

RESEARCH PAPER



## Regulation of miR-186-YY1 axis by the p53 translational isoform $\Delta 40p53$ : implications in cell proliferation

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### ABSTRACT

We have earlier shown that p53-FL and its translational isoform  $\Delta 40p53$  are differentially regulated. In this study, we have investigated the cellular effect of  $\Delta 40p53$  regulation on downstream gene expression, specifically miRNAs. Interestingly,  $\Delta 40p53$  showed antagonistic regulation of miR-186-5p as compared to either p53 alone or a combination of both the isoforms. We have elucidated the miR-186-5p mediated effect of  $\Delta 40p53$  in cell proliferation. Upon expression of  $\Delta 40p53$ , we observed a significant decrease in YY1 levels, an established target of miR-186-5p, which is involved in cell proliferation. Further assays with anti-miR-186 established the interdependence of  $\Delta 40p53$ –miR-186-5p–YY1–cell proliferation. The results unravel a new dimension toward the understanding of  $\Delta 40p53$  functions, which seems to regulate cellular fate independent of p53FL.

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### KEYWORDS

P53 isoform;  $\Delta 40p53$ ; miR-186-5p; YY1; cell proliferation

## Introduction


P53 is the tumor suppressor gene, known as the guardian of the genome. p53 has different isoforms produced by different mechanisms. One of the isoforms of p53, which is the only translational isoform, is  $\Delta 40p53$ . Previous work from our laboratory has shown the presence of two Internal Ribosome Entry Sites (IRESs) within the p53 mRNA [1]. It was shown that translation initiation from the first IRES generates the full-length p53 protein, whereas initiation at the second IRES generates  $\Delta 40p53$  [1]. We have also shown that several translation initiation factors like Polypyrimidine tract binding (PTB) protein are critical for the regulation of IRES activity [2]. The pattern of expression of p53 isoforms is tissue-specific and hence, can be specifically regulated. Moreover, p53 isoforms are expressed at a lower level than p53 protein and have distinct subcellular localizations within cells suggesting their distinct biochemical activities [3]. Cotransfection experiments of p53 protein with individual p53 isoforms suggest that wild-type p53 activity is modulated by its isoforms [3].

This can provide a probable justification for the multiple functions played by p53 protein within the cell.

$\Delta 40p53$  can form homo- and hetero-oligomer complexes with the full-length p53. p53 has a strong affinity toward this isoform [4]. This binding alters the p53 activity, probably by changing the recruitment of the co-activators [5].  $\Delta 40p53$  acts as a negative regulatory factor of p53 since coexpression of the two isoforms impairs the ability of p53 to transactivate target genes such as p21 and induce growth suppression [6]. The ratios between the levels of these two isoforms vary during the cell cycle. Recent findings suggest that the role of  $\Delta 40p53$  is most likely more complicated. It is also known that an increased amount of  $\Delta 40p53$  prolongs the pluripotency in embryonic stem cells in mice. It controls the switch from pluripotency to differentiated somatic cells by regulating the p53FL activity at critical targets such as Nanog and IGF-receptor [7].  $\Delta 40p53$  was observed to induce many apoptosis-related genes that were not induced by p53, such as P53BP2 and TIAL1 [26]. Some studies on mice also

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showed that  $\Delta 40p53$  can induce senescence even in absence of p53 full length [9]. All these studies show that  $\Delta 40p53$  is not just a negative regulator of full-length p53 but has more activity during both normal and stress conditions.

p53 protein is known to be the most frequently altered genes in human cancers [10,11]. p53 is also known to regulate different miRNAs [12–14]. Human cancer cells are often associated with a widespread decrease in miRNAs [15], which shows crosstalk between the p53 tumor suppressor pathway and the miRNA regulation system. The transcription of some pri-miRNAs is regulated by p53 through binding to consensus sites in their promoters. Also, p53 modulates miRNA processing through interaction with the Drosha-p68 complex [16]. miRNAs can target various transcripts, so they are involved in diverse processes such as cellular differentiation, metabolism, and cell proliferation [17]. miR-34 family, composed of miR-34a (from one transcript), miR-34b, and miR-34c (from another transcript) are direct transactivation targets of p53. They are induced following p53 activation, and their induction promotes apoptosis, cell-cycle arrest, and senescence. Some recent reports showed that p53 can also repress the expression of miRNAs. During senescence, p53 was shown to repress miR-106b and cause cell-cycle arrest because the miR-106b family is known to regulate p21/CDKN1A and promote cell-cycle progression [17]. Among several cancer-associated miRNAs, studies on miR-186 are intriguing since it is found to be both upregulated and downregulated in numerous cancers. It is reported to be a tumor suppressor miRNA in the majority of the studies, while in others it is validated as an oncomir. These conflicting functions of miR-186 are mostly explored in lung cancer, colorectal cancer, hepatocellular carcinoma, prostate cancer, gastric cancer, etc. Among the several cancers, it is a well-known tumor suppressor in lung cancer, inhibiting proliferation, migration, and invasion [18]. In patients with nonsmall cell lung cancer (NSCLC), low miR-186 levels in tissues are correlated with poor survival rate, suggesting miR-186 may act as a diagnostic and prognostic marker [19]. It is also found to be downregulated in cancers related to the lymphatic and skeletal system [20]. However, there are also reports of miR-186

where it enhances cell proliferation and migration by targeting tumor suppressor genes. Thus, several reports suggest the conflicting role of miR-186-5p in various cancers [21]. Differential miRNA abundance and variable target levels are major factors contributing to diverse cellular outcomes in cancer; therefore, it is important to identify the regulatory molecules behind such regulation. The contradictory role of miR-186 in cancers is a hindrance toward its promising role in therapeutics, and therefore it is crucial to explore the possible mechanisms behind such findings. Interestingly, one of the reports suggests that wild-type p53 downregulates miR-186 while mutant p53 induces its expression [22]. The role of full-length p53 in controlling the expression of miRNAs is established but the role of  $\Delta 40p53$  in regulating the miRNAs is not elucidated. Since both the isoforms have some unique transactivation features, which would further result in a differentiated and specific downstream gene expression pattern [13,17], we wanted to understand the role of  $\Delta 40p53$  in the regulation of miR-186-5p expression and its downstream consequences.

Therefore, in the present study, we have focused on the changes in the expression of miR-186-5p in cells expressing either one of the isoforms or both the isoforms, p53FL and  $\Delta 40p53$  in lung cancer and colon cancer cell lines with a different experimental approach. Results from our study showed that miR-186-5p is up-regulated only under  $\Delta 40p53$  expression as compared to p53 and the combination of both the isoforms. This shows  $\Delta 40p53$  alone could regulate miRNAs differentially independent of p53FL. Gene network analysis as well as literature reports further unraveled YY1 as the potential target for miR-186-5p. A concomitant decrease in the expression of YY1 the target of miR-186 and cell proliferation under  $\Delta 40p53$  expression elucidates that  $\Delta 40p53$  can also modulate cellular fate independent of p53FL.

## MATERIALS AND METHODS

### *Plasmids and constructs*

pGFP-hp-p53- 5'UTR-cDNA (14A): It expresses both p53FL and  $\Delta 40p53$ . It contains GFP cDNA preceding a hairpin, followed by p53 5'UTR and

cDNA in pcDNA3 backbone (a generous gift from Dr. Robin Fahraeus, INSERM, France). pGFP-hp-p53-5'UTR (A135T)-cDNA: It is the first ATG mutant (ATG/TTG), which expresses only  $\Delta 40p53$ . It is a point mutant, in which 135th nucleotide is mutated (A135T), in the context of p53 cDNA, generated in this study. pGFP-hp-p53-5'UTR(A251G/T252 C/G253T)-cDNA: It is second ATG mutant (ATG/GCT), in context of p53cDNA, which expresses only full-length p53 (p53FL) [23]. pmiRGLO-YY1-3'UTR: It contains YY1 3'UTR (region) cloned between PmeI and XbaI downstream of the luciferase gene. pmiRGLO-YY1-3'UTR mutant: It contains YY1 3'UTR with a mutation in the miR-186-5p binding site. 5'CCGGGCCTCTCCTTTGTATATTATTC TCGAGAATAATATACAAAGGAGAGGCTTTT-T-3' sequence of shRNA was used to target YY1 3' UTR (procured from shRNA library available in Dr. Gangi Shetty Subba Rao's Laboratory IISc, Bangalore).

### Cell lines and transfections

In the current study, we have used the H1299 null p53 cell line, HCT116+/+, and HCT116 -/- cell line. These cells were maintained in DMEM (Sigma) with 10% Fetal Bovine Serum (GIBCO, Invitrogen). About 70%–80% confluent monolayer of H1299 cells were transfected with various plasmid constructs using lipofectamine (Invitrogen) in OPTIMEM. Four hours later, the medium was replaced with DMEM (with antibiotic) and 10% FBS. After 48 hours, the cells were harvested for RNA isolation and western analysis.

### siRNA and anti-miR transfections

HCT116 -/- cells were transfected with 25 nM si p53 RNA (IDT). 5' - AACCUCUUGGUGAA

CCUUAGUACCT-3' is the sequence for p53 siRNA, directed against the 3'UTR of p53; therefore, it targets both p53 as well as  $\Delta 40p53$ . A nonspecific siRNA (si nsp) (Dharmacon) was used in the experiments as control. Similarly, anti-miR-186-5p obtained from Ambion (Cat#: 4464084, ID: MH11753) (Applied Biosystems) is also used (40 nM) to partially silence miR-186. A nonspecific anti-miR obtained from Dharmacon

(Cat#: IN-001005-01-05) was used in experiments as control. After 48 h, the cells were harvested in passive lysis buffer for reporter gene assays and in RIPA lysis buffer for Western blot analysis.

### Site-directed mutagenesis

First ATG mutant (ATG/TTG) and second ATG mutant (ATG/GCT) were generated using Mega Primer-A (5'GGATCTGACTGCGGCTCCTCC AAGGCAGTGACCCGGA3') and MegaPrimer-B (5'GGACAGCATCAAATCATCAGCTGCCTTG-GGACGGCAAG3') respectively, using QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, USA) as per manufacturer's protocol. DNA was isolated from transformed *E. Coli* XL-10-Gold colonies. pmiRGLO-YY1-3'UTR mutant for one of the miR-186 binding site was generated by PCR amplification of template DNA (pmiRGLO-YY1-3'UTR) using two mutagenic primers:

Forward

Primer:5'ATGAACTTCGCATCAAAAAGACAAA-AGAAAATACAACAGTGCTAAAAATGG-3'

Reverse Primer: 5'-CCATTTTTAGCACTGTTG TATTTTCTTTTGTCTTTTGTATGCGAAGTTCA-T-3', followed by DpnI digestion. DNA was isolated from transformed *E.coli* DH5 $\alpha$  colonies and was verified by DNA sequencing.

### Western blot analysis

Protein concentrations of the extracts were assayed by Bradford (Bio-Rad) and equal amounts of cell extracts were separated by SDS 12% PAGE and transferred to nitrocellulose membrane (Sigma). Samples were then analyzed by Western blotting using a rabbit-raised anti-p53 polyclonal antibody (CM1, a kind gift from Dr. Robin Fahraeus, INSERM, France and Prof. J.C. Bourdon, of University of Dundee, UK), followed by secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG; Sigma). Mouse-monoclonal anti- $\beta$ -actin antibody (Sigma) was used as a control for equal loading of total cell extracts. YY1 antibody (Santa Cruz Biotechnology, Cat. No. SC-7341 H10) was used to detect YY1 protein. For WB quantitative comparison, the intensity of immunoblots was analyzed using Mutigauge V3.0

software (Fuji Photo Film). For blots, the quantified densities of proteins were normalized against the respective actin band.

### **RNA isolation and real-time PCR**

Total RNA was extracted using TRIzol reagent (Sigma). RNA amount was quantified with Nanospectrophotometer and reverse transcribed (ABI). miRNAs were quantified with the Taqman-based method. The TaqMan microRNA Assay System was used for miRNA detection and quantification. The RT reaction was performed in a final volume of 15  $\mu$ l containing 1.5  $\mu$ l of 10X RT buffer, 5  $\mu$ l of total RNA (10 ng), 3  $\mu$ l of 5X miRNA-specific RT primer, 0.15  $\mu$ l of 100 mM dNTP, 0.19  $\mu$ l of 20 U/ $\mu$ l RNase inhibitor, and 1  $\mu$ l of MultiScribe reverse transcriptase (50 U/ $\mu$ l). The reaction condition was 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Real-time PCR was then performed in a 20  $\mu$ l reaction containing 1.33  $\mu$ l of 1:10 diluted RT product, 10  $\mu$ l of 10X TaqMan Universal PCR master mix, and 1  $\mu$ l of the primer and probe mix from the TaqMan MicroRNA Assay Kit. The reaction condition for miRNA (TaqMan) was 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The expression of the RNU6B gene was used as the endogenous control. The thermocycling conditions for SYBR green include 1 cycle of 95°C for 10 min, and 45 cycles of 95°C for 15 s, and 60°C for 30 s and 72°C for 30 s (Applied Biosystems).  $2^{-\Delta\Delta C_t}$  method algorithm was used to analyze the relative changes in expressions, where actin/RNU6B served as an endogenous control. The fold change was calculated using  $2^{-\Delta\Delta C_t}$ .  $\Delta C_t = C_t$  (a target gene)  $- C_t$  (endogenous control) and  $\Delta\Delta C_t = \Delta C_t$  (a target sample)  $- \Delta C_t$  (control sample). Melting curve analysis of every qPCR was conducted after each cycle.

### **Luciferase assay**

Firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA Cat#1910) according to the manufacturer's protocol. The transfected cells were harvested in Passive lysis buffer (PLB) after 48 hours of transfection and luciferase activity was measured.

### **Wound-healing assay**

Wound-healing assay (scratch wound-healing assay) was performed as described previously [24]. Briefly, H1299 cells were co-transfected with p53FL/ $\Delta$ 40p53/14A/GFP and anti-miR-186-5p. About 36 h post transfection, these cells were reseeded in 12 well plates to create a confluent monolayer. The cell layers were scratched using a 10  $\mu$ l pipette tip to produce a wound and further incubated for 24 h at 37°C. The rate of closure of the wound (gap reduction) was used to determine the wound-healing rate. Percentage gap reduction was calculated as follows: [(mean wound width - mean remaining width)/mean wound width] \* 100 (%).

### **BrdU cell proliferation assay**

BrdU cell proliferation assay was performed as described in BrdU cell proliferation protocol mentioned in ab126556-BrdU Cell Proliferation ELISA Kit (colorimetric). Briefly, H1299 co-transfected with p53FL/ $\Delta$ 40p53 and anti-miR-186-5p. About 36 h post transfection, these cells were reseeded in 96 well plate. After 12 h of reseeding BrdU solution was added and cells were incubated for 8 h, followed by the protocol as mentioned in the manual. Similar experiment was also performed in HCT116  $-/-$  cells transfected with anti-miR-186-5p and sh YY1.

### **Statistical analysis**

The data were expressed as mean  $\pm$  SD. Statistical significance was determined using a two-sided Student's t-test. The criterion for statistical significance was  $p \leq 0.05$  (\*) or  $p \leq 0.01$  (\*\*) or  $p \leq 0.001$  (\*\*\*) or  $p \leq 0.0001$  (\*\*\*\*).

## **RESULTS**

### **miR-186 is upregulated under $\Delta$ 40p53 expression**

To further investigate and validate the above observation in mammalian cell culture system we transfected H1299 cells (p53 null) using four different constructs: (i) vector expressing only full-length p53 protein (p53FL), (ii) vector expressing only  $\Delta$ 40p53, (iii) vector that expresses both p53

and  $\Delta 40p53$  (14A), and (iv) vector control expressing only GFP (C). Interestingly, miR-186-5p showed significant upregulation upon expression of  $\Delta 40p53$  as compared to p53 alone or the combination of both. (Figure 1a, B). The upregulation of miR-186 by  $\Delta 40p53$  expression was further validated in HCT116+/+ and HCT116-/- cells (Figure 1c), where HCT116+/+ expresses both p53FL and  $\Delta 40p53$  and HCT116-/- expresses only  $\Delta 40p53$  (Figure 1d). The upregulation of miR-186 as observed in HCT116-/- is mediated by  $\Delta 40p53$  was further confirmed by the partial silencing of  $\Delta 40p53$  using si p53 (25 nM) in HCT116-/- cells. The partial silencing of  $\Delta 40p53$  in HCT116-/- cells reduced the levels of miR-186 as compared to the nonspecific si (si nsp) (Figure 1e, F).

#### ***$\Delta 40p53$ inhibits YY1 expression mediated by miR-186***

In this study, we focused on the miR-186 mediated effect of  $\Delta 40p53$  in cell proliferation. In order to understand the physiological relevance of upregulation of miR-186 under  $\Delta 40p53$  expression, we validated the change in the levels of one of the targets of miR-186, i.e. YY1, which is known to promote cell proliferation. RNA isolated from H1299 cells expressing p53,  $\Delta 40p53$ , or both (14A) and GFP as vector control was used for determining the change in YY1 mRNA and protein levels (Figure 2a, B). But to confirm if the change in levels of YY1 under  $\Delta 40p53$  expression is because of upregulation of miR-186, a similar experiment was performed using anti-miR-186 (Figure 2c, D, E). Further to establish a direct link of miR-186 to YY1 3'UTR, a luciferase experiment was performed. H1299 cells were transfected with luciferase construct with YY1 3'UTR wild-type (pmiRGLO-YY1-3'UTR WT) and YY1 3'UTR mutant for the miR-186 binding site (pmiRGLO-YY1-3'UTR Mut) in the background of overexpression of  $\Delta 40p53$  or the vector control. The overexpression of  $\Delta 40p53$  increased the levels of miR-186 (Figure 2i) which decreased the luciferase activity as compared to the control in case of wild-type 3'UTR. But, in the mutant 3'UTR, the inhibitory effect on the luciferase activity was reduced which resulted in increased luciferase activity (Figure 2Figure 2f, G, H). This suggests that miR-186

directly binds to YY1 3'UTR thereby regulating its levels.

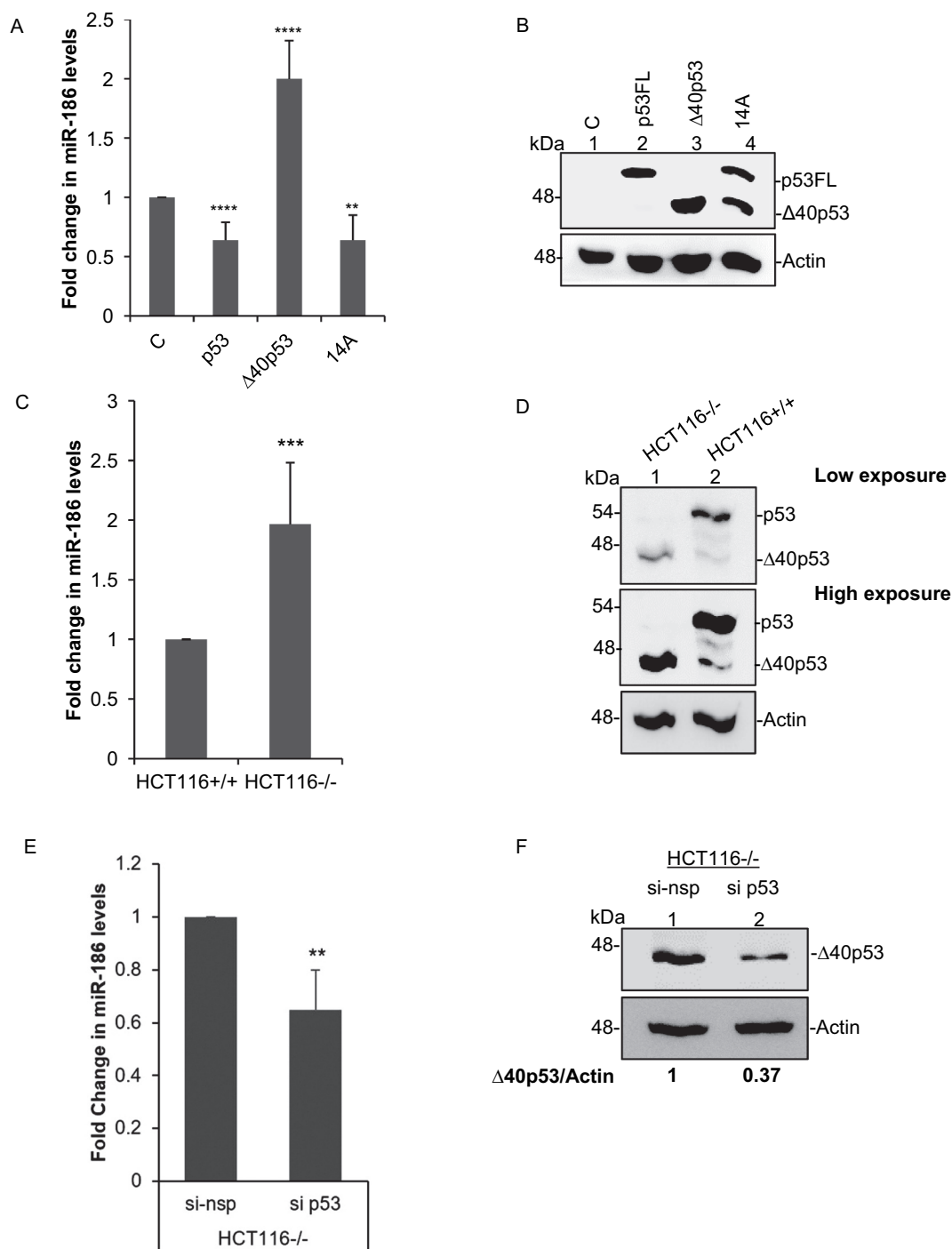
Experiments performed in HCT116+/+ and HCT116-/- cells also displayed downregulation of YY1 under  $\Delta 40p53$  endogenous expression (Figure 3, Figure 3B). The downregulation of YY1 mRNA and protein levels as observed in HCT116-/- is mediated by  $\Delta 40p53$  was further confirmed by the partial silencing of  $\Delta 40p53$  using si p53 (25 nM) in HCT116-/- cells. After partial silencing of  $\Delta 40p53$  in HCT116-/- cells the mRNA and protein levels of YY1 showed an increase as compared to the non-specific si (si nsp) (Figure 3c, D). The anti-miR-186 experiments determined that the downregulation of YY1 under  $\Delta 40p53$  expression is mediated by miR-186 (Figure 3e, F, G, H, I, J, K). The effect of anti-miR-186 on the levels of YY1 mRNA and protein was further established using a nonspecific anti-miR (Nsp anti-miR), which showed no significant change in the YY1 expression (Figure 3l, M, N).

#### ***$\Delta 40p53$ inhibits cell proliferation***

From different studies, it is known that YY1 promotes proliferation in cells and miR-186 inhibits cell proliferation by downregulating YY1 levels [24,25]. Observations from the experiment using sh YY1 in HCT116-/- cells also showed the role of YY1 in promoting cell proliferation (Figure 4 A, B). To investigate if  $\Delta 40p53$  can regulate cell proliferation, H1299 cells were co-transfected with different constructs expressing p53,  $\Delta 40p53$ , or both (14A) or GFP as vector control and anti-miR-186. p53 is a positive control of the inhibition of cell proliferation because it is known that p53 can inhibit cell growth. The wound-healing assay and BrdU cell proliferation assays were performed to demonstrate the role of miR-186 in cell proliferation and migration. The results show that  $\Delta 40p53$  can inhibit cell migration and proliferation by upregulating miR-186 which in turn inhibits YY1 expression (Figure 4 C, S1 D, E). The results were further strengthened by similar observation in HCT116-/- cells BrdU cell proliferation assay with and without anti-miR-186 (Figure 4 F).

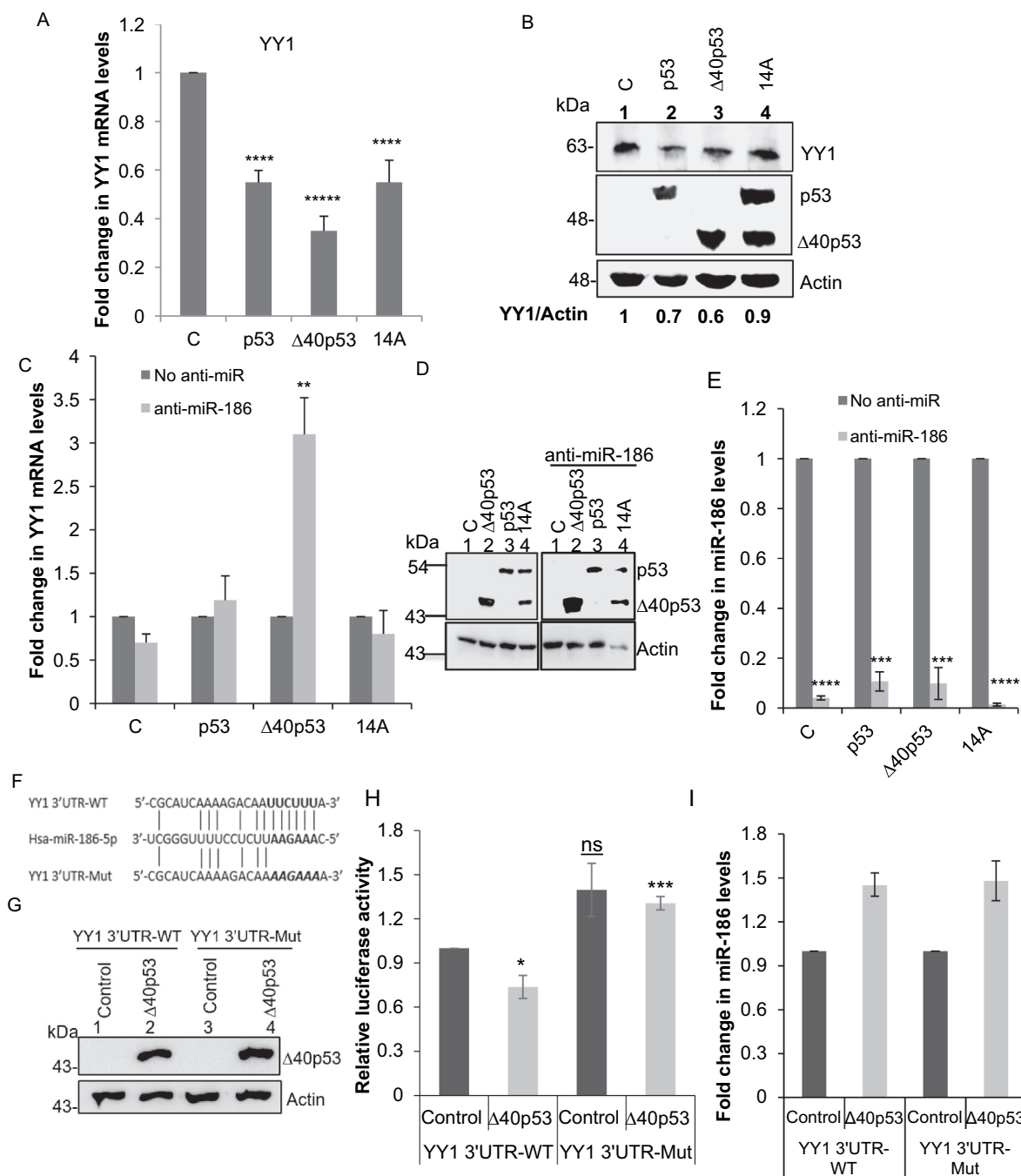
## **DISCUSSION**

There are different reports which have identified p53 regulated miRNAs and their roles [11].



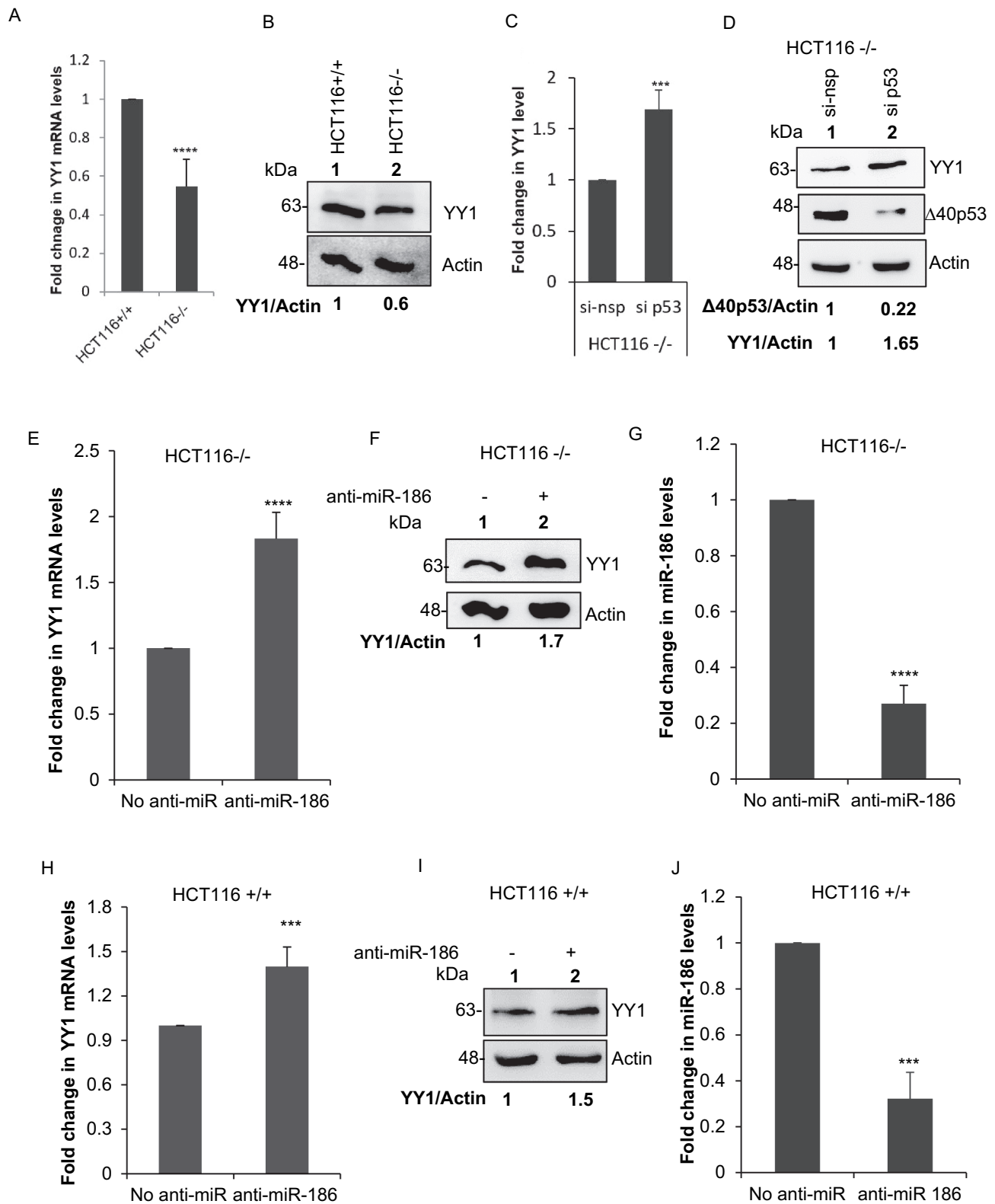
**Figure 1.** Upregulation of miR-186 under  $\Delta 40p53$  expression.

(A) Quantitative PCR for miR-186-5p ( $n = 3$ ). (B) Western blot analysis of cell extracts from H1299 cells expressing control GFP (vector control), p53FL only,  $\Delta 40p53$  only and 14A (expresses both p53FL and  $\Delta 40p53$ ), probed with CM1 after 48 h. (C) Quantitative PCR using Taqman probe for validations of miR-186-5p in HCT116+/+ (expresses both p53FL and  $\Delta 40p53$ ) and HCT116-/- cells (expresses only  $\Delta 40p53$ ) ( $n = 3$ ). (D) Western blot analysis of cell extracts from HCT116+/+ (expresses both p53FL and  $\Delta 40p53$ ) and HCT116-/- cells (expresses only  $\Delta 40p53$ ), probed with CM1. (E) Quantitative PCR using Taqman probe for validations of miR-186-5p in HCT116-/- (expresses only  $\Delta 40p53$ ) cells transfected with either si p53 (25 nM) or nonspecific si (si nsp) ( $n = 3$ ). (F) Western blot analysis of cell extracts from HCT116-/- (expresses only  $\Delta 40p53$ ) cells transfected with either si p53 (25 nM) or nonspecific si (si nsp), probed with CM1.



**Figure 2.** Overexpression of  $\Delta 40p53$  downregulates YY1 expression by upregulating miR-186-5p.

(A) Quantitative PCR of YY1 mRNA levels normalized to actin in H1299 cells transfected with C: GFP (vector control)/p53FL only/ $\Delta 40p53$  only/14A (expresses both p53FL and  $\Delta 40p53$ ), 48 h post transfection ( $n = 3$ ). (B) Western blot analysis of cell extracts from H1299 cells expressing C: GFP (vector control), p53FL only,  $\Delta 40p53$  only, and 14A (expresses both p53FL and  $\Delta 40p53$ ), probed with anti YY1 antibody, anti p53 antibody, CM1 and anti-actin antibody after 48 h. (C) Quantitative PCR of YY1 mRNA levels normalized to actin in H1299 cells cotransfected with C: GFP (vector control)/p53FL only/ $\Delta 40p53$  only/14A (expresses both p53FL and  $\Delta 40p53$ ) and anti-miR-186-5p, 48 h post transfection ( $n = 3$ ). (D) Western blot analysis of cell extracts from H1299 cells co-transfected with C: GFP (vector control)/p53FL only/ $\Delta 40p53$  only/14A (expresses both p53FL and  $\Delta 40p53$ ) and anti-miR-186-5p, probed with CM1. (E) Quantitative PCR using Taqman probe for miR-186-5p in H1299 cells cotransfected with C: GFP (vector control)/p53FL only/ $\Delta 40p53$  only/14A (expresses both p53FL and  $\Delta 40p53$ ) and anti-miR-186-5p. (F) Schematic of mutation generated in the YY1 3'UTR WT in the miR-186 binding site. (G) Western blot analysis of cell extracts from H1299 cells transfected with control and  $\Delta 40p53$  followed by transfection of YY1 3'UTR WT and YY1 3'UTR Mutant, probed with CM1. (H) Effects of  $\Delta 40p53$  mediated miR-186 expression on the activity of wild-type and mutant 3'-UTRs of YY1 was analyzed by luciferase reporter assay 48 h post transfection [The luciferase values for each condition were normalized with protein concentration. The relative luciferase activity for each condition was calculated in comparison to the YY1-3'UTR WT control samples.] (I) Quantitative PCR using Taqman probe for miR-186 from RNA extracted from H1299 cells transfected with Control and  $\Delta 40p53$  followed by transfection of YY1 3'UTR WT and YY1 3'UTR Mutant.



**Figure 3.** Endogenous  $\Delta 40p53$  inhibits YY1 expression mediated by miR-186-5p.

(A) Quantitative PCR of YY1 mRNA levels normalized to actin in HCT116+/+ (expresses both p53FL and  $\Delta 40p53$ ) and HCT116-/- cells (expresses only  $\Delta 40p53$ ) (n = 3). (B) Western blot analysis of cell extracts from HCT116-/- cells (expresses only  $\Delta 40p53$ ) and HCT116 +/+ (expresses both p53FL and  $\Delta 40p53$ ), probed with anti YY1 antibody and anti-actin antibody. (C) Quantitative PCR of YY1 mRNA levels normalized to actin in HCT116-/- (expresses only  $\Delta 40p53$ ) cells transfected with either si p53(25 nM) or nonspecific si (si nsp). (D) Western blot analysis of cell extracts from HCT116-/- (expresses only  $\Delta 40p53$ ) cells transfected with either si p53(25 nM) or



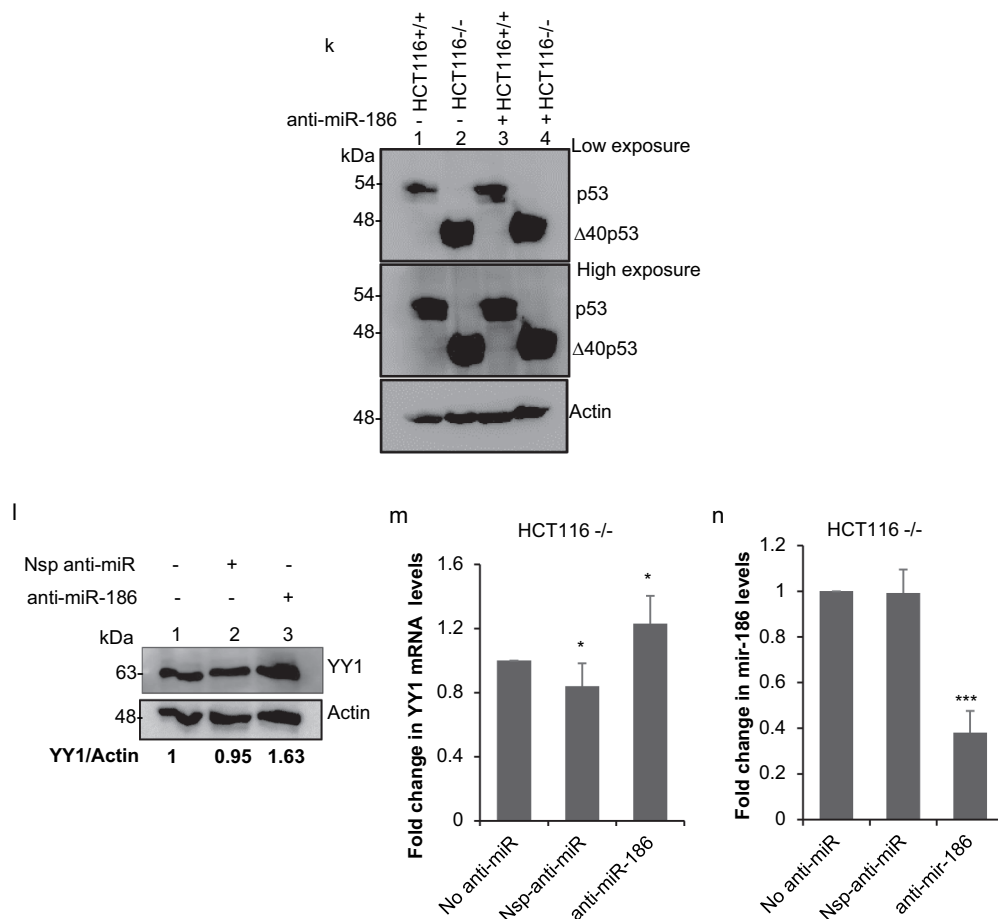
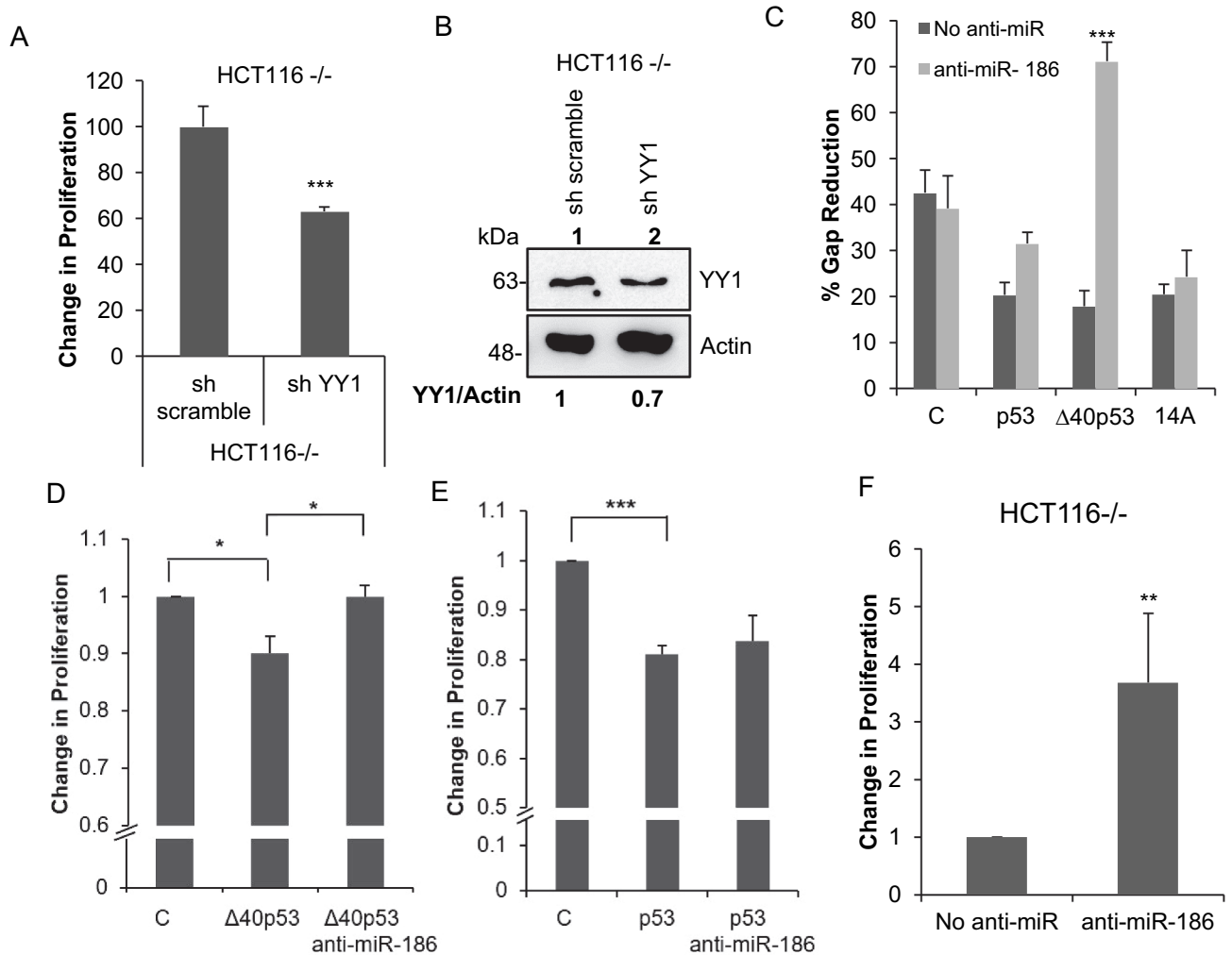


Figure 3b. continue

However, there are no studies on the shorter isoform,  $\Delta 40p53$  regulated miRNAs. In this study for the first-time function of  $\Delta 40p53$  in regulating miRNAs expression has been investigated. Several literature reports on miRNAs provided functional

significance for the selection of a specific miRNA for this study. Since miR-186 was found to be differentially regulated in different cancers we were interested to study its role in the context of p53 isoforms that orchestrate cellular fate.

nonspecific si (si nsp), probed with anti YY1 antibody and anti-actin antibody. (E) Quantitative PCR of YY1 mRNA levels normalized to actin in HCT116-/-cells transfected with anti-miR-186-5p, 48 h post transfection (n = 3). (F) Western blot analysis of cell extracts from HCT116-/- (expresses only  $\Delta 40p53$ ) cells transfected with anti-miR-186-5p, 48 h post transfection probed with anti YY1 antibody and anti-actin antibody. (G) Quantitative PCR using Taqman probe for miR-186-5p in HCT116-/-cells (expresses only  $\Delta 40p53$ ), transfected with anti-miR-186-5p. (H) Quantitative PCR of YY1 mRNA levels normalized to actin in HCT116+/+ cells transfected with anti-miR-186-5p, 48 h post transfection (n = 3). (I) Western blot analysis of cell extracts from HCT116+/+ (expresses only  $\Delta 40p53$ ) cells transfected with anti-miR-186-5p, 48 h post transfection probed with anti YY1 antibody and anti-actin antibody. (J) Quantitative PCR using Taqman probe for miR-186-5p in HCT116+/+ (expresses both p53FL and  $\Delta 40p53$ ), transfected with anti-miR-186-5p. (K) Western blot analysis of cell extracts from HCT116-/-cells (expresses only  $\Delta 40p53$ ) and HCT116+/+ (expresses both p53FL and  $\Delta 40p53$ ), transfected with anti-miR-186-5p, probed with CM1. (L) Western blot analysis of cell extracts from HCT116-/- (expresses only  $\Delta 40p53$ ) cells transfected with nonspecific anti-miR (Nsp anti-miR) anti-miR and anti-miR-186-5p, 48 h post transfection probed with anti YY1 antibody and anti-actin antibody. (M) Quantitative PCR of YY1 mRNA levels normalized to actin in HCT116-/-cells transfected with nonspecific anti-miR (Nsp anti-miR) and anti-miR-186-5p, 48 h post transfection. (N) Quantitative PCR using Taqman probe for miR-186-5p in HCT116-/-cells (expresses only  $\Delta 40p53$ ), transfected with nonspecific anti-miR (Nsp anti-miR) and anti-miR-186-5p.



**Figure 4.**  $\Delta 40p53$  inhibits cell proliferation mediated by miR-186-5p.

(A) BrdU cell proliferation assay performed in HCT116<sup>-/-</sup> cells transfected with sh YY1 and scrambled sh RNA as a nonspecific control (sh scramble) ( $n = 3$ ). (B) Western blot analysis of cell extracts from HCT116<sup>-/-</sup> (expresses only  $\Delta 40p53$ ) cells transfected with sh YY1 and scrambled sh RNA as a nonspecific control (sh scramble) probed with anti YY1 antibody and anti-actin antibody. (C) Wound-healing assay performed in H1299 cells co-transfected with C: GFP (vector control)/p53FL only/ $\Delta 40p53$  only/14A (expresses both p53FL and  $\Delta 40p53$ ) and anti-miR-186-5p. The graph represents the percentage gap reduction in the wound ( $n = 3$ ). (D, E) BrdU cell proliferation assay performed in H1299 cells co-transfected with C: GFP (vector control)/p53FL only and anti-miR-186-5p. The graph represents the change in proliferation ( $n = 3$ ). (F) BrdU cell proliferation assay performed in HCT116<sup>-/-</sup> cells transfected with anti-miR-186-5p.

A significant change was observed in the levels of miR-186-5p under overexpression of p53FL,  $\Delta 40p53$ , and both the isoforms together. This regulation of miRNAs could be at the level of their transcription or processing stage, but this study focuses on the downstream functions. The observation of upregulation of miR-186-5p by  $\Delta 40p53$  expression was further strengthened in other cell lines expressing only  $\Delta 40p53$  endogenously (HCT116<sup>-/-</sup>) as compared to the cell line expressing both the isoforms (HCT116<sup>+/+</sup>).

Not much detailed studies have been done to understand the functions of  $\Delta 40p53$ , but some studies have revealed the importance of this isoform by recognizing its role in different cellular functions like apoptosis [26], senescence [8], and differentiation [7]. Knowing that  $\Delta 40p53$  has different *trans* activation properties from the previous studies, the current study explores different functions of  $\Delta 40p53$  mediated through the miRNAs. miR-186 is known to have tumor suppressor properties. It regulates different target genes that have oncogenic properties [18,24,27]. It

is involved in functions like regulation of cell cycle, apoptosis, and cell proliferation. Literature from our laboratory has shown that synchronized,  $\Delta 40p53$  transfected H1299 cells have a higher proportion of S-phase cells 8 h and 24 h post-starvation, suggesting an anti-proliferative effect of  $\Delta 40p53$  [22]. Taken together the knowledge of previous studies for miR-186, the role of  $\Delta 40p53$  in regulating cell proliferation mediated by miR-186 is investigated. The downstream significance of miR-186 upregulation under  $\Delta 40p53$  expression was determined by checking the change in the YY1 mRNA and protein levels by overexpression of  $\Delta 40p53$  and under endogenous expression of  $\Delta 40p53$ . YY1 is a transcriptional factor that is known to target several genes involved in proliferation and differentiation. YY1 has dual roles to play and its activity varies according to the cellular conditions. YY1 comprises one of the Polycomb group proteins that maintain transcriptional repressor complexes during embryogenesis. It forms a transcription repression complex with HDACs, which when released leads to the activation of cyclin D1 gene promoter which drives G1-phase completion [28]. On the other hand, YY1 negatively regulates p53 in different ways. YY1 inhibits the transcriptional activity of p53 by inhibiting its interaction with its coactivator p300 [29]. In addition to this regulation, YY1 can also act as a negative regulator under genotoxic stress, by enhancing p53 interaction with its negative regulator Mdm2, hence leading to its ubiquitination [29,30]. Downregulation in the YY1 levels under  $\Delta 40p53$  expression and contrary upregulation in the levels after silencing of miR-186 along with the  $\Delta 40p53$  expression gave evidence of regulation of YY1 by  $\Delta 40p53$  mediated via miR-186 regulation. The change in the luciferase activity of the luciferase constructs (with YY1 3'UTR wild type and YY1 3'UTR mutant for the miR-186 binding site) under the overexpression of  $\Delta 40p53$  also validated the direct interaction of miR-186 to the YY1 3'UTR. There was a decrease in levels of YY1 under p53FL and with the 14A (a combination of p53FL and  $\Delta 40p53$ ). Also, the levels of miR-186 get downregulated under p53FL and with 14A as observed in Figure 1a. It appears that this decrease in YY1 level is not mediated through miR-186, as it did not change after anti-miR-186 treatment. As known from the literature YY1 negatively regulates p53, the decrease in steady-state

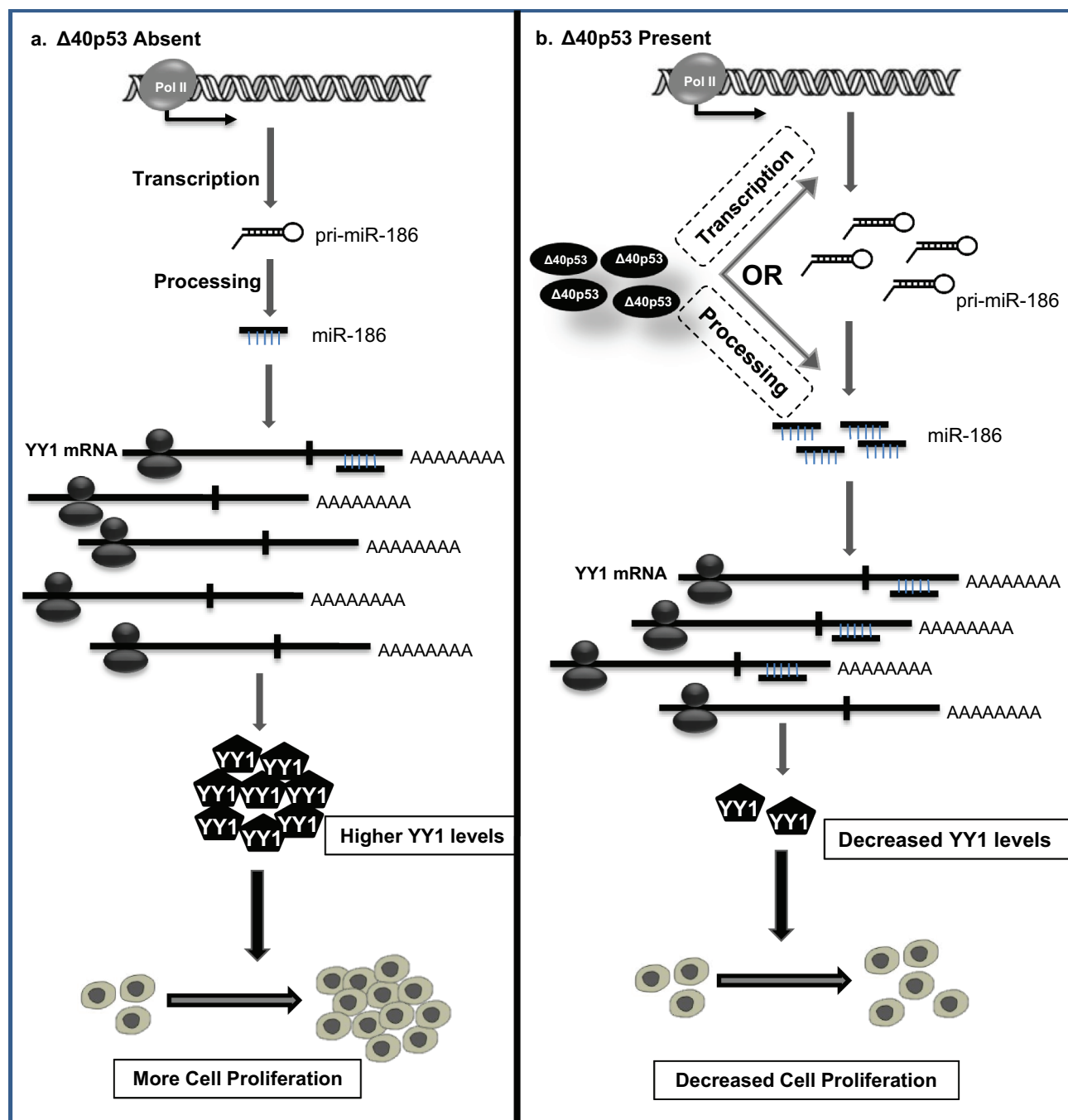
YY1 mRNA and protein levels under p53 overexpression could be a survival strategy by p53. Additionally, the decrease in the YY1 expression by both the isoforms of p53 points toward a novel strategy, where both the isoforms can regulate YY1 expression but through different mechanisms. In a consequence of downregulation of YY1 expression by  $\Delta 40p53$ , there was a reduction in cell proliferation and the reverse was observed with the silencing of miR-186 along with  $\Delta 40p53$  expression. The experiments with anti-miR-186 established the interdependence of  $\Delta 40p53$ – miR-186–5p–YY1– cell proliferation. This unfolds that  $\Delta 40p53$  can also regulate cellular fate independent of p53FL (Figure 5).

This study not only helps us understand the role of  $\Delta 40p53$  in regulating cell proliferation but also indicates toward other functions of  $\Delta 40p53$  independent of p53FL functions. The known literature of the miR-186 tells us about its other functions like regulation of cell cycle at the G1-S stage and senescence by regulating its target genes like CDK6, cyclin D1, and CK2 $\alpha$ , respectively. From our laboratory, we have shown that the IRES element 2 in p53 mRNA responsible for  $\Delta 40p53$  synthesis is more active in the G1-S phase of the cell cycle [1]. With this observation, we could speculate that  $\Delta 40p53$  can also regulate the cell cycle at the G1-S stage by regulating miR-186. Separate studies tell about the role of  $\Delta 40p53$  and miR-186 in senescence individually [8,31]. Our observation that  $\Delta 40p53$  can regulate the expression of miR-186 helps us hypothesize that one of the pathways by which  $\Delta 40p53$  regulates senescence could be mediated by miR-186.

Taken together, this study unfolds the importance of these isoforms in the differential expression of miRNAs. It also contributes to the novel role of  $\Delta 40p53$  in regulating miRNAs independent of p53 and hence the cellular fate. It will be interesting to understand the mechanism of the regulation of miRNAs by  $\Delta 40p53$  alone in future studies.

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**Figure 5.** Model depicting the regulation of cell proliferation by  $\Delta 40p53$  through miR-186-5p-YY1 axis.

(A) In absence of  $\Delta 40p53$ , miR-186-5p levels do not increase which leads to higher YY1 levels resulting in increased cell proliferation. (B) However, when  $\Delta 40p53$  is present it upregulates miR-186-5p levels probably through direct transcriptional activation or through precursor miRNA processing. Upregulated miR-186 then targets 3'UTR of YY1 leading to its decreased levels which ultimately leads to a reduction in proliferation.

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### Disclosure statement

The authors declare no conflict of interest.

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## Author contributions

AK and SD: Conception and design of studies. AK, SKT, and AP for analysis, interpretation, writing the article, and performing the experiments.

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