RESEARCH PAPER

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Transcriptional regulation of microRNA-100, −146a, and −150 genes by p53 and NFκB p65/RelA in mouse striatal STHdh^{Q7}/ Hdh^{Q7} cells and human cervical carcinoma HeLa cells

Jayeeta Ghose and N P Bhattacharyya[†,*]

Crystallography and Molecular Biology Division; Saha Institute of Nuclear Physics; Bidhannagar, Kolkata, India

[†]Present affiliation: Biomedical Genomics Center; Kolkata, India

Keywords: microRNA, miRNA gene regulation, miR-100, miR-146a, miR-150, NFκB p65/RelA, p53, transcription factor

Abbrevations: ChIP, Chromatin immunoprecipitation; Co-IP, Co-immunoprecipitation; miRNAs, microRNAs; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; p53, tumor protein 53; p65, RELA, RELA; v-rel avian reticuloendotheliosis viral oncogene homolog A; RNA POL II, RNA Polymerase II; RNA POL III, RNA Polymerase III; RLU, Relative light unit; RT-PCR, Reverse transcription polymerase chain reaction; TF, Transcriptional factor; TFBS; Transcription factor binding site; WB, Western blot

MicroRNA (miRNA) genes generally share many features common to those of protein coding genes. Various transcription factors (TFs) and co-regulators are also known to regulate miRNA genes. Here we identify novel p53 and NFκB p65/RelA responsive miRNAs and demonstrate that these 2 TFs bind to the regulatory sequences of miR-100, −146a and −150 in both mouse striatal and human cervical carcinoma cells and regulate their expression. p53 represses the miRNAs while NFκB p65/RelA induces them. Further, we provide evidence that exogenous p53 inhibits NFκB p65/RelA activity by reducing its nuclear content and competing with it for CBP binding. This suggests for the existence of a functional cross-talk between the 2 TFs in regulating miRNA expression. Moreover, promoter occupancy assay reveals that exogenous p53 excludes NFκB p65/RelA from its binding site in the upstream sequence of miR-100 gene thereby causing its repression. Thus, our work identifies novel p53 and NFκB p65/RelA responsive miRNAs in human and mouse and uncovers possible mechanisms of co-regulation of miR-100. It is to be mentioned here that cross-talks between p53 and NFκB p65/RelA have been observed to define the outcome of several biological processes and that the pro-apoptotic effect of p53 and the pro-survival functions of NFκB can be largely mediated via the biological roles of the miRNAs these TFs regulate. Our observation with cell lines thus provides an important platform upon which further work is to be done to establish the biological significance of such co-regulation of miRNAs by p53 and NFκB p65/RelA.

Introduction

MicroRNAs (miRNA) are short (~22 nucleotides) non-coding RNAs which are primarily involved in post-transcriptional regulation of gene expression. Genes coded for miRNAs are generally transcribed by RNA polymerase II (Pol II) and are often classified as Class II genes.[1] However, several miRNAs have also been observed to be transcribed by RNA polymerase III[2,3] and there are many intronic miRNAs that can be transcribed by both RNA polymerase II and RNA polymerase III.[4] Toward the beginning of miRNA research, main focus was on studying physiological and pathological functions of miRNA genes. Considering their diverse cellular roles and their implications in major biological processes, current focus on miRNA research has widely shifted toward deciphering the molecular mechanism underlying transcriptional regulation of miRNA genes. These miRNA genes share many regulatory features common to those of RNA Pol II genes. These include regulation by core promoters and distal enhancers, 5′ capping and 3′ polyadenylation of primary transcripts.[5] Similar to protein coding genes, various transcription factors (TFs) and co-regulators are known to regulate miRNA genes. It has been observed that TFs regulating miRNA transcription viz., c-myc,[6,7] p53[8-10] NFκB p65/RelA[11-13] as well as cell-type specific TFs such as MEF2, PU.1[14] and REST[15] largely overlap with those that control protein-coding genes. Growth factor stimulation triggered by platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and bone-derived neurotrophic factor can also

*Correspondence to: N P Bhattacharyya; Email: nitai_sinp@yahoo.com
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dynamically regulate miRNA gene expression.[16] Besides, similar to protein coding genes, regulation of miRNA genes by TFs is a dynamic and complex process and may involve more than one TF. There have been evidences where 2 or more TFs co-regulate the expression of miRNA genes for appropriate execution of several biological events. NFI-A has been reported to repress and C/EBPα induce the expression of miR-223 associated with granulocyte differentiation.[17] MyoD and Mef2 have been observed to activate miR-1, a process that contributes to enhancing myogenesis in skeletal muscles.[18] The pro-apoptotic p53 and the pro-survival NFκB p65/RelA have also been known to co-regulate the expression of several miRNA genes in diverse biological processes.[19-21] Since p53 and NFκB p65/RelA are mostly involved in determining cell fate, co-regulation of miRNAs by these 2 TFs, in general, may be implicated in defining final outcome of a cell undergoing cell division or apoptosis. However, till date, only in a limited number of cases the binding of the TFs to specific sites in the regulatory sequences of their target miRNA genes and the mode of such regulation have actually been experimentally determined. Hence, with a view to understanding the biological significance of combinatorial regulation of miRNAs by p53 and NFκB p65/RelA, in the present work, we first aimed at identifying novel p53 and NFκB p65/RelA responsive miRNAs which are co-regulated by these 2 TFs.

To identify miRNAs that are co-regulated by p53 and NFκB (p65/RelA), we have relied on data obtained from diverse cell types and across diverse species viz., mouse striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and human cervical carcinoma HeLa cells. Expression profile of 40 miRNAs in cells with over expressed p53 or NFκB p65/RelA and knocked down endogenous *p53* or chemically inhibited NFκB p65/RelA was determined. The selection of 40 miRNAs was based on their possible involvement in Huntington's disease (HD)[22] and several other diseases.[23] Many of these miRNAs are known to be altered in cell and animal models of HD as well as in the post-mortem brains of human HD patients[22,24] and also in diverse tumors originated from different tissues, cardiovascular diseases and other neurological diseases.[23] Among these, we identified novel p53 and NFκB p65/RelA responsive miRNAs in both human and mouse. We observed that p53 binds to the regulatory sequences in the upstream of miR-100, −146a and −150 and represses their transcription while NFκB p65/RelA sub-unit binds to the regulatory sequences in the upstream of miR-100, −146a and −150 and induces their transcription. Although elevated NFκB p65/RelA did not affect p53 nuclear level, elevated p53 was observed to reduce NFκB p65/RelA nuclear content and activity. Thus, our results provide new data about the interplay between p53 and NFκB p65/RelA in co-regulating miRNAs which have been implicated in several diseases. The combinatorial effect of the extensive physical and functional cross-talks that exist between p53 and NFκB p65/RelA has been observed to define the outcome of several biological processes. Thus, understanding the mechanisms of regulation of these altered miRNAs by p53 and NFκB p65/RelA would likely provide an opportunity for possible therapeutic intervention in such disease processes by targeting either the regulatory pathway(s) or the miRNAs themselves.

Results

Ectopic modulation of p53 alters miRNA expression in mouse striatal ST *Hdh*^{Q7}/*Hdh*^{Q7} cells and human cervical carcinoma HeLa cells

Exogenous expression of p53-CFP increased the expression of the protein (n = 3, p = 0.0017) 24 hours post-transfection in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells (**Fig. 1A**). It was observed that out of 40 miRNAs whose expressions were studied, expression levels of 7 miRNAs viz., miR-145, −34a, −148a, −199a-5p, −134, −194, −182 were increased significantly (*$P \leq 0.05$; ** $P \leq 0.01$) and 8 miRNAs viz., miR-100, −125b, −150, −221, −146a, −138, −335 and −15b were decreased significantly (*$P \leq 0.05$; ** $P \leq 0.01$) in presence of exogenous p53 in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells compared to control cells(**Fig. 1B**). Next, endogenous *p53* was knocked down in the same cells with the help of p53 siRNA construct (Imgenex, USA) which down regulates the expression of p53[25] 72 hours post transfection (n = 3, p = 0.023) (**Fig. 1C**). Real time PCR analysis to detect levels of mature miRNAs from p53 siRNA transfected ST*Hdh*^{Q7}/*Hdh*^{Q7} cells showed that expressions of miR-145, −34a, −100, −125b, −146a, −199a-5p, −150, −15b and −221 were reversed in cells with knocked down *p53* when compared to that with overexpressed p53 (**Fig. 1D**). However, expression pattern of miR-134, −148a, −182, −194, −138 and −335 were similar both in the presence of exogenous p53 as well as in cells with knocked down endogenous *p53*. Thus only 9 miRNAs whose expression patterns were reversed on knocking down endogenous *p53* could possibly be regulated by the TF. To confirm further, exogenous p53 was expressed in *p53* knocked down ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and it was observed that expression of the miRNAs could be restored back to basal level (**Fig. 1E**). Thus, p53 regulates the expression of these 9 miRNAs in mouse ST*Hdh*^{Q7}/*Hdh*^{Q7} cells.

Analysis of expression of 40 miRNAs in HeLa cells expressing exogenous p53 (**Fig. 2A**) revealed that expression of 11 miRNAs viz., let-7a, miR-107, −145, −182, −16, −34a, −148a, −134, −199a-5p, −154 and −194 were significantly increased (*$P \leq 0.05$; ** $P \leq 0.01$) and expression of 7 miRNAs viz., miR-15b, −100, −125b, −146a, −150, −205 and −221 were significantly decreased (*$P \leq 0.05$; ** $P \leq 0.01$) compared to control (**Fig. 2B**). On knocking down endogenous *p53* by siRNA (Imgenex, USA) which downregulated the expression of the protein (**Fig. 2C**), expression pattern of all the 18 miRNAs were reversed as evident in **Figure 2D**. This indicated that these 18 miRNAs are regulated by p53 in HeLa cells. Comparing the expression profile of miRNAs in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and HeLa cells, it was evident that miRNA expression may be governed by the TF differently in different cell types.

Ectopic modulation of NFκB p65/RelA alters miRNA expression in mouse striatal ST *Hdh*^{Q7}/*Hdh*^{Q7} cells and HeLa cells

In order to identify the miRNAs regulated by NFκB p65/RelA, p65 sub-unit of NFκB also known as RelA which bears the transactivation domain of the transcription factor was next

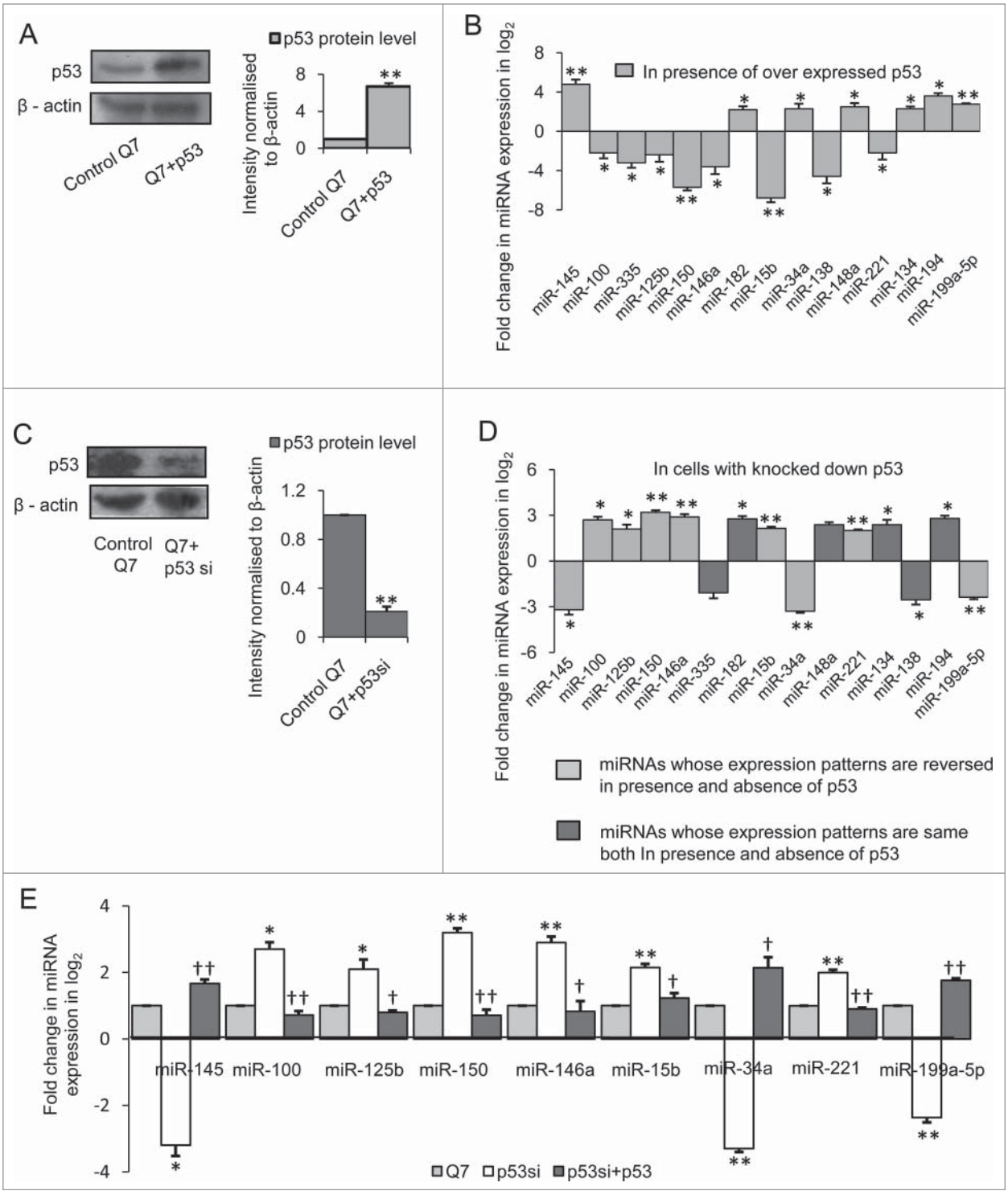

Figure 1. Regulation of miRNAs by p53 in mouse striatal ST*Hdh*^{Q7}/ *Hdh*^{Q7} cells (**A**) (**i**) Western Blot showing increased p53 protein level in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells transfected with p53-CFP; data are mean ± SD (n = 3); *p ≤ 0.05 compared to control. (**B**) Real Time PCR analysis showing changes in miRNA expression by greater than or equal to 4-fold (i.e. $-\Delta\Delta C_T \geq 2$ as shown in graph) in presence of over expressed p53 in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells compared with that of control; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**C**) Western Blot showing reduction in p53 protein level on knocking down endogenous *p53* in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells; data are mean ± SD (n = 3); *p ≤ 0.05 compared to control. (**D**) Real Time PCR analysis showing changes in miRNA expression in *p53* knocked down ST*Hdh*^{Q7}/*Hdh*^{Q7} cells; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**E**) Real Time PCR analysis showing recovery in miRNA expression on overexpressing p53-CFP in *p53* knocked down ST*Hdh*^{Q7}/*Hdh*^{Q7} cells; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control ST*Hdh*^{Q7}/*Hdh*^{Q7} cells; † $P \leq 0.05$; ††$P \leq 0.01$ compared to *p53* knocked down ST*Hdh*^{Q7}/*Hdh*^{Q7} cells (Q7 ± p53si). Error bars represent standard deviation, * or † represents statistical significance

over expressed in STHdh^{Q7}/Hdh^{Q7} cells. Twenty-four hours following transfection which increased (n = 3, p = 0.022) p65 protein level (**Fig. 3A**), miRNA expression profile was measured. It was observed that out of 40 miRNAs whose expressions were studied, 11 miRNAs viz., miR-15a, −34a, −146a, −107, −125b, −100, −150, −15b, −16, −221 and −135a were significantly up regulated (*$P \leq 0.05$; ** $P \leq 0.01$) and 6 miRNAs viz., miR-182, −214, −199a-5p, −199a-3p, −148a and −335 were decreased significantly (*$P \leq 0.05$; ** $P \leq 0.01$) in presence of exogenous NFκB p65/RelA in STHdh^{Q7}/Hdh^{Q7} cells compared to control (**Fig. 3B**). Endogenous NFκB p65/RelA activity was next reduced in STHdh^{Q7}/Hdh^{Q7} cells by treatment with aspirin[26]. It was observed that treating STHdh^{Q7}/Hdh^{Q7}cells with 2.0 mM aspirin for 24 h decreased the basal p65 activity (n = 3, p = 0.0068) significantly (**Fig. 3C**). Under such condition, 17 miRNAs whose expressions were studied, excepting miR-107, −34a, −16, −199a-5p and −335, expression pattern of the remaining 12 miRNAs were reversed in aspirin treated cells compared to their expression pattern in presence of exogenous NFκB p65/RelA (**Fig. 3D**). Though NFκB p65/RelA is known to regulate miR-199a-5p[27] and miR-34a[28] their levels remained unaltered in the presence of aspirin.

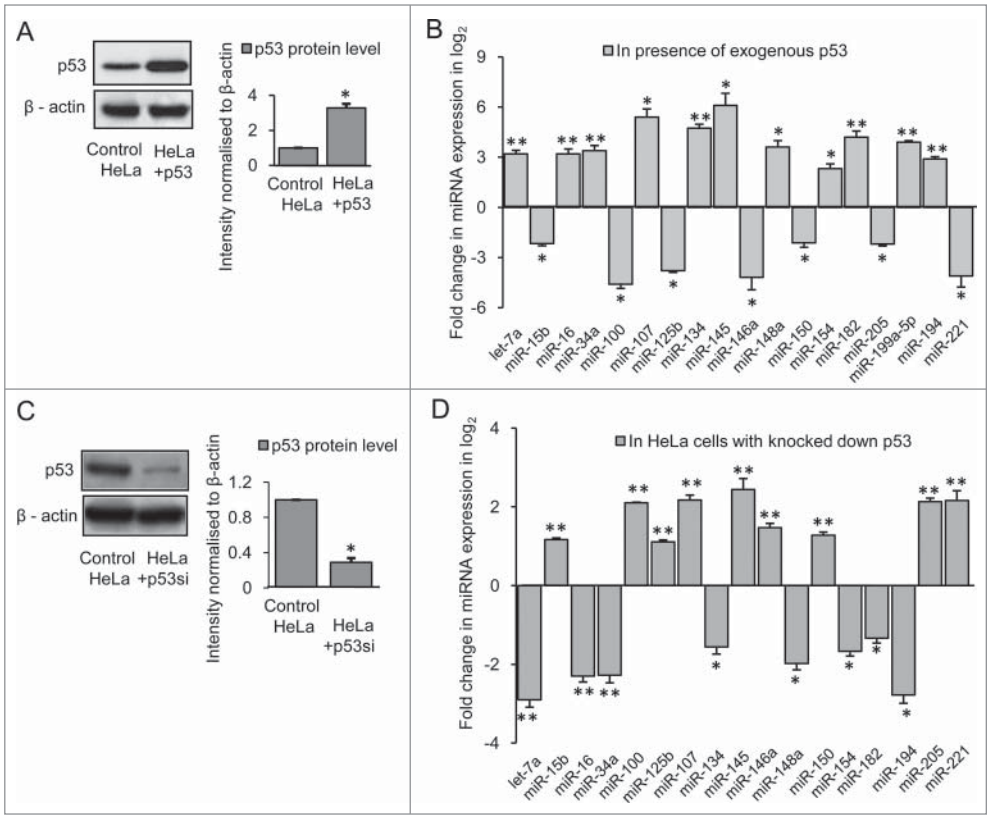

Figure 2. Regulation of miRNAs by p53 in HeLa cells (**A**) Western Blot showing increased p53 protein level in HeLa cells transfected with p53-CFP; data are mean ± SD (n = 3); *p ≤ 0.05 compared to control. (**B**) Real Time PCR analysis showing changes in miRNA expression by greater than or equal to 4-fold (i.e., $-\Delta\Delta C_T \geq 2$ as shown in graph) in presence of over expressed p53 in HeLa cells compared with that of control; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**C**) Western Blot showing reduction in p53 protein level on knocking down endogenous *p53* in HeLa cells; data are mean ± SD (n = 3); *p ≤ 0.05 compared to control. (**D**) Real Time PCR analysis showing changes in miRNA expression in *p53* knocked down HeLa cells; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. Error bars represent standard deviation, * represents statistical significance.

To further confirm that the expression changes of the mature miRNAs were specific due to the status of NFκB p65/RelA, endogenous NFκB p65/RelA activity was next reduced in STHdh^{Q7}/Hdh^{Q7} cells with the help of the peptide inhibitor SN50[29]. STHdh^{Q7}/Hdh^{Q7} cells treated with 15 μM SN50 showed decrease in basal endogenous NFκB p65/RelA activity 24 hours post treatment (n = 3, p = 0.003) (**Fig. 3E**). Under such condition, miRNA expression profile revealed changes in the expression of 12 miRNAs (**Fig. 3F**). Overexpressing NFκB p65/RelA in SN50 treated cells led to the recovery in p65-RE activity (n = 3, p = 0.03) (**Fig. 3E**) and also in miRNA expression compared to control (**Fig. 3F**). This result showed that 12 miRNAs were indeed regulated by NFκB p65/RelA in STHdh^{Q7}/Hdh^{Q7} cells.

Similarly, in order to find out the miRNAs which could be altered by NFκB p65/RelA in HeLa cells, we expressed exogenous NFκB p65/RelA in HeLa cells that increased p65 protein level (n = 3, p = 0.0042) compared to control cells (**Fig. 4A**). Under such conditions, expression of 9 miRNAs viz., miR-15a, −15b, −100, −125b, −135a, −146a, −150, −190 and −221 were significantly increased (*$P \leq 0.05$; ** $P \leq 0.01$) and expression of 7 miRNAs viz., miR-145, −148a, −199a-5p, −199a-3p, −214, −299 and −335 were significantly decreased (*$P \leq 0.05$; ** $P \leq 0.01$) in NFκB p65/RelA transfected HeLa cells compared to control (**Fig. 4B**). On inhibiting NFκB p65/RelA by treating HeLa cells with 2 mM aspirin which reduced basal p65 activity (n = 2, p = 0.0014) (**Fig. 4C**), the expression pattern of the 16 miRNAs were reversed (**Fig. 4D**) indicating that these miRNAs could be regulated by NFκB p65/RelA in HeLa cells. Comparing the results obtained in STHdh^{Q7}/Hdh^{Q7} cells and HeLa cells, it was evident that miRNA expression by NFκB p65/RelA was different in these 2 cells. These differences could be due to the differences in cell types and/or species of origin.

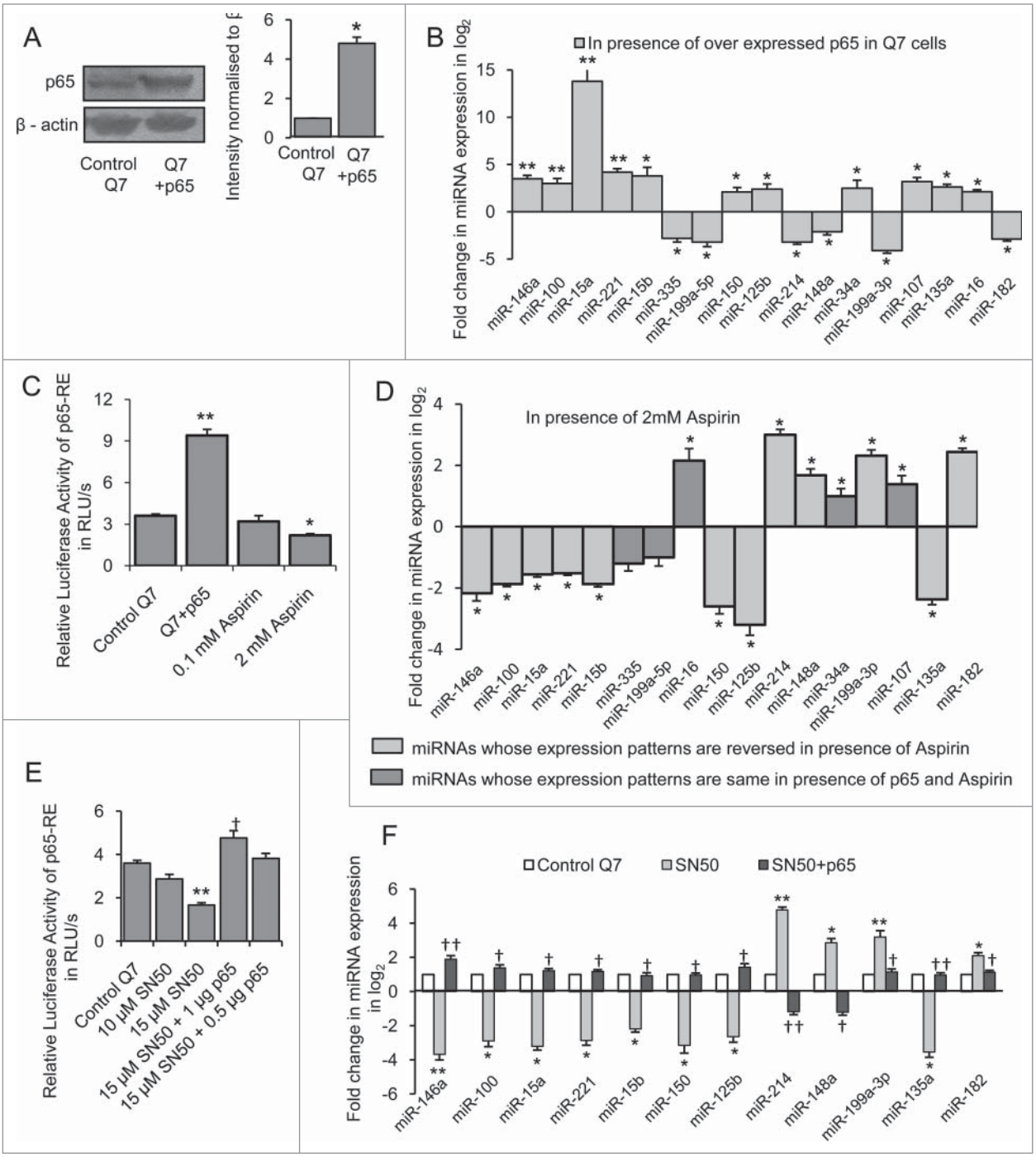

Figure 3. Regulation of miRNAs by NFκB p65/RelA in mouse striatal ST*Hdh*^{Q7}/ *Hdh*^{Q7} cells (**A**) Western Blot showing increased p65 protein level in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells transfected with p65 sub-unit of NFκB; data are mean ± SD (n = 3); *$P \leq 0.05$ compared to control. (**B**) Real Time PCR analysis showing changes in miRNA expression by greater than or equal to 4-fold (i.e. $-\Delta\Delta C_T \geq 2$ as shown in graph) in presence of overexpressed p65 in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells compared with that of control; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**C**) Changes in p65-RE activity in presence of exogenous p65 and different doses of Aspirin in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**D**) Real Time PCR analysis showing changes in miRNA expression in ST*Hdh*^{Q7}/*Hdh*^{Q7}cells with reduced p65-RE activity; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**E**) Changes in p65-RE activity in presence of different doses of SN50 and its recovery following exogenous expression of p65 in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells; data are mean ± SD (n = 3); ** $P \leq 0.01$ compared to control; † $P \leq 0.05$ compared to 15 μM SN50 treated cells (**F**) Real Time PCR analysis showing alteration of miRNA expression in ST*Hdh*^{Q7}/*Hdh*^{Q7}cells with reduced p65-RE activity and its recovery following restoration of p65-RE activity; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control ST*Hdh*^{Q7}/*Hdh*^{Q7} cells; † $P \leq 0.05$; ††$P \leq 0.01$ compared to ST*Hdh*^{Q7}/*Hdh*^{Q7} cells treated with 15 μM SN50. Error bars represent standard deviation, * or † represents statistical significance.

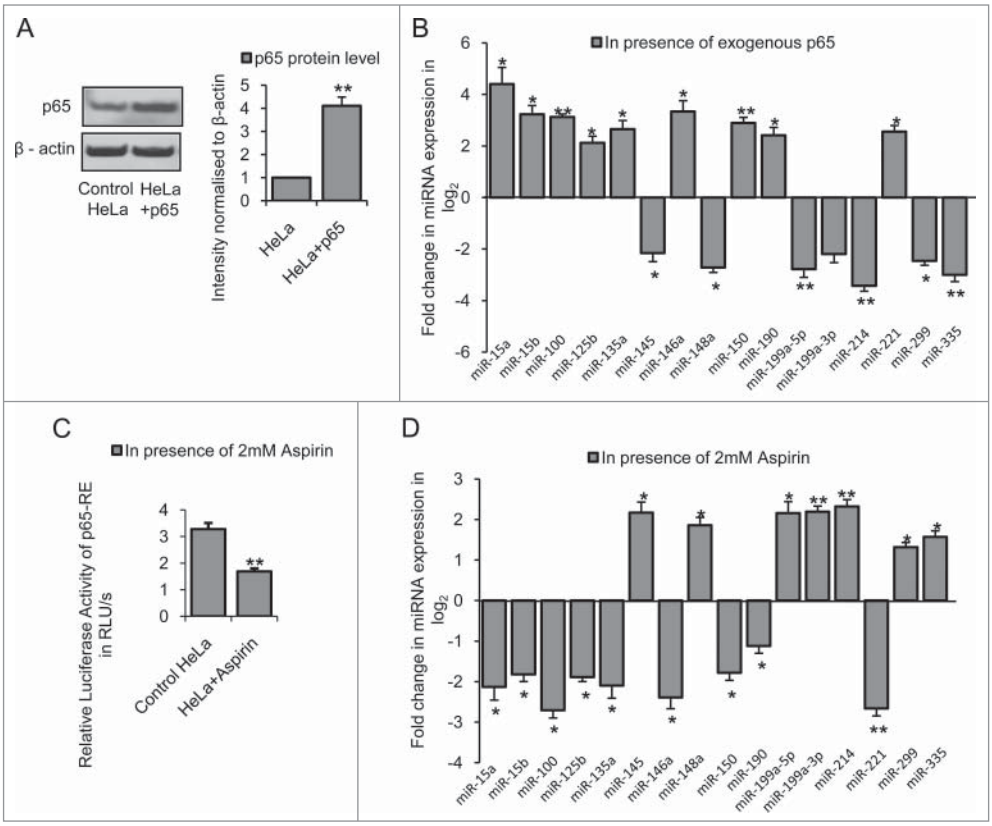

Figure 4. Regulation of miRNAs by NFκB p65/RelA in HeLa cells (**A**) Western Blot showing increased p65 protein level in HeLa cells transfected with p65 sub-unit of NFκB; data are mean ± SD (n = 3); ** $P \leq 0.01$ compared to control. (**B**) Real Time PCR analysis showing changes in miRNA expression by greater than or equal to 4-fold (i.e., $-\Delta\Delta C_T \geq 2$ as shown in graph) in presence of over expressed p65 in HeLa cells compared with that of control; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**C**) Reduction in p65-RE activity in presence of 2 mM Aspirin in HeLa cells; data are mean ± SD (n = 3); ** $P \leq 0.01$ compared to control. (**D**) Real Time PCR analysis showing changes in miRNA expression in HeLa cells with reduced p65-RE activity; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. Error bars represent standard deviation, * represents statistical significance.

miRNAs regulated by both p53 and NFκB p65/RelA in mouse striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and HeLa cells

Close examination of the results obtained revealed that out of the 40 different miRNAs, p53 could increase the expression of miR-34a, −145 and −199a-5p and decrease the expression of miR-15b, −100, −125b, −146a, −150 and −221 in both mouse striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and human cervical carcinoma HeLa cells. NFκB p65/RelA on the other hand has been observed to increase the expression of miR-15a, −15b, −100, −125b, −135a, −146a, −150 and −221 and decrease the expression of miR-148a, −199a-3p and −214 in both mouse striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and human cervical carcinoma HeLa cells. Expression of 6 miRNAs viz., miR-146a, −100, −221, −15b, −150 and −125b were increased by NFκB p65/255 RelA and decreased by p53 i.e. altered by both these TFs as schematically shown in **Figure 5A**.

p53 and NFκB p65/RelA alter transcription of the miRNAs in human cervical carcinoma HeLa cells

In order to examine whether miR-15b, −100, −125b, −146a, −150 and −221 are transcriptionally regulated by p53 and NFκB p65/RelA or whether their mature levels are altered by TFs affecting any of the miRNA processing events,[10,30] p53 and NFκB p65/RelA were separately transfected in HeLa cells. RT-PCR carried out 24 hours following transfection revealed that p53 decreased the expression of pri-miR-100, pri-miR-125b, pri-miR-146a, pri-miR-150 and pri-miR-221 and NFκB p65/RelA increased the same at the transcriptional level (**Fig. 5B-D**). GAPDH transcript level was taken as endogenous control for calculating fold changes in pri-miRNA expression[13]. The expression level of pri-miR-15b however, remained unchanged both in presence of exogenous p53 or NFκB p65/RelA. Hence it was confirmed that 5 miRNAs viz., miR-100, −125b, −146a, −150 and −221 are regulated by both p53 and NFκB p65/RelA at the transcriptional level and not in the level of miRNA processing.

p53 modulates putative promoter activity of upstream sequence constructs of miRNA genes bearing predicted p53 binding sites in HeLa cells

In order to identify the specific binding sequences upstream of the 5 miRNA genes which were observed to be regulated by both p53 and NFκB p65/RelA, we have utilized in-house search tool www.bioinformatics.org/grn/npb3 as described earlier[31] and the UCSC Genome Browser (http://genome.ucsc.edu). The details of such methods and the results obtained are shown in supplementary text. The summary of the result is shown in **Table 1**.

Region C in the upstream of miR-100 (**Fig. S1A**), region B1 in the upstream of miR-146a (**Fig. S1B**), region C in the upstream of miR-150 (**Fig. S1C**), region B in the upstream of miR-125b (**Fig. S1D**) and region B in the upstream of miR-221 (**Fig. S1E**) were PCR amplified from human genomic DNA and cloned into pGL3 basic vector and co-transfected in HeLa cells in presence of exogenous p53. It was observed that ectopic expression of p53 significantly reduced the basal luciferase activities of the constructs bearing segments of region C in the upstream of miR-100 (n = 3, p = 0.032), region B1 in the upstream of miR-146a (n = 3, p = 0.025) and region C in the upstream of miR-150 (n = 3, p = 0.019) respectively in HeLa cells (**Fig. 6A**). Conversely, knocking down endogenous *p53* in

HeLa cells by p53 siRNA markedly increased the luciferase activities of these constructs bearing the upstream sequences of miR-100 (n = 3, p = 0.0035), miR-146a (n = 3, p = 0.021) and miR-150 (n = 3, p = 0.029) respectively (**Fig. 6A**). However, although hsa-miR-125b and hsa-miR-221 are already known to be repressed by p53 in vivo,[8,9] no direct evidence of p53 regulation at the putative binding sites i.e. at region B in the upstream of hsa-miR-125b and region B in the upstream of hsa-miR-221 were observed either in presence of exogenous p53 or by knocking down endogenous *p53* in HeLa cells. Thus, it is likely that p53 might exert its suppressive effect on miR-125b and −221 through other regulatory sites. These results indicate that p53 represses the expression of miR-100, −146a and −150 in HeLa cells by inhibiting transcriptional activities at these sites in the regulatory sequences of the miRNA genes.

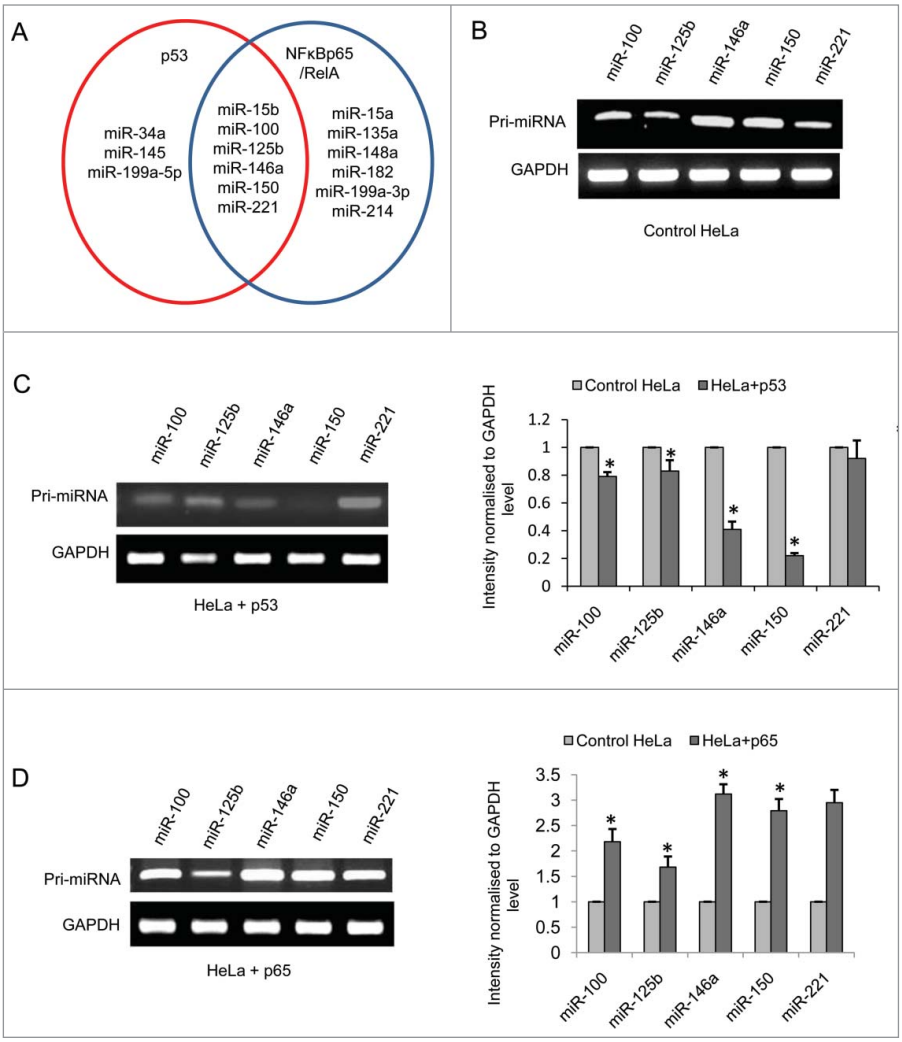

Figure 5. p53 and NFκB p65/RelA transcriptionally regulate the expression of miR-100, −125b, −146a, −150 and −221 (**A**) Schematic representation of miRNAs which are regulated by p53 only (miR-34a, −145, −199a-5p), NFκB p65/RelA only (miR-15a, −199a-3p, −148a, −135a, −182, −214) and regulated by both p53 and NFκB p65/RelA (miR-15b, −100, −125b, −146a, −150, −221) in both mouse striatal STHdh^{Q7}/Hdh^{Q7} cells and human cervical carcinoma HeLa cells; RT-PCR showing (**B**) Expression of pri-miR-15b, pri-miR-100, pri-miR-125b, pri-miR-146a, pri-miR-150 and pri-miR-221 in HeLa cells; (**C**) Decrease in the expression of the pri-miRNAs in presence of overexpressed p53-CFP; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**D**) Increase in the expression of the pri-miRNAs in presence of over expressed NFκB p65/RelA in HeLa cells; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. GAPDH mRNA level is taken as endogenous control in each case. Error bars represent standard deviation, * represents statistical significance.

Binding of p53 at regulatory sequences causes transcriptional inhibition of miR-100, −146a and −150 genes in HeLa and ST Hdh^{Q7}/Hdh^{Q7} cells

Finally, p53 was over expressed in HeLa cells and ChIP assays were performed to detect its direct interaction if any with these putative *cis*-regulatory elements. Thus, isolated chromatin from p53 transfected HeLa cells were immunoprecipitated by anti-p53 antibody, followed by PCR analysis with primers targeted to sequences in region C in the upstream of miR-100, region B1 in the upstream of miR-146a and region C in the upstream of miR-150 respectively in HeLa cells. PCR analysis with primers targeted to a region of *GAPDH* promoter[32] was taken as negative control in ChIP performed with anti p53 antibody. It was observed that p53 physically binds to regulatory sequences in the upstream of miR-100 (**Fig. 6B**), −146a (**Fig. 6C**) and −150 (**Fig. 6D**) in HeLa cells. Thus, these results demonstrate for the first time that p53 could directly bind to its response elements in the upstream sequence of miR-100, −146a and −150 and repress their transcription in human cervical carcinoma cell line viz., HeLa cells.

In order to see whether such regulation by p53 is also conserved in mouse as predicted earlier by analyzing miRNA profiling data in STHdh^{Q7}/Hdh^{Q7} cells in presence of exogenous p53, ChIP assays were carried out by immunoprecipitating isolated chromatin from both wild type STHdh^{Q7}/Hdh^{Q7} cells as well as from p53 transfected STHdh^{Q7}/Hdh^{Q7} cells with anti-p53 antibody. This was followed by PCR analysis with primers targeted to corresponding conserved region C' in the upstream of miR-100, region B1' in the upstream of miR-146a and region C' in the upstream of miR-150 respectively. It was observed that both endogenous p53 as well as ectopic p53 could physically bind to regulatory sequences in the upstream of miR-100 (**Fig. 6E**), −146a (**Fig. 6F**) and −150 (**Fig. 6G**) in mouse striatal cells as well. These results demonstrate for the first time that p53 binds to regulatory sequences in the upstream of miR-100, −146a and

Table 1. Summary of the transcription regulation of miRNAs by p53 and NFκB p65/RelA

MiRNA genes (with chromosomal coordinates)	Potential p53/ NFκB p65/RelA (p65) BS			Regulatory elements predicted based on chromatin state	
	Region (1st nucleotide of the pre-miRNA taken as +1)	Putative p53 BS [Two (G/A)(G/A)(G/A)C(T/A)(T/A)G(C/T)(C/T)(C/T) sequences separated by 0–13 base pairs] No. of sites	Putative NFκB p65/RelA BS [GG(G/A)(G/A)NN(C/T)(C/T)CC] No. of sites	Functional elements predicted (UCSC Genome Browser)	Cell line where observed (Information taken from UCSC Genome Browser)
hsa-miR-100 (chr11: 122152229–122152308 [–])	A (−720 to −978)		p65 (2)	Strong enhancer	HSMM, NHLF
	B (−2486 to −2820)		p65 (1)	Active promoter	HSMM
	C (−3493 to −3878)	p53 (1)	p65 (2)	Active promoter	HSMM
	D (−4722 to −5101)	p65 (3)	-	Active promoter	HMEC, HSMM
	E (−6621 to −6974)	p53 (1)	p65 (1)	Strong enhancer	HMEC, HSMM, NHEK, NHLF
	F (−7250 to −7628)		p65 (3)	Strong enhancer	
	G (−8658 to −9118)	p53 (2)	p65(1)	Strong enhancer	
hsa-miR-146a (chr5: 160485352–160485450 [+])	A (−19496- to 19175)	p53 (1)	p65 (2)	Weak transcription	HepG2, HMEC, NHLF
	B1 (−17583 to −17135)	p53 (1)	p65 (5)	Active promoter	GM12878, HepG2
	B2 (−14666 to −14179)	p53 (1)	p65 (3)	Active promoter	GM12878, HepG2
	C (−8337 to −10136)		p65 (5)	Weak promoter	GM12878
	D (−4537 to −6336)	p53 (3)	p65 (4)	Weak promoter	GM12878
hsa-miR-150 (chr19: 49500785–49500868 [–])	A (−143 to −736)	p53 (2)	p65 (1)	Weak enhancer	GM12878, K562, HepG2, HUVEC
	B (−2097 to −2512)		p65 (4)	Weak promoter	HepG2
	C (−2688 to −3093)	p53 (1)	p65 (4)	Weak enhancer	HepG2
	D (−4020 to −4258)	p53 (1)	p65 (4)	Weak enhancer	K562, HepG2
	E (−4567 to −5658)	p53 (1)	p65 (5)	Weak enhancer	K562, HepG2
	F (−11480 to −11640)	p53 (1)	p65 (2)	asatrong enhancer	HepG2, HUVEC, HSMM, NHLF
	G (−12795 to −14012)	p53 (1)	p65 (3)	Active promoter	HepG2
hsa-miR-125b (chr11: 122099757–122099844 [–])	A (−1039 to −1239)		p65 (2)	Active promoter	HMEC, HSMM, NHLF
	B (−10601 to −10920)	p53 (1)		Weak enhancer	HUVEC
	C (−125 to −414)	p53 (1)		Active promoter	HMEC, HSMM, NHLF
hsa-miR-221 (chrX: 45746157–45746266 [–])	A (−4493 to −4773)		p65 (1)	Strong enhancer	HMEC, NHEK, NHLF
	B (−1619 to −1997)	p53 (1)		Strong enhancer	HMEC, NHEK, NHLF, HUVEC, HSMM
	C (−4438 to −4458)	p53 (1)		Strong enhancer	HMEC, NHEK, NHLF

The genomic co-ordinates of the miRNAs were identified from miRBase (http://www.mirbase.org/).
20 kb upstream sequences of the pre-miRNAs (1st nucleotide of the pre-miRNA taken as +1) were downloaded from UCSC Genome Browser (http://genome.ucsc.edu) for both human and mouse.
The putative p53 and NFκB p65/RelA binding sites (BS) at miRNA upstream sequences were analyzed by using the in-house search tool www.bioinformatics.org/grn/npb3.
The p53 binding site is a dimer, comprising of 2 monomers, each 10 nucleotides long, with a variable spacer that can range between 0 to 13 nucleotides. The consensus sequence of the monomer is RRRCWWGYYY (R = G or A, W = T or A, Y = C or T).
The NFκB p65/RelA binding site is a monomer consisting of 10 nucleotides73 and the consensus sequence of the monomer is GGGRNNYYCC.
Presence of predicted regulatory regions based on ENCODE annotation data in the upstream sequences of the human miRNA genes were also evident from UCSC Genome Browser.
For any given miRNA the region in the 20 kb upstream with the highest score was considered. The 20 kb upstream sequence may have more than one putative p53 or p65 binding site with identical or lesser score other than the ones mentioned in the table.
Abbreviations used: GM12878: B-lymphocyte; HepG2: Hepatocellular carcinoma; HMEC: Mammary epithelial cells; HSMM: Skeletal muscle myoblasts; HUVEC: Umbilical vein endothelial cells; K562: Leukemia; NHEK: Epidermal keratinocytes; NHLF: Lung fibroblasts.

−150 in both human and mouse and represses transcription of these miRNA genes.

NFκB p65/RelA modulates putative promoter activity of upstream sequence constructs of miRNA genes bearing predicted NFκB p65/RelA binding sites in HeLa cells

In order to investigate the role of NFκB p65/RelA and to test the putative promoter activity of the upstream sequences of miRNA genes bearing putative NFκB p65/RelA binding sites region A, B and C in the upstream of miR-100 (**Fig. S1A**), region B1 in the upstream of miR-146a (**Fig. S1B**), region B and C in the upstream of miR-150 (**Fig. S1C**), region A in the upstream of miR-125b (**Fig. S1D**) and region A in the upstream of miR-221 (**Fig. S1E**) were PCR amplified from human genomic DNA, cloned into pGL3 basic vector and co-transfected in HeLa cells in presence of exogenous NFκB p65/RelA. It was observed that ectopic expression of NFκB p65/RelA significantly increased the basal luciferase activities of the

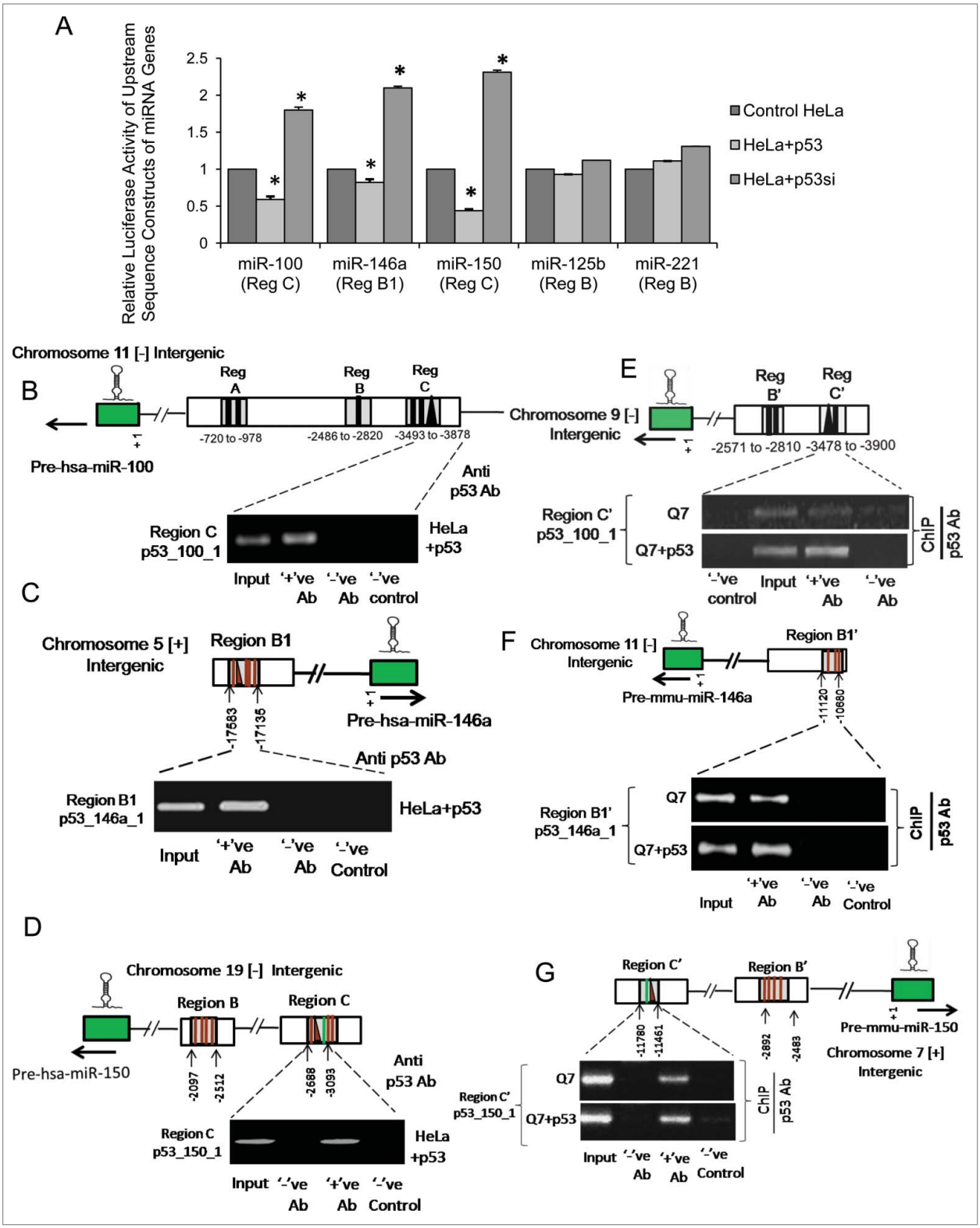

Figure 6. p53 binds to the regulatory sequences in the upstream of miR-100, −146a and −150 genes in both human and mouse and represses their transcription (**A**) Changes in relative luciferase activity of upstream sequence constructs of miR-100, −146a, −150, −125b and −221 bearing putative p53 binding sites in presence of both exogenous p53 and on knocking down basal endogenous *p53* by siRNA mediated gene silencing in human cervical carcinoma HeLa cells; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. Chromatin immunoprecipitation (ChIP) assay showing binding of ectopic p53 to its respective binding sites within the regulatory elements in the upstream of (**B**) miR-100, (**C**) miR-146a and (**D**) miR-150 in HeLa cells, (n = 3). (ChIP) assay showing binding of ectopic p53 as well as endogenous p53 to corresponding conserved regions bearing putative p53 binding sites in the upstream of (**E**) miR-100, (**F**) miR-146a and (**G**) miR-150 in mouse striatal STHdh^{Q7}/Hdh^{Q7} cells; (n = 3). Error bars represent standard deviation, * represents statistical significance. Solid lines represent putative NFκB p65/RelA binding sites and solid triangles represent putative p53 binding sites in the regulatory sequences of the miRNA genes.

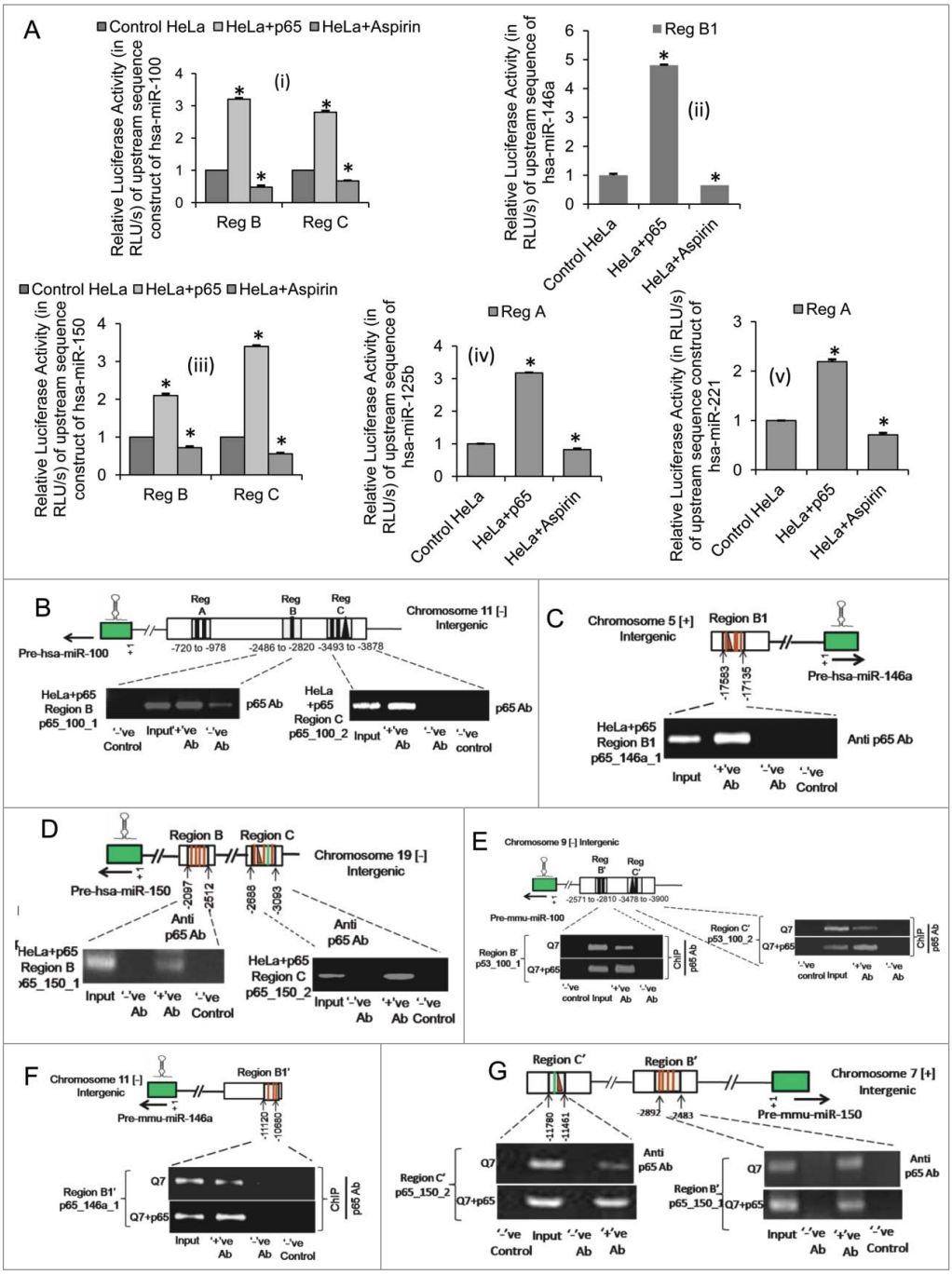

Figure 7. NFκB p65/RelA binds to the regulatory sequences in the upstream of miR-100, −146a and −150 genes in both human and mouse and activates their transcription (**A**) Changes in relative luciferase activity of upstream sequence constructs of (i) miR-100, (ii) miR-146a, (iii) miR-150, (iv) miR-125b and (v) miR-221 bearing putative NFκB p65/RelA binding sites in presence of both exogenous NFκB p65/RelA and on inhibition of basal endogenous NFκB p65/RelA activity in human cervical carcinoma HeLa cells; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. Chromatin immunoprecipitation (ChIP) assay showing binding of ectopic NFκB p65/RelA to its respective binding sites within the regulatory elements in the upstream of (**B**) miR-100, (**C**) miR-146a and (**D**) miR-150 in HeLa cells, (n = 3). (ChIP) assay showing binding of ectopic NFκB p65/RelA as well as endogenous NFκB p65/RelA to corresponding conserved regions bearing putative NFκB p65/RelA binding sites in the upstream of (**E**) miR-100, (**F**) miR-146a and (**G**) miR-150 in mouse striatal STHdh^{Q7}/Hdh^{Q7} cells; (n = 3). Error bars represent standard deviation, * represents statistical significance. Solid lines represent putative NFκB p65/RelA binding sites and solid triangles represent putative p53 binding sites in the regulatory sequences of the miRNA genes.

constructs bearing segments of region B (n = 3, p = 0.0019) and C (n = 3, p = 0.023) in the upstream of miR-100 (**Fig. 7A(i)**), region B1 in the upstream of miR-146a (n = 3, p = 0.037) (**Fig. 7A(ii)**), region B (n = 3, p = 0.0028) and C (n = 3, p = 0.0034) in the upstream of miR-150 (**Fig. 7A(iii)**), region A in the upstream of miR-125b (n = 3, p = 0.0015) (**Fig. 7A(iv)**) and region A in the upstream of miR-221 (n = 3, p = 0.0026) (**Fig. 7A(v)**) respectively in HeLa cells. Conversely, inhibiting basal endogenous NFκB p65/RelA activity by treating HeLa cells with 2mM Aspirin markedly repressed the luciferase activities of these constructs bearing the upstream sequences of miR-100 (region B, n = 3, p = 0.021; region C, n = 3, p = 0.014) (**Fig. 7A(i)**), miR-146a (region B1, n = 3, p = 0.025) (**Fig. 7A(ii)**), miR-150 (region B, n = 3, p = 0.0027; region C, n = 3, p = 0.0023) (**Fig. 7A(iii)**), miR-125b (region A, n = 3, p = 0.013) (**Fig. 7A(iv)**) and miR-221 (region A, n = 3, p = 0.0045) (**Fig. 7A(v)**) respectively. These results confirm that NFκB p65/RelA regulates the expression of miR-146a,[11] −125b[12] and −221[33] and demonstrate for the first time that NFκB p65/RelA induces the expression of miR-100 and −150 in HeLa cells by enhancing transcriptional activities at regulatory sites in the upstream sequences of the miRNA genes. It had already been reported that NFκB p65/RelA induces the expression of miR-146a by binding to and activating transcription at sites −1121 and

−3432 in the upstream sequence of miR-146a gene.[11] This report identifies a novel NFκB p65/RelA binding site at −17,290 which also contributes to miR-146a regulation by the TF.

Promoter binding of NFκB p65/RelA sub-unit is required for the transcription of NFκB dependant miR-100, -146a and -150 genes in HeLa and ST*Hdh*^{Q7}/*Hdh*^{Q7} cells

Finally, NFκB p65/RelA was overexpressed in HeLa cells and ChIP assays were performed to detect its direct interaction if any with these putative *cis*-regulatory elements. Thus, isolated chromatin from NFκB p65/RelA transfected HeLa cells were immunoprecipitated by anti-p65 antibody, followed by PCR analysis with primers targeted to sequences in region B and C in the upstream of miR-100, region B1 in the upstream of miR-146a and region B and C in the upstream of miR-150 respectively in HeLa cells. PCR analysis with primers targeted to a region on chromosome 1 (Chr1: 204,366,822–204,366,872)[6] was taken as negative control in ChIP peformed with anti-p65 antibody. It was observed that NFκB p65/RelA physically binds to regulatory sequences in the upstream of miR-100 (**Fig. 7B**), miR-146a (**Fig. 7C**) and miR-150 (**Fig. 7D**) in HeLa cells. Thus, these results identify a novel NFκB p65/RelA regulatory site in the upstream of miR-146a and demonstrate for the first time that NFκB p65/RelA could directly bind to its response elements in the upstream sequence of miR-100 and −150 and activate their transcription in human cervical carcinoma cell line viz., HeLa cells.

In order to see whether such regulation by NFκB p65/RelA is also conserved in mouse as predicted earlier by analyzing miRNA profiling data in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells in presence of exogenous NFκB p65/RelA, ChIP assays were carried out by immunoprecipitating isolated chromatin from both wild type ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and from p65 sub-unit transfected ST*Hdh*^{Q7}/*Hdh*^{Q7} cells with anti-p65 antibody. This was followed by PCR analysis with primers targeted to corresponding conserved regions B' and C' in the upstream of miR-100, region B1' in the upstream of miR-146a and region B' and C' in the upstream of miR-150 respectively. It was observed that both endogenous NFκB p65/RelA as well as ectopic NFκB p65/RelA could physically bind to regulatory sequences in the upstream of miR-100 (**Fig. 7E**), miR-146a (**Fig. 7F**) and miR-150 (**Fig. 7G**) in mouse striatal cells as well. These results demonstrate for the first time that NFκB p65/RelA binds to regulatory sequences in the upstream of miR-100, −146a and −150 in both human and mouse and induces transcription of these miRNA genes.

Functional cross-talk between p53 and p65 (NFκB/RelA) governs NFκB p65/RelA activity in mouse ST*Hdh*^{Q7}/*Hdh*^{Q7} cells

These functional p53 and NFκB p65/RelA binding sites in the upstream regulatory sequences lie in close proximity (**Figs. S1A, B, C**) and the fact that p53 binds and represses transcription and NFκB p65/RelA binds and activates transcription of these miRNAs at these binding sites suggest for the possibility of a functional cross-talk between p53 and NFκB p65/RelA in co-regulating miRNA expression. In order to validate our hypothesis of the existence of a possible cross-talk between p53 and NFκB p65/RelA, p53 was over expressed in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells. Ectopic expression of p53 in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells decreased NFκB p65/RelA response element (p65-RE) activity ($n = 3$, $p = 0.027$) 48 hours post transfection and reduction in endogenous *p53* level via p53 siRNA mediated gene silencing led to an increase ($n = 3$, $p = 0.045$) in the same (**Fig. 8A**). This observation is supported by immunoblot analysis with nuclear and cytoplasmic extract from p53 transfected ST*Hdh*^{Q7}/*Hdh*^{Q7} cells which showed reduced ($n = 3$, $p = 0.018$) nuclear content of NFκB p65/RelA in presence of overexpressed p53 (**Fig. 8B**). Similarly, expression of NFκB p65/RelA responsive chemokine *CCL5*[34] was significantly decreased ($n = 3$, $p = 0.021$) in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells in presence of ectopic p53 (**Fig. 8C**). This showed that ectopic p53 inhibited NFκB p65/RelA in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells. Conversely, over expression of NFκB p65/RelA in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells did not alter p53 nuclear content (**Fig. 8D**) or the expression of p53 responsive CASP1 gene (**Fig. 8E**) indicating that NFκB p65/RelA did not influence p53 activity in mouse striatal cells.

Moreover, elevated p53 was also observed to compete with NFκB p65/RelA for CBP binding. In a series of co-immunoprecipitation experiments using cell extracts from both control ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and ST*Hdh*^{Q7}/*Hdh*^{Q7} cells transfected with exogenous p53 pulled down with anti-CBP antibody it was revealed that p53 could bind more strongly with CBP ($p = 0.024$) in p53 transfected ST*Hdh*^{Q7}/*Hdh*^{Q7} cells compared to control ST*Hdh*^{Q7}/*Hdh*^{Q7} cells (**Fig. 8F**) and NFκB p65/RelA could bind less strongly with CBP ($p = 0.013$) under such conditions (**Fig. 8G**). In order to examine whether this reduced binding of NFκB p65/RelA with CBP in p53 transfected ST*Hdh*^{Q7}/*Hdh*^{Q7} cells was due to competition by elevated nuclear p53 for CBP, endogenous *p53* was knocked down in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and immunoprecipitation with cell extracts prepared 72 hours post transfection (which significantly decreased nuclear p53 content, $p = 0.035$) showed an increased binding of NFκB p65/RelA with CBP ($p = 0.0041$) (**Fig 8H**) and a reduced binding of p53 with CBP (**Fig. 8I**) compared to control ST*Hdh*^{Q7}/*Hdh*^{Q7} cells. Thus, the results obtained indicate that sequestration of transcriptional co-activator p300/CBP by elevated p53 in p53 transfected ST*Hdh*^{Q7}/*Hdh*^{Q7} cells lead to reduced NFκB p65/RelA binding with p300/CBP which possibly accounts for the observed p53 mediated repression of endogenous NFκB p65/RelA activity in these cells. Similar nuclear competition between p53 and NFκB p65/RelA for a limiting amount of CBP were also reported earlier.[36–38] Thus, these observations suggest for the co-regulation of miRNAs by p53 and NFκB p65/RelA where NFκB p65/RelA induces transcription of miR-100, −146a and −150 and p53 represses the same by inhibiting endogenous NFκB p65/RelA activity.

Co-regulation of miR-100, −146a and −150 by p53 and NFκB p65/RelA in mouse striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells

In order to validate our hypothesis, upstream sequence constructs bearing functional p53 and NFκB p65/RelA binding sites

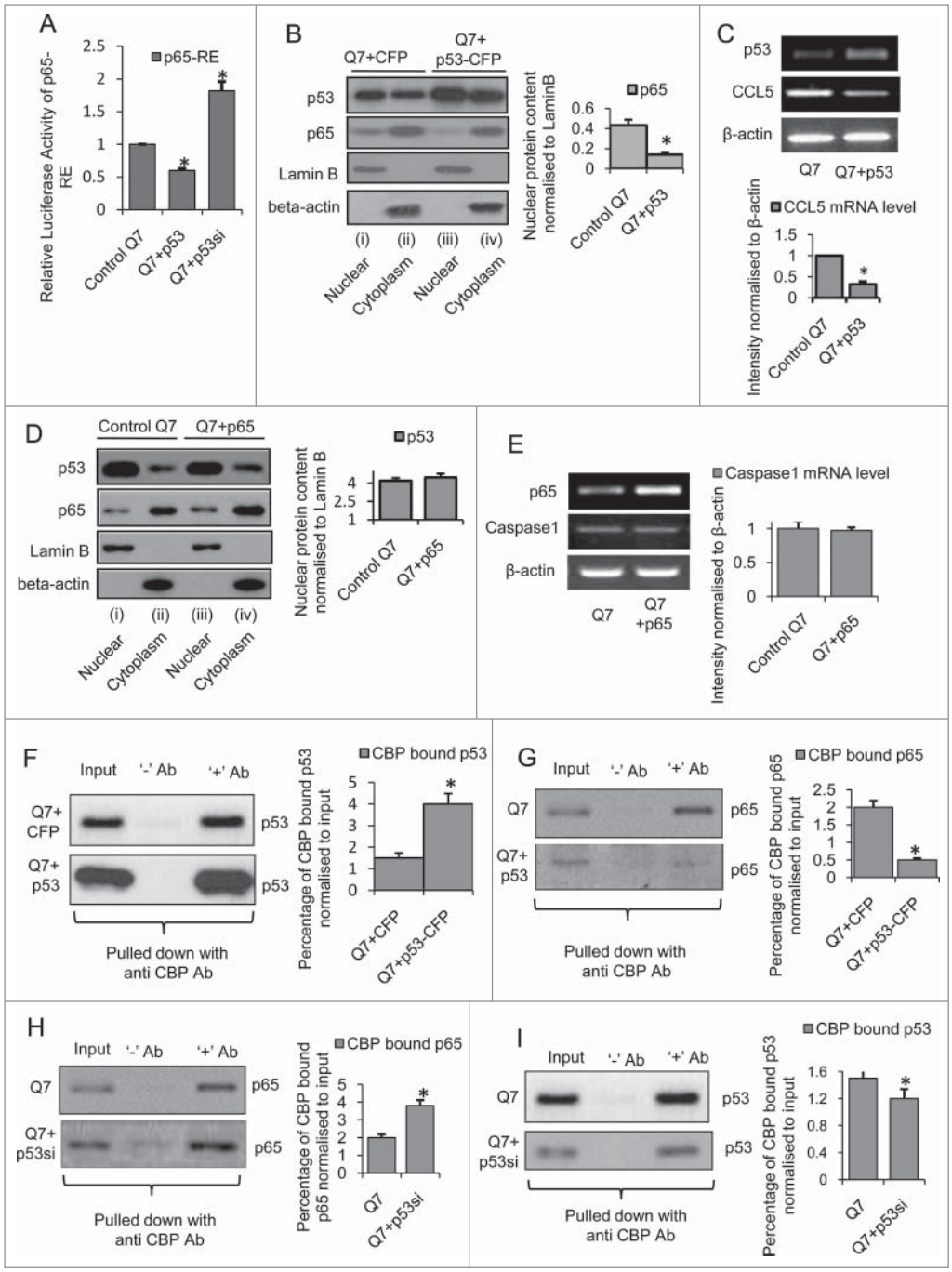

Figure 8. Ectopic expression of p53 in mouse striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells competes with NFκB p65/RelA for CBP binding and inhibits NFκB p65/RelA activity (**A**) Luciferase assay showing reduced NFκB p65/RelA response element (p65-RE) activity in presence of overexpressed p53 in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells and increased p65-RE activity in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells with knocked down *p53*; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**B**) Western Blot showing increased p53 nuclear content and decreased p65 nuclear content and (**C**) RT-PCR showing reduction in *CCL5* expression in ST*Hdh*^{Q7}/*Hdh*^{Q7} in presence of exogenous p53-CFP; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**D**) Western Blot showing no change in p53 nuclear content on exogenous expression of NFκB p65/RelA in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells, n = 3. (**E**) RT-PCR showing no change in *Caspase1* mRNA level on ectopic expression of NFκB p65/RelA in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells, n = 3. Co-immunoprecipitation assay showing (**F**) increased p53 binding and (**G**) decreased p65 binding to CBP in p53-CFP transfected ST*Hdh*^{Q7}/*Hdh*^{Q7} cells compared to wild type ST*Hdh*^{Q7}/*Hdh*^{Q7} cells; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. Co-immunoprecipitation assay showing (**H**) increased p65 binding and (**I**) decreased p53 binding to CBP on knocking down endogenous *p53* in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. Error bars represent standard deviation, * represents statistical significance.

in close proximity viz., region C in the upstream of miR-100 (**Fig. S1A**), region B1 in the upstream of miR-146a (**Fig. S1B**) and region C in the upstream of miR-150 (**Fig. S1C**) were co-transfected in mouse striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells along with p53 and/or NFκB p65/RelA. Luciferase assay with region C bearing both functional p53 and NFκB p65/RelA binding sites in the upstream of miR-100 revealed that ectopic NFκB p65/RelA could increase the luciferase activity of the construct by over 7-fold (n = 3, p = 0.042, * represents statistical significance where * $P \leq 0.05$; ** $P \leq 0.01$) of the basal value. However, such increase was significantly reduced in presence of ectopic p53 (n = 3, p = 0.029, † represents statistical significance where † $P \leq 0.05$; †† $P \leq 0.01$) (**Fig 9A**). Interestingly, on overexpressing NFκB p65/RelA along with p53, the luciferase activity of the construct could not revive and was significantly less (n = 3, p = 0.011, † represents statistical significance where † $P \leq 0.05$; †† $P \leq 0.01$) than that observed in presence of NFκB p65/RelA alone (**Fig. 9A**). These results indicate that apart from repressing miR-100 expression via inhibiting NFκB p65/RelA activity, p53 here could also decrease miR-100 expression directly by binding to its putative binding site in the upstream sequence of miR-100.

In order to investigate the role of p53 and NFκB p65/RelA in co-regulating miR-146a expression, promoter luciferase assay with the upstream construct encompassing region B1 with both functional p53 and NFκB

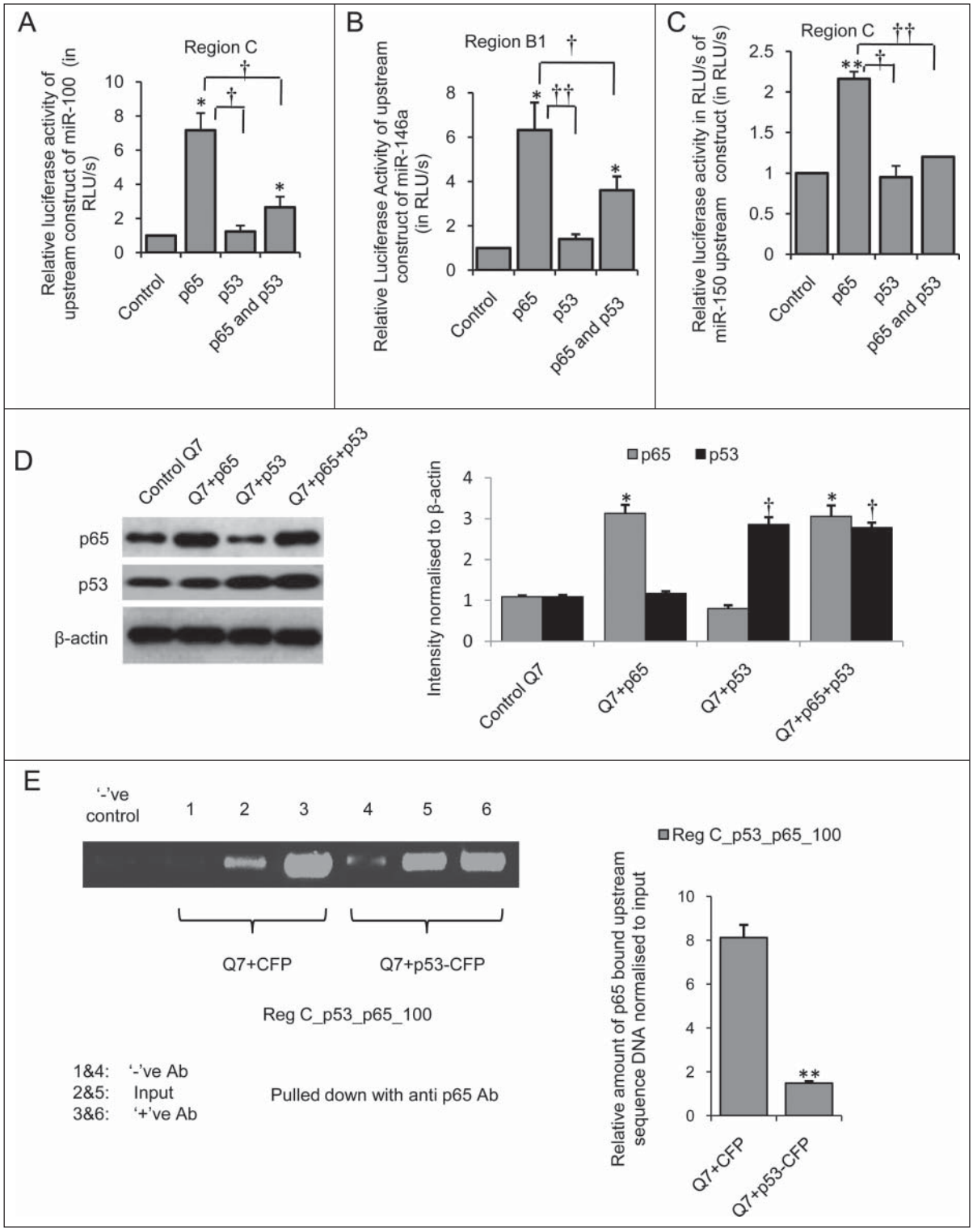

Figure 9. Functional cross-talk between p53 and NFκB p65/RelA regulate miR-100, −146a and −150 expressions in mouse striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells Changes in relative luciferase activity of (**A**) miR-100 upstream sequence construct (**B**) miR-146a upstream sequence construct and (**C**) miR-150 upstream sequence construct in presence of exogenous p53-CFP and NFκB p65/RelA in mouse striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells. In presence of exogenous NFκB p65/RelA luciferase activities of the constructs were increased whereas in presence of exogenous p53-CFP the luciferase activities of the constructs were significantly reduced; data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. (**D**) Western blot showing increase in p65 protein level by more than 3 fold (*p≤ 0.05) and increase in p53 protein level by more than two fold († p≤ 0.05) when 2.5 μg each of p65 and p53 plasmid clones was transfected either in isolation or in combination in STHdhQ7/HdhQ7 cells compared to control STHdhQ7/HdhQ7 cells; (n = 3). (**E**) Chromatin immunoprecipitation assay showing decreased NFκB p65/RelA occupancy at its binding site in miR-100 upstream sequence (Reg C) in p53-CFP transfected ST*Hdh*^{Q7}/*Hdh*^{Q7} cells compared to control ST*Hdh*^{Q7}/*Hdh*^{Q7} cells, data are mean ± SD (n = 3); *$P \leq 0.05$; ** $P \leq 0.01$ compared to control. This indicates p53 driven exclusion of NFκB p65/RelA from its binding site that partially contributes to miR-100 repression. Error bars represent standard deviation, * represents statistical significance.

p65/RelA binding sites was performed in presence of overexpressed p53 and NFκB p65/RelA. The results obtained revealed that ectopic NFκB p65/RelA could increase the expression of miR-146a by 6.4-fold of the basal level (n = 3, p = 0.039, * represents statistical significance where * $P \leq 0.05$; †† $P \leq 0.01$) (**Fig. 9B**) which was significantly reduced in presence of exogenous p53 (n = 3, p = 0.0085, † represents statistical significance where † $P \leq 0.05$; †† $P \leq 0.01$). However, on addition of NFκB p65/RelA along with p53 luciferase activity of the construct could not revive and was significantly less (n = 3, p = 0.0085, † represents statistical significance where † $P \leq 0.05$; †† $P \leq 0.01$) than that observed in presence of NFκB p65/RelA alone (**Fig. 9B**). These indicate inhibition of NFκB p65/RelA activity by p53. In order to investigate the role of p53 and NFκB p65/RelA in co-regulating miR-150 expression, promoter luciferase assay with the upstream construct containing region C with both functional p53 and NFκB p65/RelA binding sites revealed that ectopic NFκB p65/RelA could increase the luciferase activity of the construct by over 2-fold (n = 3, p = 0.0033, * represents statistical significance where * $P \leq 0.05$; ** $P \leq 0.01$) (**Fig. 9C**) which was reduced to the basal level in presence of p53) (n = 3, p = 0.021, † represents statistical significance where † $P \leq 0.05$; †† $P \leq 0.01$). On overexpressing NFκB p65/RelA along with p53, the luciferase activity of the construct could not revive and was significantly less (n = 3, p = 0.0039, † represents statistical significance where † $P \leq 0.05$; †† $P \leq 0.01$) than that observed in presence of NFκB p65/RelA alone (**Fig. 9C**). This indicated that p53 could strongly inhibit miR-150 at this upstream region.

It is to be mentioned here that under identical experimental conditions with those maintained in the luciferase assays, western blot analysis revealed an increase in NFκB p65/RelA protein level by more than 3-fold when 2.5 μg of the plasmid was transfected both in isolation (n = 3, p = 0.029, * represents statistical significance where * $P \leq 0.05$; ** $P \leq 0.01$) and in combination with equal amount of p53-CFP (n = 3, p = 0.036, * represents statistical significance where * $P \leq 0.05$; ** $P \leq 0.01$) in STHdh^{Q7}/Hdh^{Q7} cells (**Fig. 9D**). Similarly, ectopic expression of p53-CFP in isolation (n = 3, p = 0.021, † represents statistical significance where † $P \leq 0.05$; †† $P \leq 0.01$) as well as in combination with equal amount of NFκB p65/RelA (n = 3, p = 0.018, † represents statistical significance where † $P \leq 0.05$; †† $P \leq 0.01$) demonstrate a 2-fold increase in p53 protein level in both the cases (**Fig. 9D**). Thus, NFκB p65/RelA protein level remains the same when expressed isolated and in combination with p53-CFP in STHdh^{Q7}/Hdh^{Q7} cells. Therefore the partial recovery in luciferase activities of the constructs as observed in **Figure 9A-C** in presence of both NFκB p65/RelA and p53 as compared to those in presence of NFκB p65/RelA alone is not due to reduced NFκB p65/RelA protein level but due to inhibition of NFκB p65/RelA activity by p53 or due to other p53 mediated repression mechanism(s).

p53 binds to region C in the upstream of miR-100 and displaces NFκB p65/RelA from its functional binding site thereby repressing transcription of miR-100 gene

The co-regulation of miR-100 by p53 and NFκB p65/RelA as observed in the present work could be explained based on prediction in the context of nuclear cross-talk or functional interaction between p53 and NFκB p65/RelA by models previously described by Schneider et al.[39] The co-regulation of miR-100 at region C (−3493 to −3878) (**Fig. 9A**) by p53 and NFκB p65/RelA can be explained by *Exclusion model* where elevated p53 in p53 transfected STHdh^{Q7}/Hdh^{Q7} cells might have prevented binding of endogenous NFκB p65/RelA at the closely placed or overlapping p65 binding site thereby reducing miR-100 transcription. In order to validate our hypothesis, chromatin immunoprecipitation was done using anti-p65 antibody both in control STHdh^{Q7}/Hdh^{Q7} cells and in STHdh^{Q7}/Hdh^{Q7} cells transfected with exogenous p53. Over expression of p53-CFP in wild type STHdh^{Q7}/Hdh^{Q7} cells resulted in decreased p65 occupancy (p = 0.032) at region C in the upstream of miR-100 (**Fig. 9E**) 48 hours post transfection compared to control STHdh^{Q7}/Hdh^{Q7} cells transfected with empty vector CFP-C1. These results demonstrate that ectopic p53 in STHdh^{Q7}/Hdh^{Q7} cells binds to miR-100 upstream sequence and displaces NFκB p65/RelA from its nearby binding site. This may partially account for the p53 mediated repression of miR-100 expression in STHdh^{Q7}/Hdh^{Q7} cells. However, since p53 mediated suppression of miRNA expression could not be entirely retrieved by overexpressing NFκB p65/RelA, therefore co-regulation of miR-100 by p53 and NFκB p65/RelA cannot be solely explained by the *Exclusion Model*. Presently we are investigating additional mechanisms of co-regulation of miR-100 by p53 and NFκB p65/RelA.

Discussion

Like protein-coding genes, most miRNA genes are initially transcribed by RNA Polymerase II in the nucleus as primary transcripts[1] and can be regulated by transcription factors (TFs).[5] In this context, good many number of papers have been published describing transcription factors that bind to promoter regions of miRNA genes and regulate their expression either in physiological or pathological conditions.[13] In this work, it was observed that p53 could increase the expression of 3 miRNAs viz., miR-145, −34a, −199a-5p and decrease the expression of 6 miRNAs viz., miR-15b, −100, −125b, −146a, −150 and −221 (**Figs. 1 and 2**) and NFκB p65/RelA could increase the expression of 8 miRNAs viz., miR-15a, −15b, −100, −125b, −135a, −146a, −150 and −221 and decrease the expression of 4 miRNAs viz., miR-214, −199a-3p, −148a and −182 in mouse striatal STHdh^{Q7}/Hdh^{Q7} cells and human cervical carcinoma HeLa cells (**Figs. 3 and 4**). However, miR-145, −34a, −15b, −100, −125b and −221 were already known to be regulated by p53.[8,40–42] Similarly, miR-146a, −221, −15a, −34a, −214 and −125b were already known to be regulated by NFκB p65/RelA.[11,12,28,27,33] In our earlier work, we had reported that miR-146a could be regulated by p53.[43] Thus, this report demonstrates for the first time that -146a, −150 and −199a-5p could be regulated by p53 and that NFκB p65/RelA increases the expression of miR-100, −150 and −135a and decreases the expression of miR-199a-3p and −148a in both mouse and human and across

diverse cell lines. Of these miRNAs, miR-100, −125b, −146a, −150 and −221 have been observed to be transcriptionally repressed by p53 and induced by NFκB p65/RelA (**Fig. 5**). 20 kb upstream sequences of these miRNA genes also harboured putative p53 and NFκB p65/RelA binding sites (**Table 1**). Promoter luciferase assays and chromatin immunoprecipitation (ChIP) demonstrated p53 and NFκB p65/RelA to bind to their respective binding sites in the upstream sequences of miR-100, −146a and −150 and regulate their expression both in HeLa and ST*Hdh*^{Q7}/*Hdh*^{Q7} cells (**Figs. 6 and 7**). Regulation of miRNA genes by direct physical binding of p53 has been previously shown for several miRNAs viz., miR-34a,[40] miR-34b, −34c,[44] miR-192/194/215,[45] miR-17–92 cluster,[32] miR-107,[46] miR-200c/141,[47] miR-149*,[48] miR-605,[49] miR-1246[50] and miR-1204.[51] Similarly, NFκB p65/RelA has been earlier reported to bind with upstream sequence and regulate the expression of miR-146a,[11] miR-9,[52] miR-125b-1, −21, −23b, −30b,[12,13] miR-17–92, −30a, −130a,[13] miR-301,[53] miR-221,[33] miR-34a.[28] In the present work, it has been observed for the first time that p53 can bind with the upstream sequence and suppress the expression of miR-100, −146a and −150 and that NFκB p65/RelA can bind with the upstream sequence and induce the expression of miR-100 and −150 in both human and mouse. A novel NFκB p65/RelA binding site in the upstream sequence of miR-146a has also been identified in the present work.

These functional p53 and NFκB p65/RelA binding sites in the upstream regulatory sequences lie in close proximity (**Fig. S1A, B, C**) and the fact that p53 binds and represses transcription and NFκB p65/RelA binds and activates transcription of these miRNAs at these binding sites suggest for the possibility of a functional cross-talk between p53 and NFκB p65/RelA in co-regulating miRNA expression. Interestingly, elevated p53 in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells was observed to inhibit NFκB p65/RelA nuclear content and activity and repress NFκB p65/RelA dependant CCL5 expression (**Fig. 8A–C**). However, ectopic NFκB p65/RelA did not alter p53 nuclear content or p53 mediated CASP1 gene expression (**Fig. 8D & E**). Moreover, elevated p53 was also observed to compete with NFκB p65/RelA for CBP binding (**Fig. 8F-I**). Since nuclear cross-talk between 2 transcription factors (TFs) can occur at several levels where any one TF can influence the activity of the other to define the outcome of a process,[39] therefore observations in the present work support for the existence of a cross-talk between these 2 TFs in co-regulating miRNA expression. In fact, luciferase assays demonstrated p53 and NFκB p65/RelA to co-regulate miR-100, −146a and −150 expression where p53 mediated inhibition of NFκB p65/RelA activity possibly led to miRNA repression (**Fig. 9A–D**). In such case, ectopic expression of p53 in ST*Hdh*^{Q7}/*Hdh*^{Q7} cells was observed to reduce NFκB p65/RelA occupancy at Region C in the upstream of miR-100 (**Fig. 9E**). This suggests one of the possible mechanisms by which p53 represses miR-100 expression by inhibiting NFκB p65/RelA mediated activation. However, since such p53 mediated suppression of miRNA expression could not be entirely retrieved by over expressing NFκB p65/RelA as suggested by luciferase assays (**Fig. 9A–C**), this indicates the involvement of other mechanisms in the process.

Such prediction in the context of transcriptional cross-talk between p53 and NFκB p65/RelA by models were previously described by Schneider et al.[39] and illustrated in **Figure 10**. The upstream region bearing both p53 and NFκB p65/RelA binding sites through which the miRNAs can be co-regulated are indicated by region C (−3493 to −3878) in the upstream of miR-100 (**Fig. S1 A**), region B1 (−17583 to −17135) in the upstream of miR-146a (**Fig. S1 B**) and region C (−2688 to −3093) in the upstream of miR-150 (**Fig. S1 C**). p53 mediated repression of miR-100, −146a and −150 could be due to binding of p53 at these sites which could (i) lead to exclusion of NFκB p65/RelA from the nearly overlapping NFκB p65/RelA

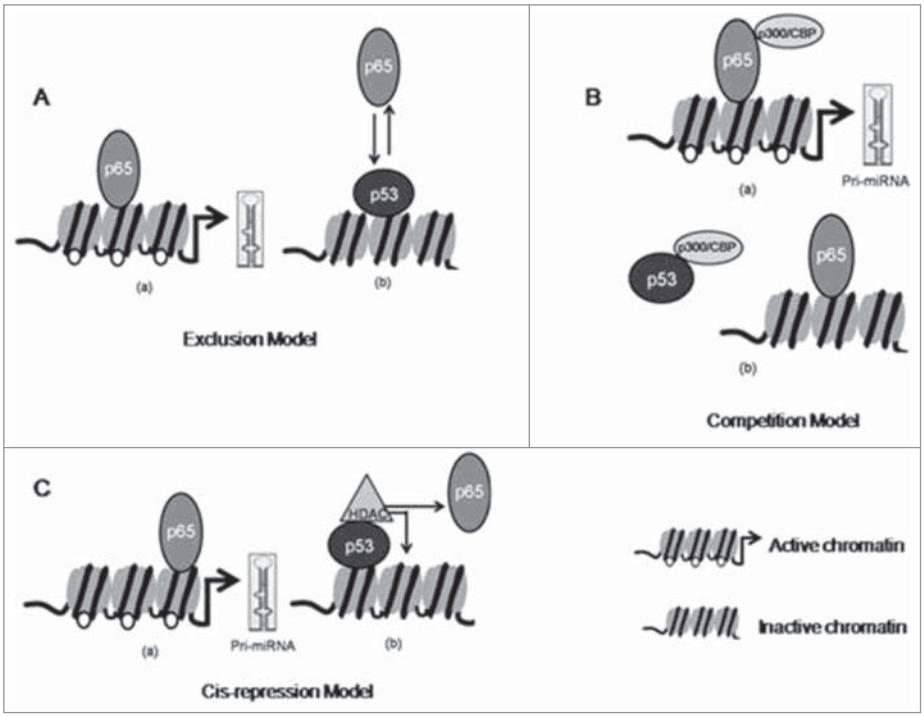

Figure 10. Probable mechanism of cross-talk between p53 and NFκB p65/RelA (p65) in regulating miRNA expression Cross-talk between p53 and p65 in regulating miRNA expression where p53 represses p65 transactivation by (**A**) Exclusion Model in which elevated p63 displaces p65 from overlapping or nearby DNA binding sites; (**B**) Competition Model in which elevated p53 competes with p65 for p300/CBP binding; (**C**) Cis-repression Model in which p53 binds with DNA and recruits HDAC and suppresses expression of genes.

binding site (*Exclusion model*) or (ii) competition with NFκB p65/RelA for limited pool of co-activator CBP (*Competition model*) thereby causing such repression or (iii) facilitate recruitment of other factors necessary for repression (*Cis-repression Model*) or (iv) by more than one of these mechanisms.

Thus, the co-regulation of miR-100 at region C (**Fig. 9A**) by p53 and NFκB p65/RelA can be partly explained by *Exclusion model* (**Fig. 10A**) where elevated p53 in p53 transfected cells might have prevented binding of low endogenous p65 at the closely placed or overlapping site thereby reducing miR-100 transcription as evident in **Figure 9D**. Besides, co-regulation of miR-100 at region C by p53 and NFκB p65/RelA can also be explained by *Cis-repression model* (**Fig. 10C**) where acetylated Histone H3 at the regulatory region C (**Fig. S1A**) might be reduced by the recruitment of HDAC by p53. Moreover, NFκB p65/RelA itself functions as a transcriptional activator or as a repressor by interacting with HAT and HDAC respectively.[54] Hence increased NFκB p65/RelA association with HDAC-containing co-repressor complexes might have also contributed to reduced miR-100 expression. Presently, we are investigating whether such *Cis-repression Model* may also explain the mechanism of co-regulation of miR-100 by p53 and NFκB p65/RelA. Similarly, the co-regulation of miR-146a at region B1 (**Fig. 9B**) can be explained by the *Exclusion model* or by Cis-repression model as evident in **Supplementary Fig. 1B**. Additionally, the presence of p300/CBP binding site at region E in the upstream of miR-100 (**Fig. S1A**) and near Region B1 in the upstream of miR-146a (**Fig. S1B**) may include the possibility of co-regulation of miR-100 and −146a by *Competition model* (**Fig. 10B**) as well. Similarly, the co-regulation of miR-150 at region C (**Fig. 9C**) by p53 and NFκB p65/RelA could be explained by *Cis-repression model* (**Fig. 10**) as evident in **Supplementary Figure 1C**. Although region A in the upstream sequence of miR-125b did not bear a putative p53 binding site (**Fig. S1D**) yet miR-125b could be regulated by p53 at this site through non-specific DNA binding following *Cis-repression Model* or by inhibiting NFκB p65/RelA transactivation following *Competition Model*. Although not verified by us, the presence of putative p53 binding site in close proximity with functional NFκB p65/RelA binding site immediately adjacent to Region A of miR-221 upstream sequence (**Fig. S1E**) may also explain for the observed repression in miR-221 expression by p53 following the *Exclusion Model* discussed or it may also occur following *Cis-repression model* at region A. Further extensive studies would be required to validate our prediction.

Thus results obtained in the present work demonstrate the existence of a functional cross-talk between p53 and NFκB p65/RelA in co-regulating miRNA expression. In fact, several instances of cross-talks between p53 and NFκB p65/RelA had been previously reported where one TF was observed to enhance or repress the activity of the other. Depending upon cellular needs, p53 may either repress transcription from NFκB p65/RelA gene promoter,[3] inhibit NFκB p65/RelA activity,[36–38,55,56] or may enhance the transcriptional activity of p65.[57,58] NFκB p65/RelA on the other hand may either increase the expression of p53[59,60] or may inhibit p53 transactivation.[37,61,62] There are even instances of transcriptional co-operation between these 2 TFs in the co-regulation of miR-224 expression in mouse ovarian granulosa cells.[20] Besides, there has been a report where p53 has been observed to act as a co-factor binding to RelA/p65 and inducing miR-21 expression in human heart failure.[19] Very recently p53 and NFκB p65/RelA have been observed to play significant role in head and neck squamous cell carcinoma (HNSCC) by regulating the expression of miR-21 and −34a/c thereby modulating downstream target genes.[21] This discrepancy in the functional relationship between p53 and NFκB p65/RelA may be attributed to different cell types and methods for activating/inactivating p53 and NFκB p65/RelA used by different investigators in their studies.

The importance of the present findings lies in the fact that the pro-apoptotic effect of p53 and the pro-survival functions of NFκB can be largely mediated via the biological roles of the miRNAs these TFs regulate. In general, p53 induced miRNAs viz., miR−34, −200, −15/16 and −192/194/215 families, −145 and −107 are mediators of tumor suppression and stress responses thereby executing these well characterized functions of p53.[30] Upon induction, these miRNAs target genes affecting important biological processes such as metabolism, cell cycle progression, migration, epithelial–mesenchymal transition, stemness, differentiation and cell survival. NFκB p65/RelA regulated miRNAs on the other hand generally target genes critical to innate and adaptive immunity, cell proliferation, inflammation, and tumor development thereby largely mediating the biological functions of the TF.[63] NFκB p65/RelA directly binds and induces the transcription of miR-146a, −125b, −9, −155, −21, −221, −222 among others while suppresses the expression of miR-199a/214 cluster.[27] The 2 TFs p53, NFκB p65/RelA and miRNAs are involved in several TF-miRNA loops. The two master TFs p53 and NFκB p65/RelA are also known to influence the transcriptional activities of one another depending on cellular context. The combinatorial effect of the extensive physical and functional cross-talks that exist between p53 and NFκB p65/RelA has been observed to define the outcome of several biological processes. This work reports for the first time the existence of a functional cross-talk between p53 and NFκB p65/RelA that resulted in regulation of miR-100, −146a and −150 expressions in diverse cell types and diverse species.

Materials and Methods

Mouse striatal STHdh^{Q7}/Hdh^{Q7} cells and human cervical carcinoma HeLa cells

Immortalized striatal STHdh^{Q7}/Hdh^{Q7} cells were established from wild type (Q7/7) Hdh knock-in mice which express full-length Huntingtin gene (*HTT*) with 7 Glu (Q) residues.[64] These STHdh^{Q7}/Hdh^{Q7} cells are primarily used as controls in experiments where STHdh^{Q111}/Hdh^{Q111} cells expressing endogenous full-length mutant *HTT* gene with 111 Glu (Q) residues are extensively used as cell model of Huntington's disease (HD) for identifying molecular alterations in the disease pathogenesis. Prof. Marcy E. MacDonald of Massachusetts General Hospital,

USA kindly donated us these cell lines. The human cervical carcinoma cell line i.e., HeLa cells were obtained from National Cell Science Center, Pune, India.

Cell culture and transfection

Immortalized striatal ST*Hdh*^{Q7}/*Hdh*^{Q7} cells were cultured in DMEM (Cat No. AT006, HiMedia) supplemented with 10% (v/v) heat inactivated FBS (Cat No. S1810–500, Biowest), antibiotics penicillin/streptomycin PS 1% (v/v) and 400 μg/ml G418 (Cat No. sc-29065A, Santa Cruz Biotechnology) at 33°C in humidified condition and 5% CO_2. Human cervical carcinoma cells (HeLa) were cultured in MEM (Cat No. AT020, HiMedia), 10% (v/v) FBS (Cat No. S1810–500, Biowest), 1% (v/v) PS at 37°C in humidified condition and 5% CO_2.

All transfections were carried on 70–80% confluent cells using Lipofectamine 2000 (Cat No. 11668–019, Invitrogen) as per manufacturer's protocol. Unless otherwise mentioned, for single transfection experiment 1 μg (30 mm plate), 2.5 μg (60 mm plate) or 5 μg (100 mm plate) i.e. amounting to 0.25 nM of plasmid DNA constructs as well as 5 μl, 10 μl or 15 μl of Lipofectamine 2000 respectively were used. Transfection efficiency was normalized by co-transfecting cells with GFP-C1 and counting and determining the percentage of GFP positive cells under the microscope.[22] For knocking down p53, cells were transfected with 2.5 μg (60 mm plate) of p53 siRNA construct (Cat No. IMG-701, Imgenex).[25] For inhibiting basal NFκB p65/RelA activity, cells were treated with 2 mM aspirin (Cat No. 027039, Central Drug House Laboratory Reagent)[26] or with 15 μM SN50 (Cat No. 481480–1MG, Millipore).[29] The transfected cells were harvested at definite time intervals and treated in accordance with experimental requirement.

Identification of putative p53 and NFκB p65/RelA (p65) binding site(s) in the upstream sequences of miRNA genes

The genomic co-ordinates of the miRNAs were identified from miRBase (http://www.mirbase.org/).[65] Next, 20 kb upstream sequences of the pre-miRNAs (1st nucleotide of the pre-miRNA taken as +1) were downloaded from UCSC Genome Browser (http://genome.ucsc.edu)[66] for both human and mouse. The putative p53 and NFκB p65/RelA binding sites (BS) at miRNA upstream sequences were analyzed by using the in-house search tool www.bioinformatics.org/grn/npb3 as described earlier.[31] The detailed procedure has been described in **Supplementary Text** and the results obtained in **Supplementary Table S1**. Presence of predicted regulatory regions based on ENCODE annotation data in the upstream sequences of the human miRNA genes were also evident from UCSC Genome Browser.[67] The corresponding conserved regions in upstream of mouse miRNA genes were identified by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

DNA constructs

For overexpression studies, full-length human *p53* cDNA was obtained by PCR from human brain cDNA library and cloned into CFP vector using *BamH1* (Cat No. R0136L, NEB) and *Sal1* sites (Cat No. R3138L, NEB). The primers used for cloning were p53_CFP_F: 5′-ACGCGTCGACGTGGAGCCGCAGTCAGATCCTA-3′ and p53_CFP_R: 5′-CGCGGATCCCAGTCTGAGTCAGGCCCTTC-3′. Full-length p65 subunit of NFkB (RelA) i.e., NFκB p65/RelA cloned into pLG3 vector was obtained as a kind gift from Dr. Susanta Roychoudhury, IICB, Kolkata. For knocking down endogenous *p53*, pSuppressorNeo p53 plasmid DNA containing p53 siRNA construct (Cat No. IMG-701, Imgenex) was used and described earlier.[25]

For functional assay of NFκB p65/RelA promoter, the plasmid NFκB luciferase (p65-RE), containing multiple copies of NFκB response elements in pGL3 vector was obtained as a kind gift from Prof. Susanta Roychoudhury, IICB, Kolkata. For promoter luciferase assays of the regulatory sequences of miRNA genes, we cloned upstream sequence regions of miR-100, −125b, −146a, −150 and −221 bearing one or more putative p53 and NFκB p65/RelA binding sites into pGL3 Basic vector (Cat No. E1751, Promega). These regions were amplified by PCR from human genomic DNA and cloned in vector using the *Mlu I* (Cat No. R0198L, NEB) and *Hind III* (Cat No. R0104L, NEB) sites. The constructs were named accordingly to indicate different regions of the upstream sequences of the different miRNA genes they harbor. The primers used to generate the upstream sequence constructs were as follow:

RegA_100_F:CGACGCGTCATGTGTGTTTCCCAGCATC and RegA_100_R: CCCAAGCTT CCCCGCAATACTGTTTGAA; RegB_100_F: CGACGCGTTGTGTTCTGAATTCCTGGAA-GA and RegB_100_R: CCCAAGCTTGAATCCGTGGGCTCTCTTAG; RegC_100_F: CGAC-GCGTAGCCCTATCAGCCGAGAGAA and RegC_100_R: CCCAAGC TTGAAAA ACATC -GGGAACCATT; RegB1_146a_F: CGACGCGTAAAAGCCAACAGGCTCATTG and RegB1_146a_R: CCCAAGCTTGGGGTAGAGGAAGGCAGCTA; RegB_150_F: CGACGC GTCTAT- GGGCCTTTCTCCAAC and RegB_150_R: CCCAAGCTTGCCCTATTGGGAGGATCAAT; RegC_150_F: CGACGCGTCACCGCTACACATCTGGCTA and RegC_150_R: CCCAAGCT- TTGTTTTAG CATGCCTCTGGA; RegA_125b_F: CGACGCGTTCATCTTCCCATCTGCCT and RegA_125b_R: CCCAAGCTTCTGCGGATTCTTTGAAGC; RegB_125b_F: CGACGCG- TG GGCAAAGTAGTGAGACCCT and RegB_125b_R: CCCAAGCTTTTCCAATCCA GGT- CAGCACT; RegB_221_F: CGACGCGTTGCATAGTCATTTACCTTCTAGAATAA and RegB_221_R: CCCAAGCTTCCTTGCACTCACGGAAGTTT

For control vectors in various transfections pEGFP-C1 (Cat No. 6084–1, Clontech), pDsRed-C1 (Cat No. 632466, Clontech), pRNAU61.Hygro (Cat No. SD1202, Genescript), pGL3 Basic vector (Cat No. E1751, Promega) and pcDNA3.1 (Cat No. V790–20, Invitrogen) were used.

RNA preparation

Total RNA was prepared from cultured cells using TriZol Reagent (Cat No. 15596–026, Invitrogen) according to

manufacturer's protocol. RNA samples were quantitated using Biophotometer (Eppendorf, Germany).

Quantitative real time PCR for amplification of mature miRNAs and their analysis

For amplification of mature miRNA sequences, cDNA was prepared following standard procedure[68] 100 ng of total RNA using Taqman miRNA specific stem-loop primers (Applied Biosystems (AB), USA) or stem loop primers designed as per described protocol,[69] Mulv-Reverse transcriptase (Cat No. EP0442, Fermentas) and dNTPs (Cat No. R1121, Fermentas). cDNA was then accordingly subjected to either Taqman Quantitative Real Time PCR which detects only mature miRNAs[69] using Taqman PCR master mix (Cat No. 430447, Applied Biosystems) or quantitative real time PCR using SYBR Green master mix (Catalog Number 4309155, Applied Biosystems) with miRNA specific forward primer and universal reverse primer. The sequences of miRNA stem loop specific primers for cDNA preparation, miRNA specific forward primers and universal reverse primer for quantitative real time PCR using SYBR Green have been given in **Supplementary Table S2**. Real time PCR was carried on 7500 Real time PCR system (AB, USA). The small RNA U6 snRNA detected in both human and mouse were used as endogenous control to calculate fold change. The fold changes were calculated as per SDS software (AB, USA).

Semi-quantitative RT-PCR for amplication of pri-miRNAs and their analysis

For detection of primary miRNA expression levels 1 μg of total RNA was subjected to DNase treatment (Sigma) followed by cDNA preparation using random hexamer primer (Fermentas), dNTPs and MuLv- Reverse transcriptase (Fermentas). Semi-quantitative RT-PCR was next performed with the help of Bioline Taq (AB, USA) using primers designed to amplify region of the primary transcripts of the respective miRNAs. The primers were designed by Primer3 software and the sequences are given in **Supplementary Table S3**. Yield of the PCR products was estimated from the integrated optical density (IOD) using Image Master VDS software (Amersham Bioscience, UK) and mRNA expression levels were normalized relative to those of GAPDH.

Luciferase assay

For promoter luciferase assay of NFκB p65/RelA response element (p65-RE) and cloned upstream sequence constructs of miRNA genes, 1×10^5 HeLa cells or 1.5×10^5 ST*Hdh*^{Q7}/*Hdh*^{Q7} cells were plated a day before transfection per well in 6-well plates. The following day, 1 μg of p65-RE or upstream sequence constructs cloned into pGL3 vector were separately transfected either in presence or absence of pCMV-p53 or NFκB p65/RelA. Cells transfected with empty vector pGL3 were taken as control. Cells were collected 24 h post transfection and luciferase assays were performed (Sirius Luminometer, Berthold detection systems, USA) according to the manufacturer's protocol using Luciferase Reporter assay system (Cat No. E1500, Promega). Three μg of total protein was taken for each assay. Transfection efficiency was normalized by co-transfecting cells with GFP-C1 and measuring the Fluoresence at 510 nm (Fluoromax-3, Jobin Yvon Horiba, USA).[22] The experiments were carried out in triplicate. For inhibiting p65-RE activity, cells were treated with 2 mM aspirin 24 hours prior to transfection and for knocking down endogenous *p53*, cells were transfected with 1 μg of p53 siRNA construct. Luciferase assay was performed in each case following the same procedure as discussed above. For measuring relative p53 and NFκB p65/RelA protein levels for luciferase assays 2.5 μg each of the plasmids was transfected either in isolation or in combination in 3×10^5 ST*Hdh*^{Q7}/*Hdh*^{Q7} cells in 60 mm plates. Immunoblot analysis with cell extracts revealed the protein levels.

Sub-cellular fractionation, immunoprecipitation and protein gel blot analysis

The methods used for sub-cellular fractionation, immunoprecipitation and western blot analysis were similar to those which have been published.[70] Beta actin and lamin B were used as loading controls for proteins in cytoplasmic and nuclear extracts respectively. Calculations for nuclear protein contents were done normalized to nuclear Lamin B level.[71] The various antibodies used for immunoprecipitation include anti P53 (Cat No. IMG-80061, Imgenex, USA) at 1:100 dilution, anti P65 (Cat No. MAB3026, Chemicon, USA) at 1:100 dilution and anti CBP (Cat No. sc-369, Santa Cruz Biotechnology, USA) at 1:100 dilution. For protein gel blot primary antibodies used were anti β actin (Cat No. A2228, Sigma Chemicals, USA) at 1:8000 dilution, anti Lamin B (Cat No. Ab8983, Abcam, USA) at 1:5000 dilution, Anti P53 (Cat No. IMG-80061, Imgenex, USA) at 1:4000 dilution, anti P65 (Cat No. MAB3026, Chemicon, USA) at 1:4000 dilution and anti CBP (Cat No. sc-369, Santa Cruz Biotechnology, USA) at 1:200 dilution. The various secondary antibodies used were Goat Anti-mouse IgG-HRP (Cat No. 105502, GENEI, India) and Goat anti-rabbit IgG-HRP (Cat No. 105499, GENEI, India) each at 1:8000 dilution. Integrated optical density (IOD) of each nuclear band compared to nuclear Lamin B was measured using Image Master VDS software (Amersham Biosciences, UK).

Chromatin immunoprecipitation and measure of promoter occupancy

Method used for ChIP experiments was described earlier.[70] For each experiment, 200 μg of isolated chromatin was immunoprecipitated with 10 μg of anti p53 or anti p65 antibody. followed by PCR amplification to detect for the presence or absence of specific upstream sequence elements of miRNA genes bearing putative p53 or NFκB p65/RelA binding sites. PCR analysis with primers targeted to a region of *GAPDH* promoter[32] and to a region on chromosome 1 (Chr1: 204,366,822–204,366,872)[6] were taken as negative control in ChIP performed with anti p53 antibody and anti-p65 antibody respectively. The various antibodies used for immunoprecipitation include Anti P53 (Cat No. IMG-80061, Imgenex, USA) at 1:20 dilution and Anti P65 (Cat No. MAB3026, Chemicon, USA) at 1:20 dilution. The various primers used in ChIP with their sequences and purpose of use are given in **Supplementary Table S4**.

Statistical analysis

All the experiments were performed at least 3 times. Statistical analysis was done with the help of on-line software Graphpad QuickCalcs, (http://www.graphpad.com/quickcalcs/index.cfm). All data were reported into 2 groups, one for control and the other for experimental. The values of mean, standard deviation (SD) and exact number of independent experiments performed (n) for each group were provided. Student's unpaired 2-tailed -t-test was then performed by the software to compare between the means of control and experimental groups to determine their statistical significance. Error bars represent standard deviation, */† represents statistical significance (*$P \leq 0.05$; ** $P \leq 0.01$) or († $P \leq 0.05$; †† $P \leq 0.01$).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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