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Repression of the *miR-17-92* cluster by p53 has an important function in hypoxia-induced apoptosis

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We here report that *miR-17-92* cluster is a novel target for p53-mediated transcriptional repression under hypoxia. We found the expression levels of *miR-17-92* cluster were reduced in hypoxia-treated cells containing wild-type p53, but were unchanged in hypoxia-treated p53-deficient cells. The repression of *miR-17-92* cluster under hypoxia is independent of c-Myc. Luciferase reporter assays mapped the region responding to p53-mediated repression to a p53-binding site in the proximal region of the *miR-17-92* promoter. Chromatin immunoprecipitation (ChIP), Re-ChIP and gel retardation assays revealed that the binding sites for p53- and the TATA-binding protein (TBP) overlap within the *miR-17-92* promoter; these proteins were found to compete for binding. Finally, we show that pri-*miR-17-92* expression correlated well with p53 status in colorectal carcinomas. Over-express *miR-17-92* cluster markedly inhibits hypoxia-induced apoptosis, whereas blocked miR-17-5p and miR-20a sensitize the cells to hypoxia-induced apoptosis. These data indicated that p53-mediated repression of *miR-17-92* expression likely has an important function in hypoxia-induced apoptosis, and thus further our understanding of the tumour suppressive function of p53.

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Introduction

The p53 tumour suppressor gene functions as a 'guardian of the genome' both by acting as a sequence-specific DNA-binding protein as well by transcription-independent mechanisms (Wang *et al*, 2001; Sharpless and DePinho, 2002; Slee *et al*, 2004). Under normal conditions, p53 has an extremely short half-life owing to rapid proteasomal degradation. On exposure to stresses such as genotoxic damage or hypoxia, post-translational modification leads to p53 stabilization; the accumulated p53 transactivates expression of a number of target genes that collectively contribute to p53-dependent cellular response. p53 can induce cells to undergo a transient arrest in G1 to allow time for repair of damaged DNA; it can also eliminate cells through mechanisms that involve prolonged arrest in G1 or apoptosis. The elimination of damaged, stressed or abnormally proliferating cells by p53 is considered to be the principal means by which p53 mediates tumour suppression.

Aside from its transcriptional activation function, p53 can also act as a transcriptional repressor. There is accumulating evidence to show that the repression of certain genes by p53 may be important for its ability to carry out its functions. For instance, ectopic expression of various p53-repressed genes, including Bcl-2 (Chiou *et al*, 1994), survivin (Hoffman *et al*, 2002), MAP4 (Murphy *et al*, 1996) and PIK3CA (Singh *et al*, 2002), was shown to inhibit p53-dependent apoptosis. The mechanism of transrepression remains a controversial area of p53 biology and may or may not be dependent on the site-specific DNA-binding activity of p53. Proposed mechanisms include interference with the function of transcriptional activators, interference with the basal transcriptional machinery, recruitment of chromatin modifying factors to reduce promoter accessibility and recruitment of transcriptional corepressors (Ho and Benchimol, 2003).

MicroRNAs (miRNAs) are 21–23 nucleotide RNA molecules that regulate the stability or translational efficiency of target messenger RNAs. The miRNAs have been shown to have critical functions in diverse functions including the regulation of cellular differentiation, proliferation and apoptosis (Bartel, 2004; Cheng *et al*, 2005; Croce and Calin, 2005). Aberrant expression of specific miRNAs has recently been described in a variety of human malignancies, including chronic lymphocytic leukemia (Calin *et al*, 2004, 2005).

The *miR-17-92* cluster comprises a cluster of seven miRNAs on chromosome 13 that is transcribed as a single polycistronic unit (Tanzer and Stadler, 2004). It has been defined as a common miRNA signature in several solid tumours (Lewis *et al*, 2003; Volinia *et al*, 2006). Specifically, expression of this cluster is induced by the oncogene c-Myc (O'Donnell *et al*, 2005) and some miRNAs are over-expressed in lung and colorectal carcinoma (Hayashita *et al*, 2005; Dews *et al*, 2006). Over-expression of *miR-17-92* in haematopoietic stem cells significantly accelerated the formation of lymphoid malignancies (He *et al*, 2005). However, in contrast to the wealth of information about the biological effects of the *miR-17-92* cluster, little is known about its regulation.

In this study, we have shown that the *miR17-92* cluster is repressed by hypoxia-induced p53. We report that p53-mediated repression of *miR-17-92* takes place at the transcriptional level; this is mediated largely through a specific interaction between p53 and a p53-binding site in the proximal region of the *miR-17-92* promoter. We provide evidence

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that p53 exerts its repressive effect by preventing the binding of the TATA-binding protein (TBP) transcriptional factor to a TATA box that overlaps with the p53-binding site. To evaluate the physiological significance of p53-mediated repression of miR-17-92, we show that pri-*miR-17-92* expression was well correlated with p53 status in colorectal carcinomas. Furthermore, over-expression of the *miR-17-92* cluster reduced apoptosis of hypoxia-treated HCT116 $p53^{+/+}$ cells, whereas inhibition of miR-20a and miR-17-5p induced apoptosis in hypoxia-treated HCT116 $p53^{-/-}$ cells, indicating that repression of *miR-17-92* expression by p53 is likely to have a function in hypoxia-induced apoptosis. These data further our understanding of the tumour suppressive function of p53.

Results

Expression of the miR-17-92 cluster is down-regulated in hypoxia-treated wild-type cells, but not in p53-null cells

Hypoxia is a key feature of the neoplastic microenvironment. Tumours with low oxygen tension tend to exhibit poor prognosis and resistance to conventional therapy (Harris, 2002). To date, however, little is known concerning the regulation of miRNAs expression during hypoxia. To investigate hypoxia-dependent miRNA-expression, Caco-2 and HCT116 $p53^{+/+}$ cells were exposed to hypoxic conditions (0.1% O_2) for 0, 24 and 48 h. RNA was extracted, and differentially expressed miRNAs were screened using an miRCURY LNA miRNA Array version 8.1 (Castoldi *et al*, 2006). We used a two-fold change threshold and statistical comparisons (analysis of variance; $P<0.05$) to identify miRNAs differentially expressed between hypoxia-treated and -untreated cells.

Eleven miRNAs were up-regulated and 46 miRNAs were down-regulated significantly under hypoxic conditions (48 h) in both cell lines (Supplementary Table S1). The miRNAs have been reported to respond to hypoxia in earlier studies (Hebert *et al*, 2007; Kulshreshtha *et al*, 2007), including the up-regulated miR-26a, 210, 21, 637 and 192, and the down-regulated miR-122a, 186, 320 and 197, but some miRNA responses differed between these studies. For example, miR-181b was down-regulated in our studies, but was reported to be up-regulated in another study (Kulshreshtha *et al*, 2007). This may reflect different cellular backgrounds or the microarrays used.

Intriguingly, we found that the expression of miR-18a, miR-19a, miR-20a and miR-19b was down-regulated in hypoxia-treated HCT116 $p53^{+/+}$ cells, but there were no significant changes in p53-null Caco-2 cells (Figure 1A; Supplementary Table S2). These four miRNAs belong to the same miRNA cluster, *miR-17-92*, which includes seven miRNAs and is located on chromosome 13 (Figure 1B). In contrast, the expression levels of homologues of the *miR-17-92* cluster, including miR-18b and miR-363 within the *miR-106a-363* cluster (chromosome X) and miR-10b and miR-25 within the *miR-106b-25* cluster (chromosome 7), were unchanged in both hypoxia-treated Caco-2 and HCT116 $p53^{+/+}$ cells. As ascertained by miCHIP analysis, the expression of the remaining three miRNAs in the *miR-17-92* cluster was unchanged by hypoxia. This may be due to cross-hybridization with substantially homologous sequences of miR-17-5p and miR-106a. The microarray used in these experiments did not contain probes capable of distinguishing between miR-17-5p and miR-106a transcripts; in addition, the signals generated by miR-17-3p and 92a-1 were difficult to detect.

Confirmation of the hypoxic repression of miR-17-92 expression by real-time RT–PCR

To validate miRNA expression as determined by miCHIP analysis, miRNA-specific quantitative real-time RT–PCR (miR-qRT–PCR) was performed on RNA isolated from Caco-2 and HCT116 $p53^{+/+}$ cells treated as described above. To exclude the possibility that the changes in miRNA recovery are not because of the effects of hypoxia, we also examined the expression of miR-210, an miRNA shown earlier to be induced by hypoxia in several studies (Hebert *et al*, 2007; Kulshreshtha *et al*, 2007; Camps *et al*, 2008) and confirmed by the miCHIP results of this study (Supplementary Table 1). As shown in Figure 1C and D, most miRNA-expression changes revealed by miCHIP analysis were confirmed by qRT–PCR. Expression of miR-17-5p, miR-17-3p, miR-18a, miR-20a, miR-19a and miR-19b-1 in the *miR-17-92* cluster were down-regulated in hypoxia-treated HCT116 $p53^{+/+}$ cells, but expression levels (except those of *miR-17-5p*) were unchanged in hypoxia-treated Caco-2 cells. With the possible exception of miR-106a, both the miCHIP and qRT-PCR results indicated that miR-18b and miR-363 (encoded by the *miR-106a-363* cluster) are not down-regulated by hypoxia. In addition, both approaches showed that expression levels of miR-106b, miR-93 and miR-25 (encoded by the *miR-106b-25* cluster) were unchanged, whereas expression of the positive-control miR-210 was significantly induced in both hypoxia-treated Caco-2 and HCT 116 $p53^{+/+}$ cells.

It has been reported that the Caco-2 cell line is deficient in functional p53 protein: one allele is deleted, whereas the other contains a nonsense E204X mutation (Djelloul *et al*, 1997); in contrast, HCT116 $p53^{+/+}$ cells contain two wild-type p53 alleles (see western-blot analysis; Figure 1C and D). This raises the question of whether differential p53 status might underlie the differences in *miR-17-92* cluster expression in response to hypoxia.

To exclude a possible effect of cellular background, we compared the expression of *miR-17-92* in hypoxia-treated isogenic HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines (Bunz *et al*, 1999). As expected, hypoxia down-regulated levels of *miR-17-92* expression in cells wild type for p53, whereas expression levels were unaffected by hypoxia in HCT116 $p53^{-/-}$cells (Supplementary Figure S1).

To confirm that repression of the *miR-17-92* cluster by hypoxia was not restricted to tumour cells, we used primary human hTERT-immortalized retinal pigment epithelial cells (hTERT RPE1), which are normal human cells immortalized by the expression of the reverse-transcriptase subunit of telomerase. These cells have an intact p53 pathway as evidenced by cell-cycle arrest with elevated levels of p21 in response to DNA damage (Uetake and Sluder, 2007). Expression of miR-17-5p, miR-17-3p, miR-18a, miR-20a and miR-19a in the *miR-17-92* cluster were down-regulated in hypoxia-treated hTERT RPE1 cells (Supplementary Figure S2), showing that selective repression of *miR-17-92* cluster miRNAs by hypoxia is not a function of cell type. These findings suggest that variations in p53 status may explain the differential responds of *miR-17-92* expression to hypoxia in the different cell lines.

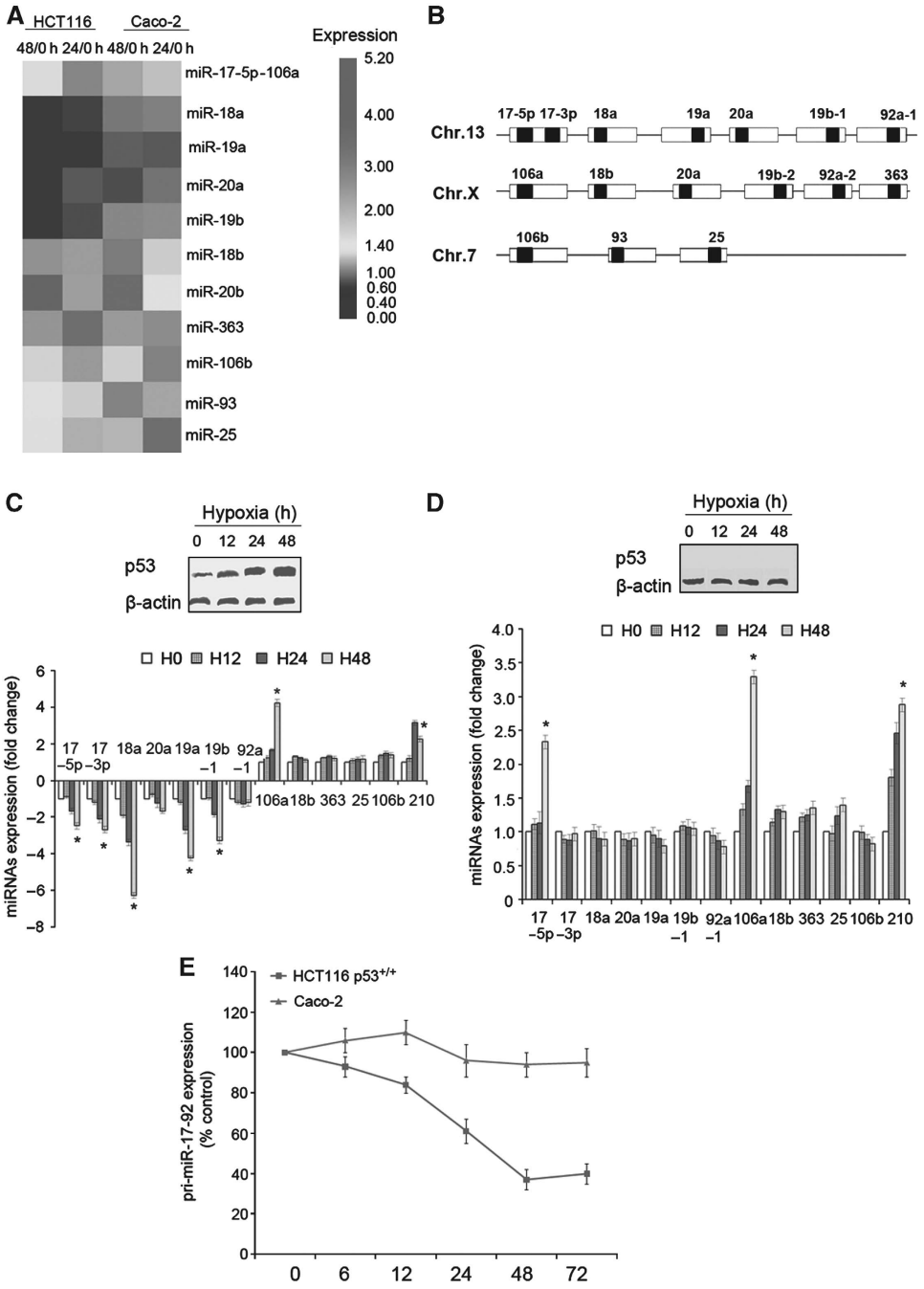

Figure 1 Down-regulation of *miR-17-92* cluster in hypoxia-treated p53-wt cells, but not in p53-null cells. (**A**) Hierarchical clustering analysis showed down-regulation of miR-18a, -19a, -20a and -19b in hypoxia-treated HCT116 $p53^{+/+}$ cells for 48 h (blue), but not in hypoxia-treated Caco-2 cells. Expression data were normalized to expression at time zero. (**B**) Genomic organization of three paralogous *miR-17-92* miRNA clusters. Black boxes indicate mature miRNAs embedded in precursor miRNAs (pre-miRNAs, white boxes) according to Tanzer and Stadler (2004). HCT116 $p53^{+/+}$ cells (**C**) and Caco-2 cells (**D**) were treated with 0.1% O_2 for 0, 12, 24 and 48 h, respectively. p53 protein levels were also analysed by western-blot analysis and normalized to β-actin. Expression of miRNAs in *miR-17-92* cluster and its paralogous clusters were confirmed by miRNA-specific quantitative RT–PCR and normalized to time point zero. Hypoxia-induced miR-210 was used as a positive control. The data shown are mean ± s.e.m. of three independent experiments. Star (*) indicates that the miRNA-expression level is significantly changed after 48 h hypoxia treatment compared with untreatment controls. Time course of pri-*miR-17-92* (**E**) repression in HCT116 $p53^{+/+}$ cells and Caco-2 cells treated with 0.1% O_2 for 0, 6, 12, 24, 48 and 72 h, respectively. The data shown are mean ± s.e.m. of three independent experiments. A full-colour version of this figure is available at *The EMBO Journal* Online.

Kinetics of pri-miR-17-92 expression under hypoxia

As p53 commonly acts as a transcriptional factor and regulate target genes at the transcriptional level, we hypothesized that p53 might be able to repress *miR-17-92* transcription under hypoxic conditions. Therefore, we next examined the kinetics of *pri-miR-17-92* expression in response to hypoxia. HCT116 $p53^{+/+}$ and Caco-2 cells were cultured under hypoxic conditions (0.1% O_2) for 0, 6, 12, 24, 48 and 72 h. The expression of pri-*miR-17-92* was analysed by qRT–PCR. When HCT116 $p53^{+/+}$ cells were exposed to hypoxia for 24 and 48 h, the

levels of pri-*miR-17-92* decreased to 59 and 36% of control levels. In contrast, pri-*miR-17-92*-expression levels were unchanged in hypoxia-treated Caco-2 cells (Figure 1E).

To study the oxygen dependence of the regulation of pri-*miR-17-92* expression, further experiments were performed in HCT116 $p53^{+/+}$ and Caco-2 cells exposed to a range of oxygen tensions for 24 h (0.1, 1, 3 and 5%). Significant repression of the pri-*miR-17-92*-expression level was seen with 0.1% O_2, whereas more modest regulation was seen with 3 and 5% O_2 (data not shown). Therefore, in the following studies of the effects of hypoxia treatment, we cultured the indicated cells with 0.1% O_2 for 24 or 48 h.

Repression of pri-miR-17-92 by hypoxia is p53 dependent

We addressed whether the repression of *miR-17-92* under hypoxic conditions is mediated by p53. The expression of p53 in HCT116 $p53^{+/+}$ and Lovo cells was down-regulated by transfection with small-interfering RNAs (siRNAs) targeting the p53 gene. siRNA-transfected cells were exposed to normoxic or hypoxic conditions for 24 h. Specific anti-p53 siRNAs significantly decreased p53 protein levels, whereas scramble control was unable to inhibit the accumulation of p53 under hypoxic conditions (Figure 2A, western blot). In cells transfected without (mock) or with scramble siRNA, expression levels of pri-*miR-17-92* were significantly reduced after hypoxia treatment. In contrast, pri-*miR-17-92* levels failed to respond to hypoxia in cells transfected with anti-p53 siRNAs (Figure 2A). Similar differential results were also observed in Lovo cells transfected with anti-p53 siRNAs versus scramble control (Figure 2B).

These results support the hypothesis that down-regulation of *miR-17-92* expression under hypoxic conditions is mediated by hypoxia-induced p53.

Knockdown c-Myc unable to inhibit the p53-mediated repression of miR-17-92

As *miR-17-92* is transcriptionally regulated by c-Myc (O'Donnell *et al*, 2005) and c-Myc is repressed by p53 activation under some stress conditions (Ho *et al*, 2005),

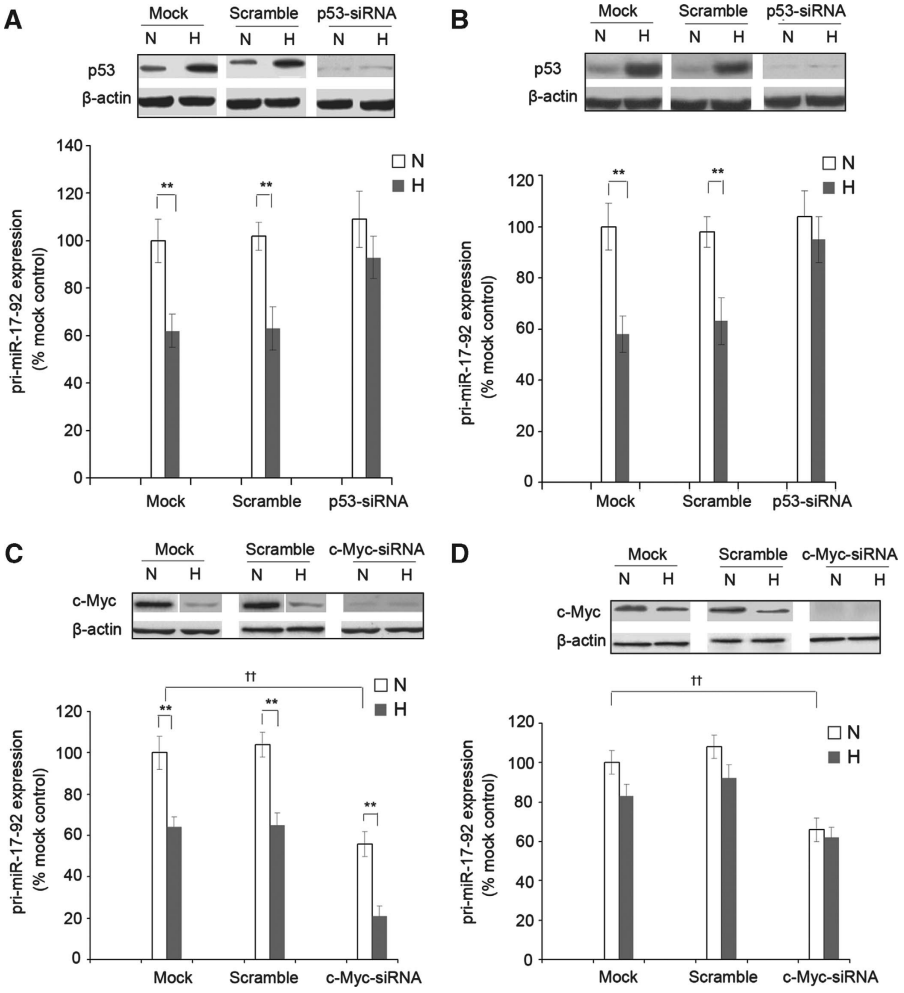

Figure 2 Repression of *miR-17-92* is p53 dependent. HCT116 $p53^{+/+}$ cells (**A**) and Lovo (**B**) cells were transfected with the transfection agent, but no siRNA (mock), siRNA against wild-type p53 (p53-siRNA) or scramble-control siRNA (scramble) for 24 h, then the cells were exposed to normoxic and hypoxic conditions for another 24 h. Reduced p53 expression by siRNAs was shown by western-blot analysis and normalized to β-actin. Expression of pri-*miR-17-92* transcripts was quantified by real-time RT–PCR. The data shown are mean ± s.e.m. of three independent experiments. $^{**}P<0.01$ versus normoxia-treatment control. HCT116 $p53^{+/+}$ cells (**C**) and $p53^{-/-}$ cells (**D**) were transfected with the transfection agent, but no siRNA (mock), siRNA against c-Myc or scramble-control siRNA (scramble), then the cells were exposed to normoxic (N) or hypoxic conditions (H) for another 24 h. c-Myc expression was demonstrated by western-blot analysis and normalized to β-actin. Expression of *miR-17-92* pri-miRNA transcripts was quantified by real-time PCR. The data shown are mean ± s.e.m. of three independent experiments. $^{\dagger\dagger}P<0.01$ versus mock control; $^{**}P<0.01$ versus normoxia-treatment control. A full-colour version of this figure is available at *The EMBO Journal* Online.

it is unknown whether p53-mediated *miR-17-92* repression is mediated by repression of c-Myc or by a mechanism independent of c-Myc. To address this question, we used a *c-myc* siRNA that effectively abrogated *c-myc* expression in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells (western-blot results, Figure 2C and D). Suppression of *c-myc* by siRNA down-regulated *miR-17-92* expression to 58% of the baseline value in $p53^{+/+}$ and to 62% in $p53^{-/-}$ cells ($^{\dagger\dagger}P<0.01$ versus mock control) under normoxic conditions, thereby confirming that *miR-17-92* expression was regulated by c-Myc. However, when the *c-myc*-deficient cells were exposed to hypoxia for 24 h, *miR-17-92* expression was repressed significantly in HCT116 $p53^{+/+}$ cells ($^{**}P<0.01$ versus normoxic-treatment control), but not in $p53^{-/-}$ cells. These data indicated that knockdown of c-Myc was unable to inhibit p53-mediated repression of *miR-17-92* under hypoxic conditions.

Hypoxia-induced p53 represses miR-17-92 promoter activity

To map the region in the *miR-17-92* promoter that responds to the p53-mediated repression, we introduced various lengths of the *miR-17-92* 5′ flanking region (−1.5,−1.1,−0.8, −0.5 and −0.3 kb) into the promoterless luciferase pGL vector. Luciferase levels were measured after transient transfection into $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells and exposed to normoxic or hypoxic conditions for 24 h.

As shown in Figure 3A and B, under normoxic conditions, all promoter constructs behaved similarly in both cell lines. The longest construct (contained within pGL3-1.5 kb), and extending from −1260 bp upstream to 263 bp downstream of the transcription start site, showed robust transcriptional activity. Luciferase activities were increased by, respectively, 24.4- and 25.6-fold in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells versus levels in the same cells transfected with the promoter-

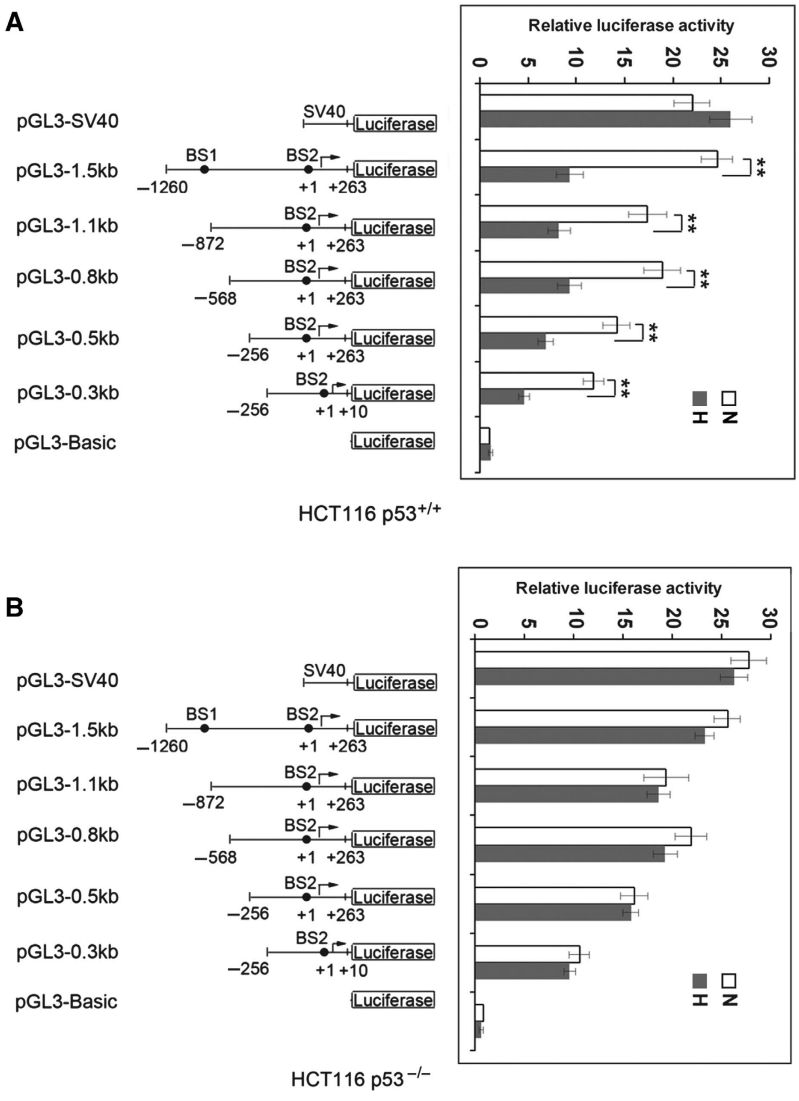

Figure 3 Mapping the region within *miR-17-92* 5′ flanking promoter responds to p53-mediated repression. HCT116 $p53^{+/+}$ (**A**) and HCT116 $p53^{-/-}$ (**B**) cells were transiently transfected with the pGL3 sequence-deleted promoter reporter (400 ng/well) and pRL-TK (*Renilla* luciferase, Promega) (100 ng/well). A simian virus 40/pGL3 (pGL3-control) construct and the reporter plasmid without insert (pGL3-Basic) were transfected separately as positive and negative references, respectively. The cells then grew in normoxic conditions (N) or exposed to hypoxic conditions for 24 h (H). Cell lysates were assessed for luciferase activity, which was normalized to *R. luciferase* activity for each transfected well. For each experimental trial, wells were transfected in triplicate and each well was assayed in triplicate. Activity was defined as firefly/*Renilla* ratio normalized to negative-control vector transfection. The data shown are mean ± s.e.m. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ versus normoxia-treatment control. A full-colour version of this figure is available at *The EMBO Journal* Online.

less construct. Promoter activity of the shortest construct, pGL3-0.3 kb, was substantially retained: luciferase activities were increased, respectively, by 11.8- and 10.6-fold in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. When transfected cells were exposed to hypoxic conditions, however, the activities of the 1.5, 1.1, 0.8, 0.5 and 0.3 kb *miR-17-92* promoter constructs were greatly reduced in HCT116 $p53^{+/+}$ cells. Hypoxia treatment of transfected HCT116 $p53^{-/-}$ cells failed to reduce luciferase expression. These results indicate that all promoter segments tested are responsive to hypoxia and suggest that a key *cis*-regulatory p53/hypoxia response element is contained within the 0.3 kb proximal region of the *miR-17-92* promoter.

Earlier studies have shown that a conserved 'CATGTG' sequence, located 1434 bp downstream of the transcript start site, is the key-binding site for c-Myc-regulated *miR-17-92* expression (O'Donnell *et al*, 2005). This site is outside our promoter constructs; therefore, these data provide more evidence that p53 can repress *miR-17-92* expression independent of c-Myc.

The p53-binding site at −20 to −44 is involved in transcriptional repression of miR-17-92

We then used the MAPPER Search Engine (Marinescu *et al*, 2005) to search the *miR-17-92* 5′ flanking region (−1260 to +263 bp) for potential p53 regulatory elements. This program detects p53-binding motifs by scanning for the p53 consensus DNA-binding sequence 5′-RRRCWWGYYY ($N = 0$–13) RRRCWWGYYY -3′, where R = G or A, W = T or A, Y = C or T and N = any base) (Deiry *et al*, 1992). Two potential p53-binding sites, BS1 (nt −691 to −716) and BS2 (nt −20 to −44), were indentified within the proximal region of the *miR-17-92* promoter (Figure 4A).

We used luciferase reporter assays to assess the functional legitimacy of the two potential p53-responsive elements. Earlier results (Figure 3A) showed that loss of the BS1-binding site did not affect p53-mediated repression activity, indicating that BS2-binding site may be the key element mediating hypoxia responsiveness. To address this possibility, the BS1 and BS2 sites were separately mutated and the transcriptional activities of the mutated promoters were determined. As shown in Figure 4B, mutation of the BS1 site did not affect the p53-mediated repression after hypoxia treatment. However, mutation of the BS2 site led to complete loss of p53 repression. These findings show that the p53-binding site BS2 located −20 to −44 nt upstream of the transcription start site is a major determinant of *miR-17-92* transcriptional repression.

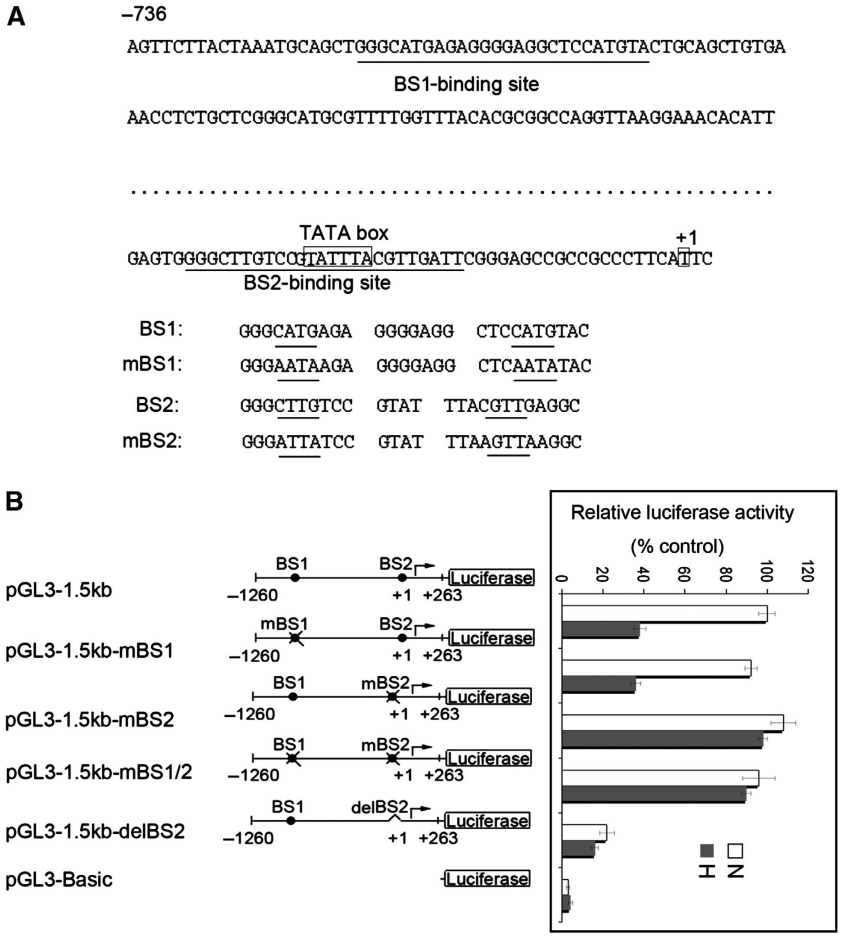

Figure 4 The p53-binding site between −20 and −44 is involved in transcriptional repression of *miR-17-92*. (**A**) Schematic diagram of the *miR-17-92* promoter 5′ flanking region. The *arrow* indicated the transcription start site, and numbering is related to the first residue of exon 1. The two p53-binding sites and the mutated sites in BS1 and BS2 are *underlined*, and the TATA box is shown in *open box*. (**B**) The indicated regions of *miR-17-92* promoter were linked to the luciferase coding region (*open boxes*). HCT116 $p53^{+/+}$ cells were transiently transfected with the pGL3 promoter reporter (400 ng/well) and pRL-TK (*R. luciferase*, Promega) (100 ng/well). pGL3-Basic vector was transfected as negative control. The cells then grew in normoxic conditions or exposed to hypoxic conditions for 24 h. Luciferase activity was plotted relative to the activity of the pGL3-1.5 kb in the normoxic conditions (100%). *, $P<0.05$, **$P<0.01$ versus normoxia-treatment control. A full-colour version of this figure is available at *The EMBO Journal* Online.

The p53-binding site overlapping the TATA box is responsible for p53-mediated repression

It was found earlier that a non-consensus TATA box (TATTTA) within the *miR-17-92* promoter is important for transcription (Woods *et al*, 2007). Intriguingly, sequence analysis revealed that the BS2-binding site (5′-TGGGGCTTGTCCG**TATTTA**CGTTGAGGC-3′), extending from −20 to −44 within the *miR-17-92* promoter, contains the non-consensus TATA box (boldface) flanked by two p53 half-sites (underlined) (Figure 4A). Therefore, we hypothesized that the overlap between the p53-binding site and the TATA box site might be responsible for p53-mediated repression.

Quantitative chromatin immunoprecipitation (ChIP) assay was used to investigate the *in vivo* physical-binding activities of p53 and TBP to the BS2-region-binding site. HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells hypoxia treated for 0, 24 and 48 h were analysed. In contrast to the accumulation of p53 after hypoxia treatment, protein levels of TBP were unchanged by hypoxia treatment in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells, in agreement with earlier findings that TBP is a housekeeping gene stably expressed under hypoxic conditions (Fink *et al*, 2008). As a positive control, MAP4 is greatly reduced in hypoxia-treated HCT116 $p53^{+/+}$ cells, but unchanged in hypoxia-treated $p53^{-/-}$ cells (Figure 5A).

As expected, the relative amount of DNA bound by the anti-p53 antibody was significantly increased by hypoxia treatment (4.4% at 24 h and 4.1% at 48 h versus 0.6% untreated control, $P<0.01$) (Figure 5B and C). In contrast, the amount of DNA bound by the anti-TBP antibody was greatly reduced under hypoxic conditions (1.9% at 24 h and 0.6% at 48 h versus 2.6% untreated control, $P<0.01$). No DNA was precipitated by anti-p53 antibody in HCT116 $p53^{-/-}$ cells with or without hypoxia treatment, and the level of DNA precipitation by the anti-TBP antibody was unchanged by hypoxia treatment (Figure 5C).

The specificity of ChIP was verified using control IgG-precipitated chromatin, for which no PCR-amplified product was visible, or the negative control *GAPDH*, the DNA of which is not precipitated by the anti-p53 antibody (data not shown). The specificity of this assay was also verified using the high-affinity p53-binding site in the *GADD153* promoter as a positive control. Hypoxia increased the amount of precipitated *GADD153* DNA from 0.4 to 3.2 and 2.8% in $p53^{+/+}$ cells exposed to hypoxic conditions for 24 and 48 h, respectively. Concomitantly, the *GADD153* mRNA level was induced by 2.4- and 4.2-fold compared with that of untreated cells, which is consistent with the results of a published report (Liu *et al*, 2007) (Figure 5D).

Proteins binding in close apposition at composite regulatory elements can act in an additive or cooperative manner or, because of mutually exclusive binding, may direct opposite expression patterns. A question of considerable interest was, therefore, whether p53 and the TBP are capable of binding to the same DNA element simultaneously. To address this point, we performed an Re-ChIP assay. Here, chromatin is first enriched by specific interaction with one antibody (ChIP); retained chromatin is then eluted and used in a second immunoprecipitation assay (Geisberg and Struhl, 2004). Sequential reaction with anti-p53 and anti-TBP antibodies indicated that there was no co-occupancy of p53 and TBP at the *miR-17-92* promoter under either normoxic or hypoxic conditions. Reciprocal Re-ChIP analysis, in which the order of the antibodies was inverted, generated identical results (Figure 5E). No detectable DNA was immunoprecipited by control IgG.

To further confirm that p53 and TBP compete for binding to the *miR-17-92* promoter, gel-shift assays were performed. We prepared labelled oligonucleotides corresponding to nt −20 to −44 of the promoter; these were incubated with purified human p53, TBP or with both. As shown in Figure 5F, incubation of recombinant p53 and TBP with the labelled probe led to two distinct retarded bands (lanes 2 and 4). Super-shifted bands were detected when the anti-p53 antibody (lane 3) or anti-TBP antibody (lane 8), notably, the formation of TBP-DNA complexes, was inhibited by the addition of increasing amounts of p53 (100, 200 and 500 ng) (lanes 5, 6 and 7).

Taken together, *in vivo* ChIP, Re-ChIP and *in vitro* gel retardation analyses show that overlap of the TATA box and the p53-binding site within the proximal region of the *miR-17-92* promoter leads to mutually exclusive binding. Competitive binding of p53, with displacement of TBP from the promoter, affords a novel mechanism for p53-mediated repression of *miR-17-92* expression under hypoxic conditions.

Pri-miR-17-92 expression correlates well with p53 status in colorectal cancer

Hypoxia occurs in all solid tumours, which can vary from 0 to 8%. Graeber *et al* (1996) showed that p53 positive tumours underwent significant hypoxia-induced apoptosis, whereas match p53-null tumours did not. This finding led to the conclusion that hypoxia acts as a selection pressure for cells with diminished apoptotic potential, for example a loss of p53 function. To evaluate the potential physiological significance of p53-mediated repression of *miR-17-92*, we correlated pri-*miR-17-92* expression with p53 status in colorectal carcinomas. The levels of pri-*miR-17-92* were analysed by real-time RT–PCR in paired colorectal cancer and normal samples. p53 expression was first detected by immunohistochemistry staining and 18 tumours showed positive staining. As tumours with a positive immunostaining for p53 not always indicate loss of function (Greenblatt *et al*, 1994), we screened mutation in p53 gene from these p53-positive samples by DHPLC and sequencing analysis. Finally, 10 colorectal tumours were identified containing pathogenic p53 mutations (Supplementary Table 4). As shown in Figure 6A, pri-*miR-17-92* was over-expressed by 2.6-fold compared with non-tumour samples ($P=0.0048$, Wilcoxon matched-pairs test). However, only 8 of the 32 tumours (25%) showed greater than five-fold change, which is consistent with the results of earlier studies (He *et al*, 2005). Of the eight tumours with higher expression of *pri-miR-17-92*, six (75%) tumours contain p53 mutations. Moreover, the relative fold change (tumour/non-tumour, T/N) of pri-*miR-17-92* in p53-mutant tumours was much higher than that in p53-wt tumours ($P=0.00018$, Wilcoxon rank sum test) (Figure 6B). These findings indicated that the expression levels of pri-miR-17-92 were correlated well with p53 status in colorectal cancers, implying that p53-mediated repression of miR-17-92 expression might have a function in the tumour suppressive function of p53.

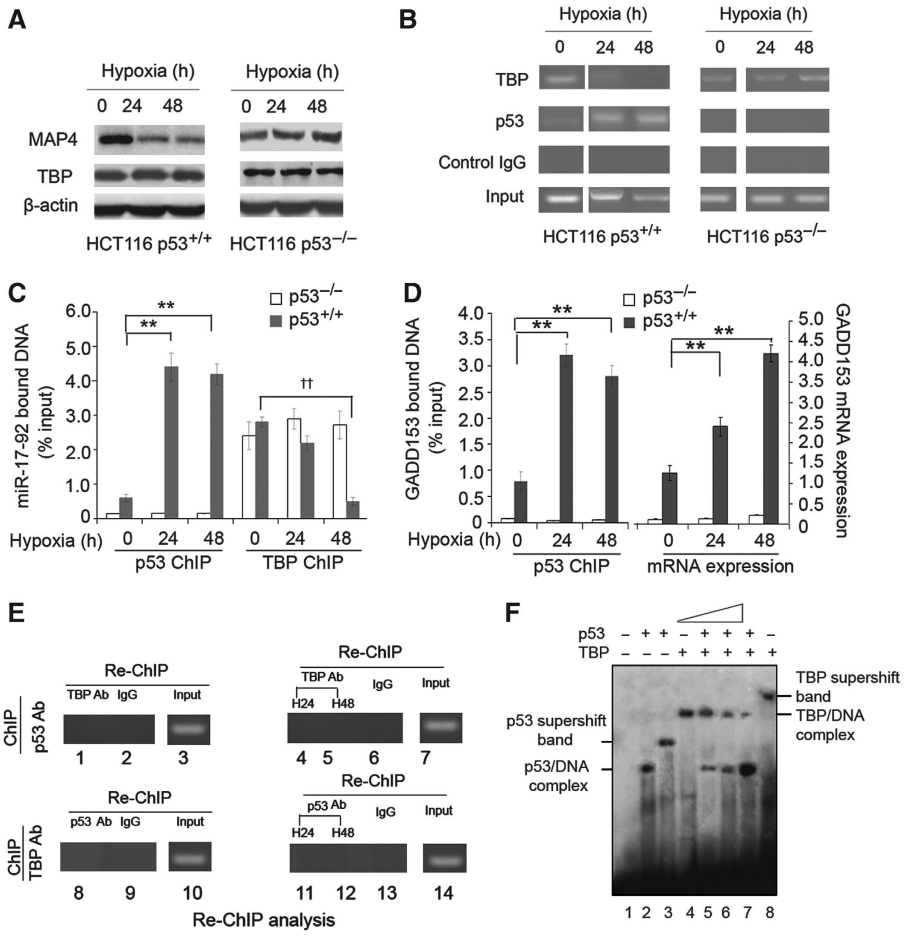

Figure 5 ChIP and Re-ChIP analysis for the occupancy of TBP and p53 in BS2-binding site under hypoxic conditions. HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with hypoxia for 0, 24 and 48 h. (**A**) Protein levels of TBP and MAP4 was determined by western-blot analysis and normalized to β-actin. (**B**) ChIP assay. The cells were cross-linked with 1% formaldehyde. Cell lysates were prepared and equal amounts of cell lysates were immunoprecipitated with anti-p53 antibody, anti-TBP antibody or control IgG. The amount of DNA bound to the *miR-17-92* promoter immunoprecipitated by p53 and TBP antibody was interrogated with primers specific for the overlap p53/TATA-binding site (Supplementary data). Amplified products were resolved in 1.5% agarose gel and visualized by ethidium bromide staining. The result is representative of an experiment repeated with three separate preparations. (**C**) The amount of DNA bound to the *miR-17-92* promoter immunoprecipitated by p53 and TBP antibody was quantified by quantitative PCR of a fragment containing the BS2 site (Supplementary data). The amount of ChIP DNA PCR product was divided by that of the input to calculate the percentage of input. Data shown were mean ± s.e.m. of three separate experiments. ** or $^{\dagger\dagger}P<0.01$ versus control (untreated with hypoxia). (**D**) The amount of DNA bound to the *GADD153* promoter immunoprecipitated by p53 antibody was assessed by quantitative ChIP analysis. Corresponding relative mRNA levels are also indicated. Data shown were mean ± s.e.m. of three separate experiments. $^{**}P<0.01$ versus control (untreated with hypoxia). (**E**) Re-ChIP assay. Chromatin was prepared from HCT116 $p53^{+/+}$ cells treated with hypoxia for 0, 24 and 48 h. ChIP was first performed using anti-p53 or anti-TBP antibody as indicated. The eluant of each immuno-complex was subjected to further immunoprecipitatin using the second antibody (anti-TBP or anti-p53). The precipitated chromation DNA was used for PCR amplification. *Lane 1–7*: ChIP with anti-p53 antibody and Re-ChIP with anti-TBP antibody. No DNA was amplified from either hypoxia untreated cells (*lane 1*) or hypoxia treated for 24 (H24, *lane 4*) and 48 h (H48, *lane 5*). *Lane 8–14*: ChIP with anti-TBP antibody and Re-ChIP with anti-p53 antibody. No DNA was amplified from either hypoxia untreated cells (*lane 7*) or hypoxia treated for 24 (H24, *lane 11*) and 48 h (H48, *lane 12*). *Lanes 2,6, 9* and *13* represent PCR amplification of control IgG. *Lanes 3, 7, 10, 14* represent PCR amplification of 10% input DNA. Amplified products were resolved in 1.5% agarose gel and visualized by ethidium bromide staining. (**F**) p53 competes with TBP for binding to the *miR-17-92* promoter. Gel shift analysis was performed using a fragment of *miR-17-92* promoter extending from −14 to −44 bp. The oligoduplexes were end labelled with [γ-^{32}p]ATP and incubated with recombinant human p53, TBP and both proteins. *Lane 1*, free probes; *lane 2*, p53 (100 ng); *lane 3*, p53-antibody + p53 (100 ng) (supershift binding); *lane 4*, TBP (1 pfu per reaction, Promega); *lane 5*, p53 (100 ng) and TBP (1 pfu per reaction); *lane 6*, p53 (200 ng) and TBP (1 pfu per reaction); *lane 7*, p53 (500 ng) and TBP (1 pfu per reaction); *lane 8*, TBP-antibody + TBP (1 pfu per reaction) (supershift binding). A full-colour version of this figure is available at *The EMBO Journal* Online.

p53-mediated repression of miR-17-92 has a function in hypoxia-induced apoptosis

The important function of p53 in mediating apoptosis in the hypoxic regions of tumours has been effectively shown earlier (Graeber *et al*, 1996). Several studies have indicated that some of the miRNAs in *miR-17-92* cluster have anti-apoptotic activities (He *et al*, 2005; Matsubara *et al*, 2007; Takakura *et al*, 2008). As p53 down-regulates *miR-17-92* levels in both HCT116 $p53^{+/+}$ and Lovo cells, we hypothesized that *miR-17-92* cluster might have a function in p53-induced apoptosis under hypoxia. To address this question, we first sought to determine whether over-expression of *miR-17-92* cluster was able to reduce the p53-induced apoptosis under hypoxia. HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells were transfected with an *miR-17-92*-expression vector (pcDNA3-*miR-17-92*) or an empty vector (pcDNA3). Transfected cells were exposed to

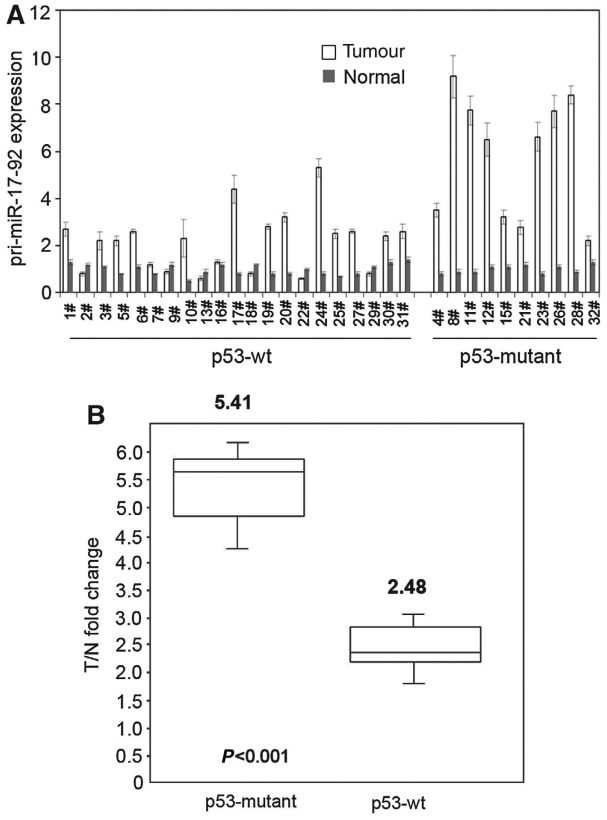

Figure 6 Correlation of p53 status and expression of pri-miR-17-92 in colorectal carcinomas. (**A**) Expression of pri-*miR-17-92* in paired tumours and normal samples was analysed by TaqMan RT–PCR and normalized to *GAPDH*-expression. Data shown were mean ± s.e.m. of three separate experiments. (**B**) Correlation of p53 status and the expression of pri-*miR-17-92*. Each box represents the range of T/N fold changes. The ends of the boxes represent the 25th and 75th percentiles, the bars indicate the 10th and 90th percentiles and a line shows the median. The number shows the median fold changes in p53-mutant samples compared with the wild-type samples. The statistically significant differences were calculated using a Wilcoxon rank sum test. A full-colour version of this figure is available at *The EMBO Journal* Online.

normoxic or hypoxic conditions for 24 h. The predicted changes in the levels of pri-*miR-17-92* were validated by qRT–PCR analysis (data not shown). As revealed by immunostaining analysis, hypoxia treatment of transfected cells led to a significant induction of p53 expression and nuclear accumulation in HCT116 $p53^{+/+}$ cells, which is consistent with earlier studies (Koumenis *et al*, 2001) (Figure 7A). The extent of apoptosis was monitored by flow cytometry for annexin V-FITC/PI staining. In control pcDNA3-transfected HCT116 $p53^{+/+}$ cells, there was a significant increase in the fraction of apoptotic cells after hypoxia treatment. In contrast, in pcDNA3-*miR-17-92*-transfected cells, the number of apoptotic cells was significantly reduced (23.8 versus 7.1%, $P<0.05$) (Figure 7A and C). In HCT116 $p53^{-/-}$ cells, no p53 expression was seen by immunostaining analysis (Figure 7B); accordingly, only a small fraction of cells were apoptotic after hypoxic treatment. Although there was a trend to reduced level of apoptosis in the pcDNA3-*miR-17-92*-transfected cells, the differences versus HCT116 $p53^{-/-}$ cells transfected with pcDNA3 were not significant (Figure 7B and C).

To further address the function of p53-mediated repression of *miR-17-92* in hypoxia-induced apoptosis, also to avoid potential over-expression artefacts, we determined the consequences of blocking the function of some miRNAs in the *miR-17-92* cluster under hypoxic conditions. As miR-17-5p and miR-20a (Matsubara *et al*, 2007) have been suggested to be the major miRNAs in *miR-17-92* cluster involved in cell apoptosis, we transfected HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells with LNA-modified antisense oligoribonucleotides (ONs) against miR-17-5p, miR-20a or pooled (miR-17-5p + miR-20a). To monitor the level of miRNA inhibition, we constructed two reporter plasmids, miR-17-5p-reporter and miR-20a-reporter, in which two sites perfectly complementary to miR-17-5p or miR-20a were inserted into the 3′-untranslated region (3′-UTR) of *Renilla luciferase* gene. As shown in Figure 7D, when introduced into HCT116 $p53^{+/+}$ cells, the luciferase activities were reduced by 60–80% compared with pGL3-control vectors, indicating the efficient down-regulation by endogenous miR-17-5p and miR-20a. When co-transfection of these plasmids with LNA modified antisense oligonucleotides, but not the scrambled oligonucleotides, it significantly enhanced the luciferase activities, indicating an effective inhibition of these miRNAs. As shown in Figure 7E and F, when both miR-20a and miR-17-5p were suppressed, the fraction of apoptotic cells was significantly increased both in hypoxia-treated HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells.

Taken together, hypoxia significantly induces apoptosis in HCT116 $p53^{+/+}$ cells, but only small fraction of apoptotic cells was induced in $p53^{-/-}$ cells, indicating that hypoxia-induced apoptosis in HCT116 cells is largely p53 dependent. Over-expression of *miR-17-92* was able to reduce the hypoxia-induced apoptosis in $p53^{+/+}$ cells; when *miR-17-92* was inactivated, hypoxia induced apoptosis both in hCT116 $p53^{+/+}$ and $p53^{-/-}$ cells; these data substantially supported that repression of *miR-17-92* by p53 has a vital function in hypoxia-induced apoptosis.

p53-mediated repression of miR-17-92 during DNA damage

As p53 was also accumulated under other stress conditions, we further determine whether p53-mediated repression of *miR-17-92* was limited to hypoxia or a general phenomenon. As shown in Figure 8A, treatment of HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells with 0.3 μM adriamycin, a DNA-intercalating drug known to induce p53 function, significantly induced the expression of p53. As a positive control, p21 expression was induced after adriamycin treatment, which is consistent with earlier studies (Krieg *et al*, 2006). Corresponding to the accumulation of p53, the level of *pri-miR-17-92* was greatly reduced and reached the lowest level (52% of the untreated control) after adriamycin treatment for 12 h. Similar results were also observed in Lovo cells (data not shown).

We next performed quantitative ChIP to determine that whether the adriamycin-induced p53 could competitively bind to the overlap p53/TBP-binding site in the *miR-17-92* promoter. As shown in Figure 8C and D, the relative amount DNA bound by the anti-p53 antibody was significantly increased by adriamycin treatment. In contrast, the amount of DNA bound by the anti-TBP antibody was greatly reduced, similar to what was observed in the treatment with hypoxia. These data indicated that p53-mediated repression of *miR-17-92* is not only limited to hypoxia, but also occurs under other stresses (e.g. DNA damage), which further supported that

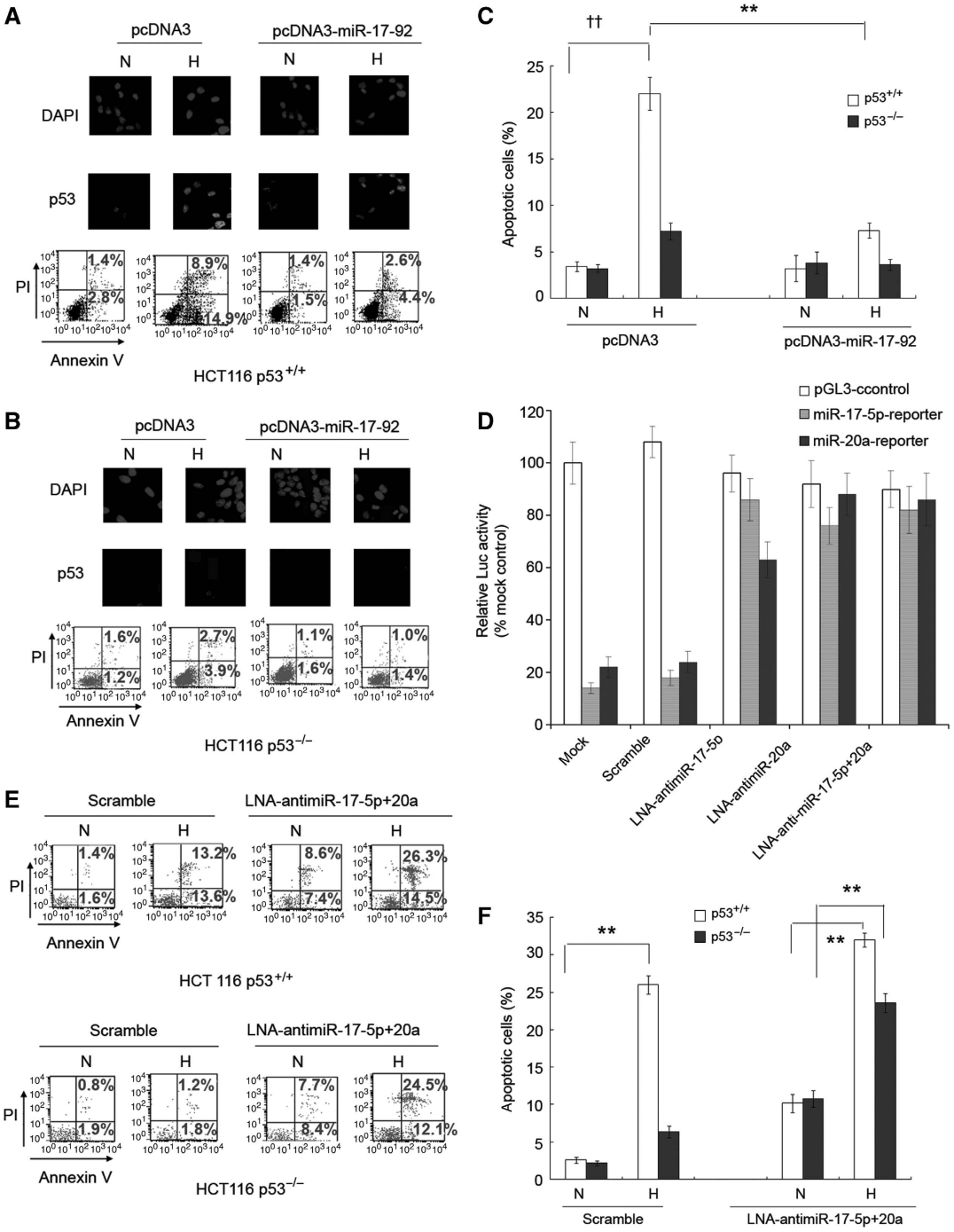

Figure 7 miR-*17-92* cluster has a function in the p53-mediated apoptosis under hypoxia. Over-expression of the *miR-17-92* cluster inhibited hypoxia-induced apoptosis. HCT116 $p53^{+/+}$ cells (**A**) and HCT116 $p53^{-/-}$ cells (**B**) were transfected with the *miR-17-92*-cluster-expression vector pcDNA3-*miR-17-92* or a control vector and then grown under normoxic (N) or hypoxic conditions (0.1% O_2) for 24 h (H). Nuclei were visualized with DAPI staining. p53 expression was demonstrated using the DO-1 monoclonal antibody and a fluorescein-conjugated mouse secondary antibody. Apoptotic cells were monitored by annexin V-FITC/PI staining and flow-cytometry analysis. The right-lower quadrant of each plot shows early apoptotic cells, whereas the right upper quadrant shows late apoptotic cells. Each experiment was performed in triplicate and similar results were obtained each time. (**C**) Quantification of apoptotic cells by flow-cytometry analysis. The values are mean ± s.e.m. $^{\dagger\dagger}P<0.01$ versus normoxia control. $^{**}P<0.01$ versus emptor-vector-transfected control. Suppression of *miR-17-5p* and *miR-20a* expression sensitized cells to hypoxia-induced apoptosis. (**D**) Inhibition of *miR-17-5p* and *miR-20a* by LNA-modified antisense ONs. The reporters or pGL3-vector control were transfected into HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells alone (mock) or with 40 nM the following ONs: scrambled nucleotides, LNA-antimiR-17-5p, LNA-antimiR-20a and pooled LNA-antimiR-17-5p + LNA-antimiR-20a (LNA-antimiR-17-92 + 20a). (**E**) HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were transfected with 40 nM scrambled nucleotides or 40 nM of a mixture of LNA-miR-17-5p and LNA-miR-20a, and grown under normoxic (N) or hypoxic conditions (0.1% O_2) for 24 h (H). Apoptotic cells were monitored by annexin V-FITC/PI staining and flow-cytometry analysis. Quantification of apoptotic cells by flow cytometry (**F**). The values are mean ± s.e.m. $^{**}P<0.01$ versus normoxia-treatment control. A full-colour version of this figure is available at *The EMBO Journal* Online.

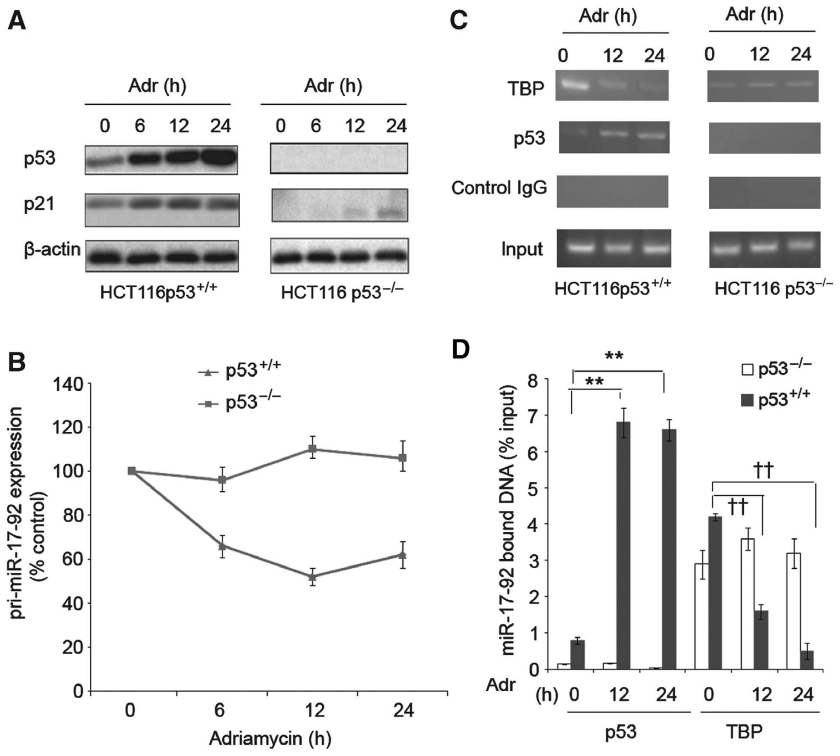

Figure 8 p53-mediated repression of miR-17-92 under DNA damage. (**A**) HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with 0.3 μM adriamycin (Adr) for 0, 12 and 24 h, respectively. Cells were collected and the protein levels of p53 and p21 were analysed by western-blot analysis and normalized to β-actin. (**B**) Expression of *miR-17-92* pri-miRNA transcripts was quantified by real-time PCR. The data shown are mean ± s.e.m. of three independent experiments. (**C**) Chromatin immunoprecipitation of *miR-17-92* promoter. The amount of DNA bound to the miR-17-92 promoter immunoprecipitated by anti-p53 and anti-TBP antibody was interrogated with primers specific for the overlap p53/TATA-binding site (Supplementary data). Amplified products were resolved in 1.5% agarose gel and visualized by ethidium bromide staining. The result is representative of an experiment repeated with three separate preparations. (**D**) The amount of DNA bound to the miR-17-92 promoter immunoprecipitated by anti-p53 and anti-TBP antibody was quantified by quantitative PCR. The amount of ChIP DNA PCR product was divided by that of the input to calculate the percentage of input. Data shown were mean ± s.e.m. of three separate experiments. ** or $^{\dagger\dagger}P<0.01$ versus control (untreated with adriamycin). A full-colour version of this figure is available at *The EMBO Journal* Online.

repression of *miR-17-92* might have a vital function in the tumour suppression function of p53.

Discussion

This study shows for the first time that the *miR-17-92* cluster is a novel target for p53-mediated transcriptional repression by specific binding under hypoxic conditions. This conclusion is based on the following observations: (1) hypoxia treatment reduced *miR-17-92* gene expression in cells containing wild-type p53 (HCT 116 $p53^{+/+}$ and Lovo), but had no effect in p53-null cells (Caco-2 and HCT 116 $p53^{-/-}$); (2) inhibition of endogenous p53 by siRNA blocked repression of *miR-17-92* expression in hypoxia-treated cells; (3) activity analysis of the sequence-deleted and mutated promoter constructs indicated that p53-mediated repression maps to BS2, a p53-binding site located in the proximal promoter region of *miR-17-92* and (4) we further showed that the p53-binding site overlaps with the TATA box of *miR-17-92* within this region of the promoter. *In vivo* ChIP analysis indicated that the BS2 site was mainly occupied by TBP under normoxic conditions, but during hypoxic conditions hypoxia-induced p53 inhibits TBP binding to this site. Further Re-ChIP assays revealed that p53 and TBP were unable to bind the same genomic region simultaneously. Furthermore, *in vitro* gel retardation analysis indicated that TBP–BS2 complexes were inhibited by increasing amounts of p53. We, therefore, propose that overlap between the p53 and TBP-binding sites results in competitive binding and p53-mediated displacement of TBP from the *miR-17-92* promoter.

This finding is of great importance to understanding the mechanisms by which p53 induces apoptosis under hypoxic conditions. Although it is generally accepted that p53 accumulation during severe hypoxia leads to rapid apoptosis (Weinmann *et al*, 2004; Hammond and Giaccia, 2005), the mechanisms underlying p53-mediated apoptosis are not yet well understood. Yu *et al* (2003) indicated that hypoxia-induced p53-dependent apoptosis is mediated through Puma and its effects on Bax; HCT116 cells lacking Puma are resistant to hypoxia-induced apoptosis. However, transformed mouse embryonic fibroblasts lacking Bax expression show no increased resistance to hypoxia-induced apoptosis (Alarcon *et al*, 2001). Other studies have indicated that, although *Bax* is implicated in hypoxia/reoxygenation-induced apoptosis, the level of apoptosis under hypoxic conditions in *Bax*-deficient cells is quantitatively and qualitatively similar to that in controls (Saikumar *et al*, 1998; Stempien-Otero *et al*, 1999). More recently, Fas/CD95 has been reported to be induced in response to hypoxia in a p53-dependent manner; this was suggested to be a critical regulator of p53-dependent apoptosis during hypoxia (Liu *et al*, 2007). With respect to these p53-transactived genes, Koumenis *et al*

(2001) showed that p53 acts as a transrepressor by complexing with *mSin3a* and inducing apoptosis.

Here, we found that *miR-17-92* is repressed by p53 under hypoxic conditions. It has been suggested that *miR-17-92* is involved in blockade of tumour cell apoptosis through E2F1 targeting (O'Donnell *et al*, 2005). Other studies have indicated that some of the miRNAs in the *miR-17-92* cluster, such as miR-17-5p and miR-20a, but not miR-18a and miR-19a, have anti-apoptotic activities (He *et al*, 2005; Matsubara *et al*, 2007; Takakura *et al*, 2008). Studies on the targeting genes revealed that *miR-17-92* can reduce c-Myc-induced apoptosis by targeting BCL-2-like 11 (BIM) and PTEN (Xiao *et al*, 2008), thereby increasing the level of anti-apoptotic BCL2. We found that *miR-17-92* expression in p53-mutant colorectal tumours is higher than that in p53-wt tumours (Figure 6). In addition, over-expression of *miR-17-92* was able to reduce the apoptosis level in hypoxia-treated $p53^{+/+}$ cells, and suppressed miR-17-5p and miR-20a expression sensitized the cells to hypoxia-induced apoptosis (Figure 7). These data confirmed that the repression of *miR-17-92* by increased levels of p53 likely has a function in hypoxia-induced apoptosis.

Although the ability of p53 to repress transcription at various viral and cellular promoters has been known for some time, the underlying mechanisms and functional consequences of transcriptional repression have been little investigated. p53-mediated transcriptional repression is generally thought to function through one of the following mechanisms: (1) interference with the functions of DNA-binding transcriptional activators; (2) interference with the basal transcriptional machinery and (3) alteration of chromatin structure of the promoters of target genes by the recruitment of proteins such as histone deacetylases (Ho and Benchimol, 2003). In this study, we have shown that overlap between the p53- and TBP-binding sites within the *miR-17-92* promoter proximal region results in competitive-binding and p53-mediated displacement of TBP. It, therefore, seems likely that p53-mediated repression of *miR-17-92* involves specific binding to the promoter, consistent with the mechanism of p53-mediated repression of the Cox-2 cyclooxygenase gene (Subbaramaiah *et al*, 1999).

Interestingly, we found that the accumulated p53 by other stresses, such as adriamycin treatment, were also able to competitively bind to the p53-binding site in the *miR-17-92* promoter and consequently down-regulated the pri-*miR-17-92* expression in HCT116 $p53^{+/+}$ cells (Figure 8). This indicated that p53-mediated repression of *miR-17-92* was not specific to hypoxia, but a general phenomenon. As earlier studies have indicated that *miR-17-92* is positively regulated by c-Myc, we propose that *miR-17-92* is able to be regulated at least by two important transcriptional factors: positively induced by c-Myc and negatively repressed by p53.

As the key 'genome gatekeeper', p53 has an important function in cancer prevention by maintaining genomic integrity through cell-cycle arrest and/or apoptotic cell death. p53 mutations are found at high frequencies in most of the common types of human cancer (Hollstein *et al*, 1991). Similar to p53, the c-Myc oncoprotein is a transcription factor that promotes cell growth and proliferation, as well as apoptosis under certain conditions. As p53 and c-Myc are involved in many of the same cellular processes, it is not surprising that they affect similar targets. Recently, a series of studies have indicated the existence of a fascinating and unexpected network of interactions involving c-Myc, E2F transcription factors and the *miR-17-92* cluster (Coller *et al*, 2007; Aguda *et al*, 2008). In addition, *miR-17-92* can increase Myc-enhanced proliferation by targeting p21 (Fontana *et al*, 2008) and consequently activating the CyclinD1/CDK4 complex to release retinoblastoma's inhibition on E2F. In this study, we found that p53 was also involved in this network, and maintains the balance between proliferative versus apoptotic responses to different stress conditions and determines the cell fate.

Materials and methods

Cell culture and treatments

Caco-2, Lovo, Human HCT116 $p53^{+/+}$ colon cancer cell lines and primary human hTERT RPE1 cells were obtained from the American Type Culture Collection. Isogenic human HCT116 $p53^{-/-}$ cell line was kindly provided by Dr Bert Vogelstein, Johns Hopkins University, Baltimore, MD. All the cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C. Hypoxia (0.1% O_2/5% CO_2/94.8% N_2) treatments were carried out in a Forma 1029 Anaerobic Chamber (Thermo Scientific). The medium of cell culture and the treatment in separate experiment are described in Supplementary data.

RNA isolation, miCHIP

Total RNA from Caco-2 and HCT116 $p53^{+/+}$ cells hypoxia treated for 0, 24 and 48 h was prepared using Trizol (invitrogen, CA). miRNA microarray including labelling, hybridization, scanning, normalization and data analysis was performed by KangChen Bio-tech (Supplementary data).

Real-time RT–PCR

Expression of mature miRNAs was determined using miR-qRT–PCR (Applied Biosystems, Foster City) and was normalized using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) relative to U6 endogenous control. *miR-17-92* pri-miRNA and *MAP4* expression was quantified by TaqMan RT–PCR and normalized to *GAPDH*-expression (Applied Biosystems, Foster City) (Supplementary data).

Patients and colorectal cancer samples

A total of 64 snap frozen colorectal patient biopsy specimens were selected (32 paired normal and tumour specimens). RNA extraction, cDNA synthesis and real-time RT–PCR analysis of pri-*miR-17-92* expression was performed as described in Supplementary data. p53 status was first detected by immunohistochemistry staining and 18 tumours showed positive staining. The 18 p53-positive samples were screened for mutation by DHPLC and sequencing analysis (Loyant *et al*, 2005) (Supplementary data).

ChIP and Re-ChIP assays

ChIP assays were performed using reagents and protocols from the ChIP kit (Upstate Biotechnology, Inc.) following manufacturer's protocol. For each ChIP, either 3 μg anti-p53 antibody (Clone: DO-1, Thermo Scientific) or anti-TBP antibody (1TBP18, ChIP grade, Abcam), 3 μg of non-specific immunoglobulin G (IgG; Sigma, St Louis, MO) were used. The Re-ChIP assays were performed using reagents and protocols from Re-ChIP-IT (Active Motif, CA) (Supplementary data).

Plasmid constructs and luciferase activity assays

All the promoter constructs of *miR-17-92* promoter are shown in Figures 3A and 4A. The miR-17-5p-reporter and miR-20a-reporter were constructed by ligating oligonucleotides containing two sites with perfect complementary to miR-17-5p or miR-20a into the *Xba*I site of the pGL3-control vector (Promega). The plasmid construction and luciferase activity assay were performed as described in Supplementary data.

Western-blot and immunofluorescent staining

Cells were treated as indicated and the harvested cells were lysed. A total of 40 to 50 μg of protein were used for western transfer and immunobloting. Antibody against p53 (Clone PAB240, Epitope: 211–220, Chemicon International, Inc), TBP (sc-40, santa cruz

biotechnology, Inc) and MAP4 (#56087, Abcam) were used and normalized to β-actin control (1:5000; Sigma).

For immunofluorescent staining analysis, cells were fixed with methanol–acetone (1:1) at −20°C for at least 10 min and rehydrated in PBS for 15 min at room temperature. The primary anti-p53 antibody (Clone: DO-1, Thermo Scientific) was used (Supplementary data).

Electrophoretic mobility shift assays

Electrophoretic mobility shift analysis (EMSA) was performed using a gel shift assay system (Promega) (Supplementary data).

Determination of apoptotic cells

Quantitation of apoptotic cells under hypoxic and normoxic conditions was obtained using the Annexin V-FITC detection kit (Beyotime) according to the manufacturer's protocol (Supplementary data).

Statistical analysis

Data are expressed as mean ± s.e.m. of n experiments performed in triplicate. Statistical comparisons were made with Student's t-test (two treatment groups) or one-way analysis of variance. The statistically significant difference of *pri-miR-17-92*-fold change in T/N between p53-mutant and p53-wt tumours was calculated using a Wilcoxon test. Any difference for which $P<0.05$ was regarded as statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Conflict of interest

The authors declare that they have no conflict of interest.

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