTGTGCTTGTGCTCT-3'.

**Generation of small RNA libraries.** The small RNA fraction from H1299 cells was isolated using a mirVana-microRNA isolation kit (Ambion). Small RNA libraries were generated by Vertis Biotechnology AG (Freising-Weihenstephan, Germany). In brief, RNA was separated on a denaturing 12.5% polyacrylamide gel and stained with SYBRgreenII. After passive elution, RNAs with a length of 15–30 bases were concentrated by ethanol precipitation and dissolved in water. Next, RNAs were poly(A)-tailed using poly(A) polymerase and an adapter was ligated to the 5' phosphate of the

miRNAs: (5' end adapter (43 nucleotides): 5'-GCCTCCCTCGCGCCATCAGCTNNNNGACCTTGGCTGTCACTCA-3'). NNNN represents a "barcode" sequence for the two samples: CAGA for RNAs originating from "p53 off" H1299 cells and CACA for "p53 on" cells. Next, first strand cDNA synthesis was performed using an oligo(dT)-linker primer and M-MLV-RNase H<sup>-</sup> reverse transcriptase (3' end oligo (dT) linker primer (61 bases): 5'-GCCTTGCCAGCCCGTCTCAGACGAGACATCGCCCCGC(T)<sub>25</sub>-3'). The resulting cDNAs were PCR-amplified in twenty-two cycles using the high fidelity Phusion polymerase (Finnzymes). 120–135 bp amplification products were confirmed by polyacrylamid gel electrophoresis (PAGE) analysis. Both cDNA pools were mixed in equal amounts and subjected to gel fractionation. The 120–135 bp fraction was electro-eluted from 6% PAA-gels. After isolation with Nucleospin Extract II (Macherey and Nagel) cDNA pools were dissolved in 5 mM Tris/HCl, pH 8.5 with a concentration of 10 ng/ $\mu$ l and used in single-molecule sequencing. Massively parallel sequencing was performed by 454 Life Sciences (Branford, USA) using the Genome Sequencer 20 system.

**Analysis of parallel sequencing results.** Base calling and quality trimming of sequence reads was performed with 454 software. The 5'-adapter sequences flanking the RNA-derived sequences were discarded and the following 25 bp sequences were extracted for further comparative analysis. The frequencies of reads in the two samples were determined using a Perl script designed for this purpose (available upon request). Sequences detected only in one sample or once in both samples were eliminated to avoid inclusion of sequencing errors. Total numbers of reads considered as miRNAs were 35076 and 39270 for the "p53-on" and "p53-off" libraries, respectively. The remaining reads (35654 for "p53-on" and 57996 for "p53-off") were reads with non-exact matches to miRNAs (with gaps, mismatches or more than three nucleotides mismatching at 3' end) and non-miRNAs RNA fragments of diverse origin. miRNAs were identified using the *H. sapiens* miRNA database (miRBase). In most cases miRNAs matched to the first 18–25 bp of the sequence reads with several mismatches at the 3' end. These mismatches could be the result of the cloning procedure, preferential degradation at the 3'-end, template-independent addition of nucleotides to the 3' end of miRNAs and miRNAs\* or sequencing errors.<sup>19</sup> Sequence read to miRNA pairings lacking gaps or mismatches were included with a maximum of 1–3 nucleotide mismatches at the 3'-end. For detection of miRNAs\* the antisense seed regions of previously reported miRNAs were compared with the obtained sequence reads.

**qPCR detection of miRNAs.** RNA samples were isolated using the mirVana miRNA Isolation kit (Ambion). For detection of *miR-34a* the mirVana qPCR detection kit in combination with a primer set for *miR-34a* (Ambion) including endogenous control 5S rRNA were utilized according to the manufacturer's protocol. cDNA was synthesized from 15–20 ng of RNA enriched for small RNAs. RT-qPCR was performed using the iQ5 Real-Time PCR Detection System (Bio-Rad).

**Northern blot analysis.** 15  $\mu$ g of total RNA were resolved by electrophoresis on 1.2% denaturing agarose gels, stained with ethidium bromide and photographed. After overnight capillary transfer onto a Hybond nylon membrane (Amersham Biosciences) the RNA was crosslinked using a Stratilinker UV illuminator (Stratagene). For the generation of a Northern probe, a 416 bp DNA fragment corresponding to nucleotides 1233–1648 of the *miR-34a* primary transcript was PCR-amplified

using 5'-CATTGGTGTCTGTTGTGCTCT-3' (forward) and 5'-TGGTGAGAATGGAAGCAGGCTCCCTCCCC-3' (reverse) primers and cDNA derived from H1299p53i cells as a template, cloned into pCR4 vector using TOPO cloning kit (Invitrogen) and verified by sequencing. Radioactive labeling of the probe with  $\alpha$ -<sup>32</sup>P-dCTP was performed using random hexamer primers and Klenow polymerase. Hybridization was performed in QuickHyb solution (Stratagene) at 68°C for 2 hours. Signals were detected with a BAS2500 phosphorimager (Fuji).

**Determination of *pri-miR34a* 5' and 3' mRNA ends.** 2  $\mu$ g of total RNA isolated from H1299 cells after induction of ectopic p53 were used for the synthesis of first strand cDNA. 5' RACE (Rapid Amplification of cDNA Ends) was performed using the 5' RACE System, Version 2.0 (Invitrogen). First strand cDNA was generated using the GSP1 primer (5'-CGTCACCTCTTGGCTTGA-3') complementary to an EST. RACE-PCR was carried out by using the tailed cDNA as template and GSP2 (5'-TTGGCATCTGTAGGTGAAGC-3') and AAP (Invitrogen) primers according to the supplied protocol (Invitrogen). The resulting PCR-products were used as templates for a second round of PCR with GSP4 (5'-ATCTTGAATTCTGGGCCCAACAGTGCAG-3') and UAAP (Invitrogen) primers. The obtained PCR-amplified fragments were cloned (TOPO TA cloning Kit, Invitrogen) and sequenced using T3 and T7 oligonucleotides.

For characterization of the 3'-end, the reaction was performed with oligo-dT primer containing a T7 promoter at its 5' end using SuperScript III reverse transcriptase (Invitrogen) according to manufacturer's instructions. 10% of this reaction was used for thirty cycles of PCR amplification with a gene specific primer 5'-GGAGCCTGCTTCCATTCTCACCCTCTGAA-3' and 5 units of Platinum Taq polymerase (Invitrogen). A single band of ~2 kbp size was obtained but not in a control PCR without RT. The product was gel-purified, cloned into TOPO-Ta vector (Invitrogen) and sequenced in both directions using M13 primers.

**Chromatin immunoprecipitation (ChIP) assay.** p53 was induced in MCF-7 cells by treatment with 20  $\mu$ M etoposide for 6 hours and in H1299 by addition of 100 ng/ml doxycycline for 12 hours. For cross-linking, formaldehyde (Merck) was added to the medium at a final concentration of 1%. After 4 (MCF-7) or 10 minutes (H1299) incubation at room temperature the reaction was stopped by addition of glycine at a final concentration of 0.125 M followed by additional incubation for 5 minutes. The fixed cells were washed twice using TBS buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl) and harvested in SDS buffer (50 mM Tris/HCl, pH 8.1, 0.5% SDS, 100 mM NaCl, 5 mM EDTA and protease inhibitors (Complete Mini, Roche)). Cells were pelleted by centrifugation at 300 $\times$ g for 5 min at 4°C and resuspended in 2 ml IP buffer (67 mM Tris/HCl, pH 8.3, 100 mM NaCl, 5 mM EDTA, 0.33% SDS, 1% Triton X100, and protease inhibitors (Complete Mini, Roche)). Chromatin was sheared by sonication (Sonifier: Bandelin HD70 Sonopuls) four times (MCF-7) or two times (H1299) for 20 seconds (power setting: continuous, MS72D) to generate DNA fragments with an average size of 300–1000 bp. For each immunoprecipitation, lysates were precleared by addition of 15  $\mu$ l/ml preblocked Fast Flow protein G sepharose beads (Amersham; 0.4 mg/ml fatty acid free BSA, (Sigma); 1.5 mg/ml sheared salmon sperm DNA in PBS (Promega)). Lysates were incubated for 20 hours at 4°C with a p53 antibody (clone DO-1 Santa Cruz SC-126) or mouse IgG2a (M5407, Sigma). **Washing and reversal of cross-linking** was done as described previously. Precipitated DNA was analyzed by RT-qPCR and fold enrichment of p53 binding to the respec-

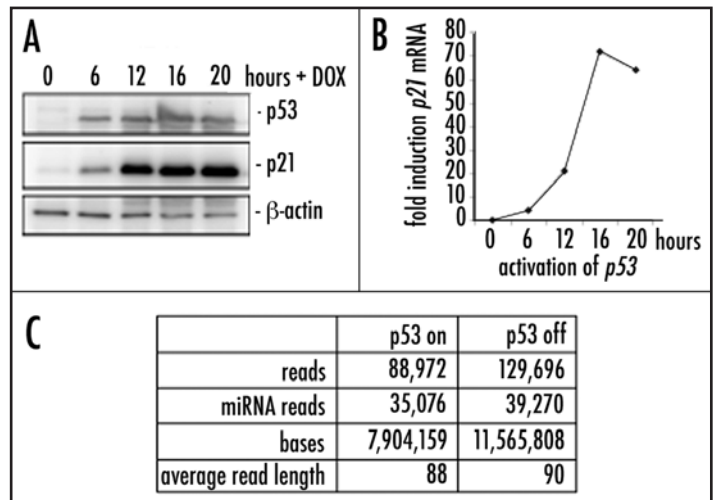


Figure 1. Characterization of differential expression of small RNAs by parallel sequencing analysis after conditional p53 expression. (A) p53 expression was induced by addition of doxycycline (DOX) in H1299 lung carcinoma cells. After the indicated periods lysates were obtained and subjected to immunoblot analysis for p53 and p21.  $\beta$ -actin served as a loading control. (B) In parallel RNA was isolated and subjected to RT-qPCR analysis. For normalization the expression of  $\beta$ -actin was determined. (C) Summary of the parallel sequencing results obtained from libraries generated from small RNAs isolated sixteen hours after p53 activation. For assessment of miRNA expression by quantification of sequence reads see also Supplementary Table 1.

tive DNA sequences was calculated as described previously.<sup>18,20</sup> For normalization of input DNA amplification of a fragment on chromosome 16q22 was used. **Primer sequences: chromosome 16q22**, Frw.: 5'-CTA CTC ACT TAT CCA TCC AGG CTA C-3', Rev.: 5'-ATT TCA CAC ACT CAG ACA TCA CAG-3'; **p21 promoter**, Frw.: 5'-CCT TTG GAT GGT TTG GAT GTA-3'; Rev.: 5'-GGG AGG ACA GGC TTC TTT CT-3'; **miR34a BDS-1**, Frw.: 5'-CGC ACG GAC TGA GAA ACA C-3', Rev.: 5'-CAC GAG CAG GAA GGA GGA C-3'; **miR34a BDS-2**, Frw.: 5'-CCA GCC GAT GCT TAC ACA GT-3', Rev.: 5'-GTT GCA GAT CAC ACC AGT G-3'; **miR34a BDS-3**, Frw.: 5'-TCC AGG GAA GTG CTG AC TCT-3', Rev.: 5'-AGG ATC ACT TGA GGC GAG AA-3'.

**Transfection and FACS analysis.** Cells were transfected in 6-well plates using HiPerFect transfection reagent (Qiagen) with 200 nM final concentration of siRNA duplexes. After 24 hours medium was changed. FACS analysis was performed as described previously.<sup>17</sup> Duplexes were as follows (sense/antisense):

miR-34a, 5'-UGGCAGUGUCUAGCUGGUUGUU-3'/  
5'-CAAUCAGCAAGUAUACUGCCCUA-3';  
control siRNAs, 5'-UUGUCUUGCAUUCGACUAAUU-3'/  
5'-UUAGUCGAAUGCAAGACAAUU-3'.

## RESULTS

**A genome-wide screen for p53-regulated miRNAs.** Pools of H1299 lung cancer cells, which harbor a homozygous deletion of the p53 locus, carrying a p53-encoding, episomal pRTS vector were generated. The pRTS vector includes all components of a dual tet-repressor/activator system,<sup>16</sup> which allows tight control of p53 expression by addition of doxycycline (DOX). In the absence of DOX expression of p53 and the GFP marker was not detectable in H1299 cells (Fig. 1A and data not shown). To determine the optimal

Table 1 Differential miRNA expression after p53 activation detected by massively parallel sequencing

miRNA (Name)	p53 On (Reads)	p53 Off (Reads)	Fold Change (Normalized)	Reported Silencing Targets	miRNA (Name)	p53 On (Reads)	p53 Off (Reads)	Fold Change (Normalized)	Reported Silencing Targets
induced miRNAs:									
hsa-miR-34a	69	3	33.4	E2F3, NOTCH1, DLL1	hsa-miR-132	64	46	2.0	
hsa-miR-15b	944	166	8.2		hsa-miR-128a	330	242	2.0	
hsa-miR-20a	197	37	7.7	E2F1	hsa-miR-584	139	102	2.0	
hsa-miR-26b	139	35	5.8		hsa-let-7a	407	309	1.9	RAS, HMGA2
hsa-miR-15a	148	41	5.2	BCL2	hsa-miR-190	118	91	1.9	
hsa-miR-27a	367	103	5.2		hsa-miR-23b	221	192	1.7	
hsa-miR-106a	71	20	5.1		hsa-miR-30a-3p	93	85	1.6	
hsa-miR-224	150	44	4.9		repressed miRNAs:				
hsa-miR-155	95	29	4.8		hsa-miR-99b	26	60	0.6	
hsa-miR-21	382	118	4.7		hsa-miR-221	1536	3597	0.6	
hsa-miR-106b	1566	501	4.5		hsa-miR-100	394	928	0.6	
hsa-miR-424	71	23	4.5		hsa-miR-362	47	113	0.6	
hsa-miR-182	33	11	4.4		hsa-miR-324-3p	37	89	0.6	
hsa-miR-17-5p	486	178	4.0	E2F1	hsa-miR-151	642	1550	0.6	
hsa-miR-23a	7309	3062	3.5	SDF-1, BRN-3b	hsa-miR-125b	392	1010	0.6	
hsa-miR-27b	365	157	3.4		hsa-miR-17-3p	317	870	0.5	
hsa-miR-183	34	16	3.1		hsa-miR-378	99	275	0.5	
hsa-miR-16	184	91	2.9	BCL2	hsa-miR-615	150	427	0.5	
hsa-miR-92	101	50	2.9		hsa-miR-22	529	1843	0.4	
hsa-miR-24	105	54	2.8		hsa-let-7b	52	184	0.4	
hsa-miR-29a	651	342	2.8		hsa-miR-671	10	38	0.4	
hsa-let-7c	419	229	2.7	RAS	hsa-miR-422b	796	3349	0.3	
hsa-miR-191	330	189	2.5		hsa-miR-320	1412	6204	0.3	
hsa-miR-25	7328	4613	2.3		hsa-miR-501	21	113	0.3	
hsa-miR-103	959	619	2.2						
hsa-miR-532	29	19	2.2						
hsa-let-7e	95	66	2.1						

Sixteen hours after p53 activation in H1299 lung carcinoma cells RNA was isolated. Two libraries representing small RNAs were generated and subjected to 454-sequencing. The frequencies of detected miRNAs were normalized by a factor of 1.45 correcting for the different total number of reads from the two samples. Identified miRNAs, the number of matching reads and reported targets for down-regulation are depicted. Only those miRNAs with a significant difference in read numbers ( $p < 0.05$ ; Fisher exact test) are listed. For a complete list of detected miRNAs see Supplemental Table 1.

time point for isolation of p53-regulated miRNAs, we measured the induction of *p21*, a well-characterized p53 target gene, after addition of DOX (Fig. 1A and B). Six hours after addition of DOX expression of p53 and p21 protein was easily detectable. *p21* mRNA showed a peak of induction at 16 hours. Therefore, this time point was chosen for the isolation of small RNAs and compared to cells not exposed to DOX. DNA libraries representing small RNAs were generated and subjected to emulsion PCR and subsequent pyrosequencing.<sup>15</sup> Thereby, ~89.000 reads were obtained representing a state of activated p53 and ~130.000 reads for untreated control cells (Fig. 1C). We employed this relatively high sequence read number to maximize the sensitivity. Subsequently, the frequency of sequence reads corresponding to miRNAs was determined. In total sixty-four different previously annotated miRNAs were represented in the two libraries (Supplementary Table 1). In addition, eighteen sequences corresponding to complementary arms of the hairpins, indicated as

miRNAs\*, were identified. 34 miRNAs were found to be induced more than 1.5 fold ( $p < 0.05$ ), whereas sixteen miRNAs were repressed after p53 activation ( $p < 0.05$ ) (listed in Table 1). Twenty-four miRNAs were not significantly affected in their abundance by activation of p53. The expression levels of miRNAs induced after p53 activation were distributed over three orders of magnitude: among the thirty-four induced miRNAs three were represented by more than 1000 reads in the “p53 on” library indicating a relatively high level of expression. 21 miRNAs were present in the range between 100 to 1000 reads and ten were below 100. On average, miRNAs\* were under-represented. Only miR-181a-3p\* and miR-532\* were more abundant than the corresponding miRNAs. For miR-140\* a corresponding miRNA was not detected.

*MiR-34a* is encoded by a p53-responsive gene. The most pronounced increase in miRNA abundance after p53 activation was observed for *miR-34a* which was induced 33.4 fold according to

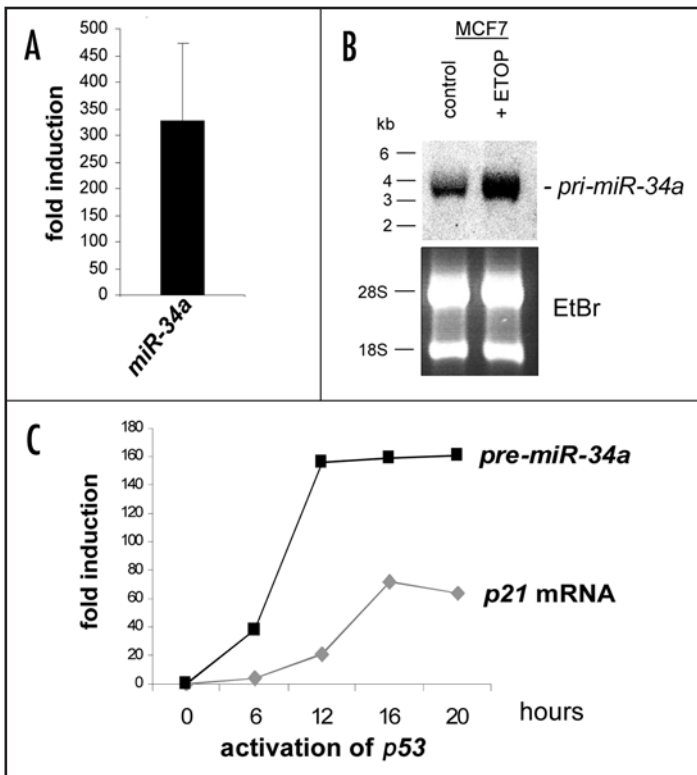


Figure 2. Regulation of miRNA expression by p53 and DNA damage. (A) Levels of processed *miR-34a* sixteen hours after *p53* activation in H1299 cells were determined by RT-qPCR. Determinations were performed in biological triplicates. The error bar represents the standard deviation. (B) Determination of the *miR-34a* primary transcript size by Northern blot analysis. MCF-7 cells were treated with etoposide (ETOP) for twelve hours. An autoradiograph of a Northern blot performed with a *pri-miR-34a* specific probe is shown along with a picture of the Ethidium bromide (EtBr)-stained gel from which the blot was made. On the left the positions of a RNA standard are shown. (C) Comparison of *miR-34a* and *p21* induction by p53. *p53* expression was induced by addition of DOX for the indicated periods in H1299 cells. RNA was isolated and subjected to RT-qPCR analysis for *p21*, *miR34* pre-miRNA and  $\beta$ -actin (for normalization) as described previously.<sup>18</sup>

the sequencing data. Quantification by RT-qPCR at the processed miRNA level revealed a more than 300-fold induction for *miR-34a* after *p53* activation (Fig. 2A). Due to its exceptional induction after *p53* activation we focused on the analysis of *miR-34a*. The organization of the *miR-34a* gene has not been described previously. According to our analyses of the NCBI data base several ESTs matched to a region surrounding the *miR-34a* hairpin, indicating that a larger primary transcript may be induced by p53 (data not shown). These searches also indicated that *miR-34a* is encoded by its own transcription unit as these ESTs did not encode proteins. In the cell line MCF-7, which expresses wild-type p53, the addition of the DNA-damaging agent etoposide induced the expression of the *miR-34a* primary transcript, indicating that activation of endogenous p53 presumably leads to transactivation of *miR-34a* (Fig. 2B). According to Northern blot analysis the size of the *miR-34a* primary transcript is ~3.7 kbp (Fig. 2B). The expression of the predicted *miR-34a* primary transcript was analyzed by RT-qPCR (Fig. 2C). Thereby a dramatic induction of the *miR-34a* primary transcript after p53 activation was detected. Furthermore, the induction of the *miR-34a* primary transcript was more pronounced and occurred earlier than the induction of *p21* mRNA (Fig. 2C).

**Characterization of p53 binding elements in the *miR-34a* gene.** Using RACE (rapid amplification of cDNA ends) we identified the first exon and the presumptive transcription start site (TSS) and the 3' end of the *miR-34a* pri-miRNA (data not shown; GenBank accession: EF570048 (*pri-miR-34a*); EF570049 (*mir-34a* locus)). According to the genomic position of the first exon the *miR-34a* gene has one relatively large intron of ~30 kbp (Fig. 3A). By inspection of the sequence we identified several candidate p53 binding sites (BDS-1 to 3) in the promoter region and the intron. The organization of the *miR-34a* locus and the positioning of putative p53 binding sites is largely conserved in the mouse and rat genome (Fig. 3B and C). The positioning and composition of BDS-1 was highly conserved among the three species implying functional relevance. By chromatin-immunoprecipitation (ChIP) analysis we could detect occupation by p53 for BDS-1, which is located 39 bp upstream of the TSS (Fig. 3D). This association was more pronounced than the binding of p53 to the previously described p53 binding site in the *p21* promoter. The 2 intronic sites (BDS2+3) showed relatively weak occupation by p53. This weaker association with p53 is presumably because BDS-2 and BDS-3 represent single half sites, whereas BDS-1 is a perfect match to the previously described p53 consensus binding site, which has two palindromic half sites facilitating binding of a p53 tetramer.<sup>21</sup> For all tested binding sites the occupation with p53 was enhanced after induction of DNA damage by treatment with etoposide (Fig. 3D). Also after activation of a conditional *p53* allele in H1299 cells the region surrounding BDS-1 in the *miR-34a* promoter showed enhanced occupation by p53 to a similar extent as the well documented association of p53 with the *p21* promoter (Fig. 3E). In summary, these results demonstrate that *miR-34a* is a direct p53 target gene.

**p53-dependent induction of *miR-34a* after DNA damage.** Northern blot analysis confirmed the induction of *pri-miR-34a* after activation of a conditional *p53* allele in H1299 cells (Fig. 4A). Furthermore, *pri-miR-34a* was induced after addition of adriamycin to human diploid fibroblast (HDF). To determine whether p53 is required for the induction of *pri-miR-34a* following DNA damage we analyzed HCT116 cells rendered *p53*-deficient by homologous recombination.<sup>22</sup> In HCT116 cells expressing wild-type p53 an induction of *pri-miR-34a* was detected 12 hours after addition of etoposide by Northern blot analysis that was similar to the induction of the p53-target gene *p21* (Fig. 4A). However, in p53-deficient HCT116 cells *pri-miR-34a* was not induced and the basal expression level of *pri-miR-34a* was reduced. This pattern was also found for the *p21* mRNA. Therefore, the induction of *pri-miR-34a* is p53-dependent. When RT-qPCR was used to quantify *pri-miR-34a* expression after DNA damage in this isogenic pair of HCT116 cell lines the requirement of p53 for the *pri-miR-34a* induction was confirmed (Fig. 4B). Taken together, these results demonstrate that p53 is a critical mediator of *pri-miR-34a* activation following DNA damage.

***MiR-34a* induces apoptosis.** To determine the function of p53-induced *miR-34a* we transfected H1299 cells with duplex siRNAs corresponding to processed *miR-34a*. The functionality of the *miR-34a* siRNA was confirmed in a reporter assay (data not shown). After 48 hours cells were harvested and subjected to DNA content analysis by flow cytometry (Fig 5A). Thereby we detected an increase in the fractions of cells with a sub- $G_1$  DNA content, which is indicative of apoptosis, whereas the number of cells in S-phase and  $G_2$ /M-phase was decreased. Therefore, activation of *miR-34a* by p53 may contribute to induction of apoptosis.

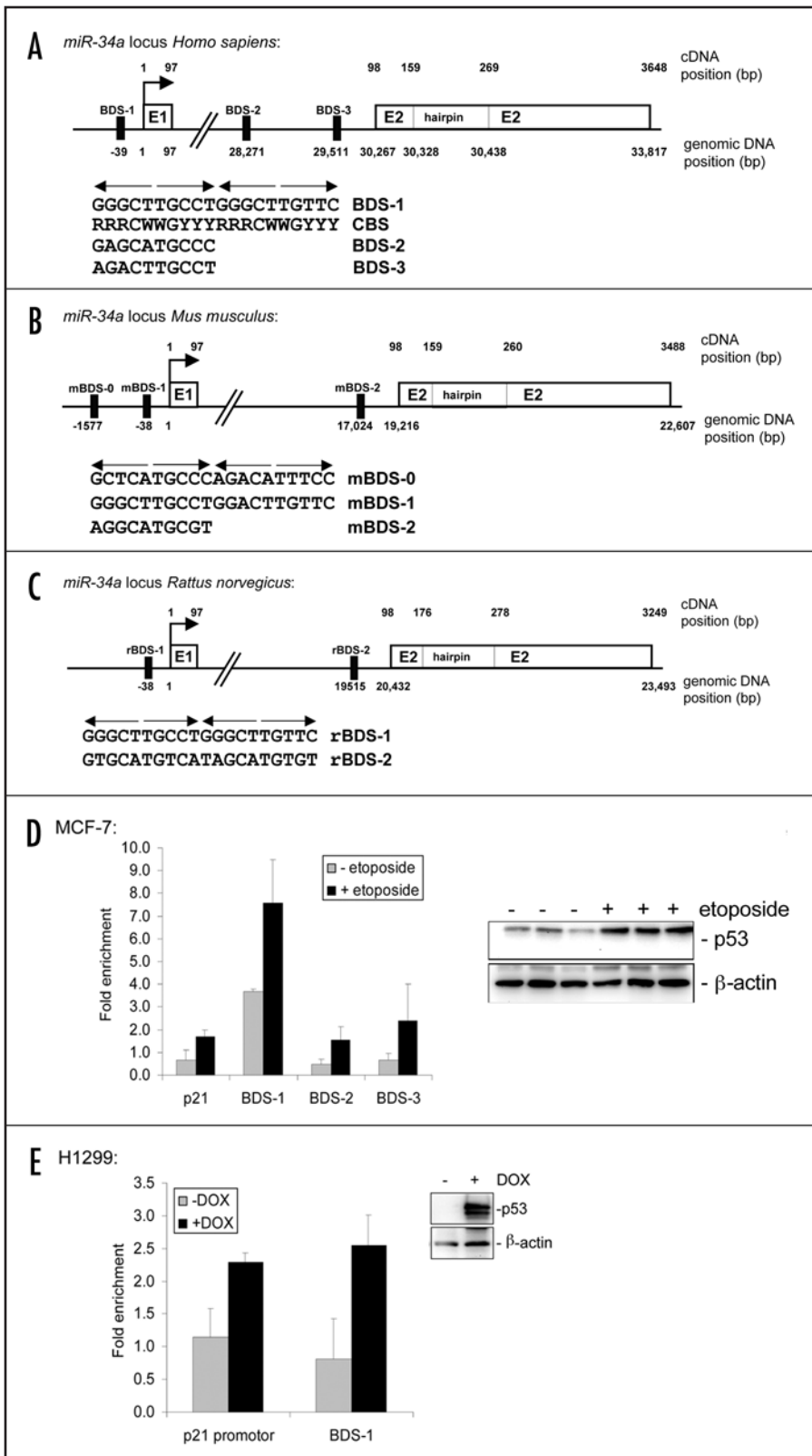


Figure 3. Characterization of *miR-34a* as a direct p53 target gene. (A) Genomic structure of the human *miR-34a* promoter. GenBank accession number: EF570049. Open rectangles indicate exons, closed represent the putative p53 binding sites BDS-1 to BDS-3. p53 binding sites were identified with the MAPPER program. The arrow on exon 1 indicates the transcription start site. CBS: consensus binding site for p53. A comparison of the putative p53 binding sites in the *miR-34a* promoter region to the previously characterized consensus binding site (CBS),<sup>21</sup> with (R) = purine, (Y) = pyrimidine, (W) = A or T, is depicted. Arrows indicate the palindromes which serve as contact sites for single p53 molecules organized in a tetramer. (B) Genomic structure of the mouse *miR-34a* promoter; GenBank accession number: EF606691. (C) Genomic structure of the rat *miR-34a* promoter; GenBank accession number: EF606690. (D) Chromatin-immunoprecipitation (ChIP) analysis of MCF-7 cells after DNA damage by exposure to etoposide for 6 hours. Immunoprecipitated DNA was analyzed by RT-qPCR as described previously.<sup>18,20</sup> Relative enrichment of p53-bound DNA in comparison to control antibody IP at the indicated genomic regions is shown. For normalization of input DNA PCR amplification of a region on chromosome 16q22 was used. The assay was performed in triplicates for BDS-1-3. Error bars represent standard deviations. For the p53 binding site in the *p21* promoter the average of 2 experiments with standard error is depicted. Right panel: confirmation of increased p53 levels after exposure to etoposide by immunoblotting. (E) ChIP analysis of H1299 cells after activation of ectopic p53 expression. Comparison of BDS-1 of *miR-34a* with the p53 binding site in the *p21* promoter. The graph shows the average of duplicate experiments, error bar: standard error. Right panel: detection of p53 protein after addition of DOX by immunoblotting.

*Pri-miR-34a* induces a G<sub>1</sub>-arrest and suppresses colony formation. Next we analyzed the effect of ectopic expression of the full length *pri-miR-34a* transcript in U-2OS cells. For this purpose *pri-miR-34a* was inserted into the pEMI vector, which allows the conditional expression of microRNAs as we have previously shown,<sup>17</sup> and pools of U-2OS cells harboring this episomal plasmid were generated.

The miRNAs identified here may contribute to tumor suppression by p53 by downregulation of proteins involved in numerous tumorigenic processes. Good candidates for miRNA targets represent proteins involved in cell cycle progression and cell survival. Their downregulation by RNA interference would contribute to cell cycle arrest and apoptosis, which is a common result of p53 activation

## DISCUSSION

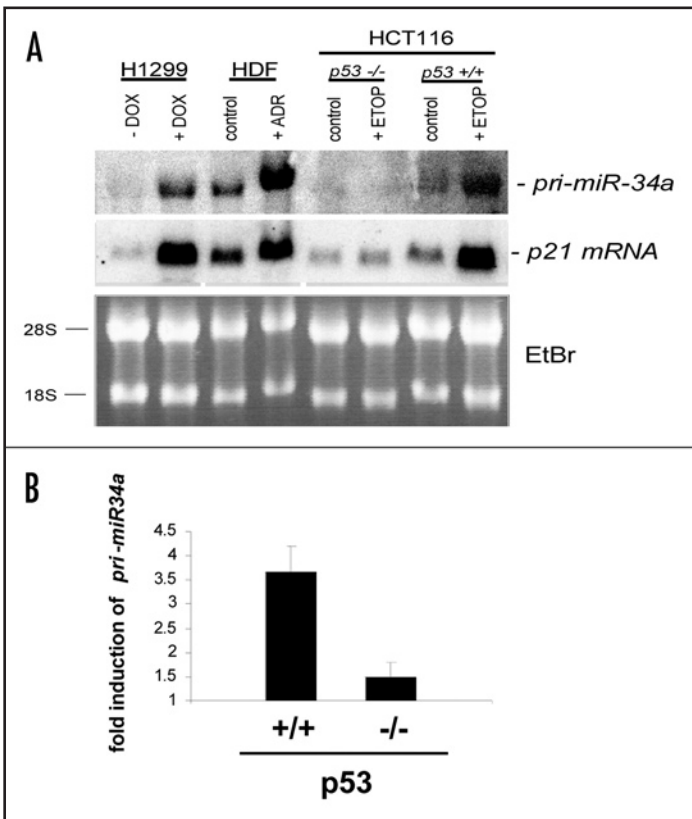


Figure 4. Induction of *pri-miR34a* after DNA damage is p53-dependent. (A) Comparison of *pri-miR-34a* and *p21* expression in cells with varying status or activity of p53. From left to right: Ectopic p53 was induced by DOX addition for 16 hours in H1299 cells. HDF (human diploid fibroblasts) were treated with adriamycin for 6 hours. HCT116 cells with p53 knock-out (*p53*<sup>-/-</sup>) or control cells (*p53*<sup>+/+</sup>) were treated with etoposide for 12 hours. "control" indicates treatment with vehicle alone. Autoradiograph of Northern blots performed with *pri-miR-34a* and *p21* specific probes are shown along with a picture of the Ethidium bromide (EtBr)-stained gels from which the blot was made. Northern blot analysis was performed as described previously.<sup>34</sup> (B) RT-qPCR analysis of *pri-miR-34a* expression in HCT116 cells with varying *p53* status. HCT116 cells with *p53* knock-out (*p53*<sup>-/-</sup>) or control cells (*p53*<sup>+/+</sup>) were treated with etoposide for 12 hours. RT-qPCR analyses were performed in biological triplicates. Error bars represent standard deviations.

following DNA damage. Recent evidence suggests that the activation of oncogenes in the early phases of tumorigenesis may cause replication-stress which induces DNA damage (ref. 20 and references therein). DNA damage is therefore presumably a common inducer of p53's tumor suppressive activity.

In mammals it is difficult to directly predict relevant downstream targets of a miRNA, as a perfect complementarity between miRNAs and protein coding genes almost never exists. Several algorithms were developed that predict miRNA targets, e.g., TargetScan, PicTar, and miRanda.<sup>23-25</sup> However, the number of targets predicted for each miRNA by programs is in the hundreds. The identification of microRNA targets relevant for tumor suppression by p53 will therefore represent an interesting, but complex, task for the future. Several interesting targets of the p53-induced miRNAs identified here have been characterized by others previously. The NOTCH1 receptor and its ligand DLL1 represent confirmed targets for down-regulation by *miR-34a*.<sup>26</sup> Interestingly, activation of NOTCH1 has been implicated in tumor formation.<sup>27,28</sup> In addition ectopic *miR-34a*

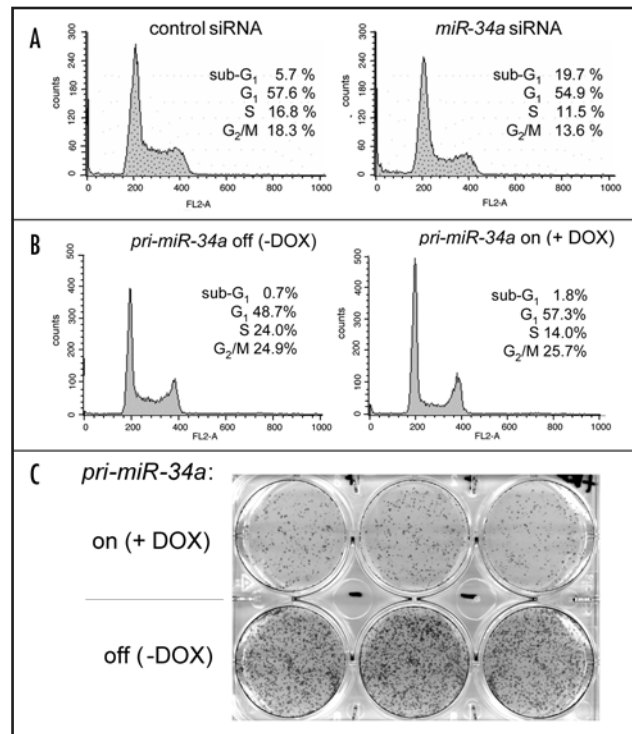


Figure 5. Ectopic *miR-34a* induces apoptosis and suppresses cell proliferation. (A) DNA content analysis of H1299 cells transfected with siRNAs corresponding to *miR-34a* and control (non-silencing) siRNAs. 48 hours after transfection cells were collected, stained with propidium iodide and subjected to flow cytometry. 10,000 cells were evaluated in each sample. Representative results are depicted. The indicated percentages are the average of duplicate experiments. (B) U-2OS cells harboring a conditional *pri-miR-34a* allele were collected 48 hours after addition of DOX, stained with propidium iodide and analyzed by flow cytometry. Ten thousand cells were evaluated in each sample. Representative results are depicted. The indicated percentages are the average of triplicate experiments. (C) U-2OS cells harboring a conditional *pri-miR-34a* allele were seeded at low density. After six days in the presence or absence of DOX colony formation was determined by staining with crystal violet. Triplicate results are shown.

reduces the levels of E2F3 by targeting its mRNA.<sup>29</sup> The *miR-34a* gene itself may be a tumor suppressor gene since it maps to a region on chromosome 1p36 which is commonly deleted in neuroblastoma and other tumors.<sup>29</sup> Several other p53-induced microRNAs may have tumor suppressive activity: *miR20a* and *miR17-5p* target the mitogenic transcription factor E2F1,<sup>30</sup> and *miR15a* and *miR16* downregulate the anti-apoptotic proto-oncogene Bcl2.<sup>31</sup> Furthermore, let7a suppresses the expression of RAS<sup>12</sup> and HMGA2.<sup>32</sup> miRNAs repressed after p53 activation represent candidate oncogenes, when their expression is rendered constitutive by amplification or translocation. Whether other miRNAs up-regulated after p53 activation, besides *miR-34a*, are direct p53 target genes is currently unknown. It is conceivable that indirect mechanisms also contribute to their up-regulation. E.g., changes in the cell cycle distribution which occur after p53 activation may result in changes in miRNA processing. Similar to miRNAs induced by the c-MYC transcription factor p53-induced miRNAs may serve to fine tune the response to p53 activation.<sup>30</sup>

This study has unraveled a new layer of complexity in the p53-network. The detailed mechanisms of the differential miRNA expression caused by p53 activation, the resulting changes in target protein abundance and the relevance for tumor suppression by p53

provide a fertile ground for future investigations. Furthermore, siRNAs corresponding to p53-induced miRNAs may have potential as cancer therapeutic agents as RNA interference based therapies are currently emerging.<sup>33</sup>

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**Data deposition.** GenBANK accession: EF570048 (human *pri-miR-34a*); EF570049 (human *miR-34a* locus); EF606691 (mouse *miR-34a* locus); EF606690 (rat *miR-34a* locus)

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