The "LINC" in between Δ40p53-miRNA axis in the regulation of cellular homeostasis

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ABSTRACT

Recently, we showed that Δ40p53, the translational isoform of p53, can inhibit cell growth independently of p53 by regulating microRNAs. Here, we explored the role of Δ40p53 in regulating the lncRNA-miRNA-cellular process axis and specifically focused on LINC00176 for further exploration. Interestingly, LINC00176 levels were affected predominantly by the overexpression/stress-mediated induction and knockdown of Δ40p53 compared to p53 levels. Additional assays revealed that Δ40p53 alone (and not p53) transactivates LINC00176 transcriptionally; however, both the proteins can directly bind to its RNA. Pulling down the RNA revealed that LINC00176 sequesters several putative miRNA targets, which could further titrate several mRNA targets involved in different cellular processes. Therefore, to understand the downstream effect of the regulation, we ectopically overexpressed and knocked down LINC00176 in HCT116 p53-/- (harboring only Δ40p53) cells which affected proliferation, cell viability, and the expression of epithelial markers. Our results provide essential insights into the pivotal role of Δ40p53 in regulating the novel LINC00176 RNA-miRNA-mRNA axis independent of FL-p53 and maintaining cellular homeostasis.

Keywords: p53 isoform; Δ40p53; LINC00176; miRNA; cellular effect of Δ40p53

INTRODUCTION

The tumor suppressor p53 is a master transcriptional regulator that regulates several critical events during cell fate determination, including transcriptional regulation of multiple genes involved in cell survival, cell cycle regulation, apoptosis, and metabolism (1). p53 has 12 isoforms created by alternate promoters, splicing sites, and translation initiation sites. These different isoforms have unique functions and can modulate p53 activity (2). The Δ40p53 isoform is the only translational isoform of p53. Our laboratory has shown that translation initiation from the first IRES (Internal Ribosomal Entry Site) of *TP53* generates full-length p53 protein, whereas translation initiation from the second IRES generates Δ40p53 (3). Various ITAFs (IRES *trans* acting factors) are required for the IRES-mediated translation of the two isoforms under different stress conditions (4-6). We have also shown how a protein and miRNA crosstalk at the TP53 3′-UTR influences differential expression of p53 and Δ40p53 (7).

These two isoforms have a robust affinity toward each other as both contain the oligomerization domain and can therefore form homo- and hetero-tetramers (8). Δ40p53 can both positively and negatively regulate p53 activity: it can alter the recruitment of p53 co-activators, increasing its activity (9), but it can negatively regulate the transactivation of p53 target genes, such as p21 (10). Δ40p53 also has p53-independent functions. It controls the switch from pluripotency to differentiation in somatic cells and helps maintain pluripotency in embryonic stem cells. It suppresses the Nanog and IGF-receptor and maintains pluripotency in embryonic stem cells (11). It also regulates proliferation and glucose homeostasis in mice (12).

Moreover, it uniquely regulates apoptosis-related genes such as p53BP2 and TIAL1(13) and can also independently induce senescence (14). In certain cancers, full-length p53 is insufficient to protect against tumorigenesis, and Δ40p53 is essential for mediating apoptosis through the selective activation of specific target genes (15). Recent observations also suggest a role for Δ40p53 as a tumor suppressor in hepatocellular carcinoma (16). Our latest studies showed that Δ40p53 can regulate miR-186-5p independent of full length p53 (17). However, some studies have examined the functional role of Δ40p53; there has been little detailed investigation regarding intermediate molecules (such as RNAs or proteins) through which Δ40p53 exerts its influence on cells.

Some RNAs in the p53 response pathway are involved in tumor suppression (18). Among the cellular pool of RNAs, lncRNAs (long non-coding RNAs) have gained attention in cancer because of their transactivation functions. LncRNAs are a heterogeneous class of non-coding RNAs >200 nucleotides in length with very low or no coding potential. Several lncRNAs crosstalk with p53, thereby regulating cellular processes including cell proliferation, differentiation and development, chromosomal imprinting, and genomic stability (19-21). LncRNAs also function as competitive endogenous RNAs (ceRNAs), which sequester miRNAs and alter the expression of their downstream target genes (mRNAs) by competing for their common binding regions. ceRNA network links the function of protein-encoding mRNAs to the function of non-coding RNAs. Growing evidence indicates that such networks formed by lncRNA/miRNA/mRNA interactions have implications in broad spectrum of biological processes including disease conditions like carcinogenesis (22-25). In cancer cells, several aberrantly expressed long non-coding RNAs (lncRNAs) with miRNA responsive element (MREs) competitively sequestrate miRNAs and impairs the interaction between miRNA and mRNA, thereby attenuating the repression on the downstream mRNAs (22). Thus, this emerging network is crucial for diverse cellular processes like cancer (26,27).

In this study, we focused on the regulation of long non-coding RNAs by Δ40p53 and their impact on ceRNA network. To understand the potential role of Δ40p53-mediated regulation of lncRNAs, we investigated a preliminary screen (unpublished observation) in our laboratory in H1299 cells (a p53-null cell line) with overexpression of full-length p53, Δ40p53, or both isoforms. Based on the results obtained from our preliminary experiments and literature survey, we decided to focus on LINC00176 and its differential regulation by p53 isoforms. LINC00176 is a comparatively novel, non-coding RNA reported in few cancer conditions, but it is pretty underexplored in literature. Recent studies have demonstrated that LINC00176 regulates the expression of microRNAs in hepatocellular carcinoma (28) and promotes ovarian cancer progression by downregulating ceruloplasmin (29). By contrast, a report on genome-scale CRISPR screening has emphasized its possible role as a tumor suppressor lncRNA (30). From the above reports, it is clear that LINC00176 is regulated through several pathways, possibly involving different upstream regulators and downstream targets. Therefore, in this study, we explored the role of Δ40p53/p53-LINC00176 axis in microRNA-mediated regulation of mRNAs and their effect on cell growth and proliferation.

MATERIALS AND METHODS

Bioinformatic Analysis

GEPIA (http://gepia.cancer-pku.cn/detail.php?gene=LINC00176) was used for analysing differential LINC00176 levels in cancer. LINC00176 was queried in the database and the results were generated by selecting Cancer versus Normal Analysis (31). starBase v2.0 database (https://starbase.sysu.edu.cn/starbase2/browseNcRNA.php) was used to identify potential miRNA targets of LINC00176 (32). catRAPID fragments software was used to predict possible protein binding regions in LINC00176 (http://service.tartaglialab.com/page/catrapid_group_old), and the interaction score between LINC00176 and the Δ40p53 was predicted by RPISeq software (http://pridb.gdcb.iastate.edu/RPISeq/results.php)(33). PROMOALGEN has been used to predict transcription factor binding site in *LINC00176* gene (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). miRTarBase has been used to identify mRNA targets of miRNAs (https://mirtarbase.cuh_k.edu.cn/~_miRTarBase/_miRTarBase_2022/php/index.php)(34). dbDEMC2.0 has been used to perform a meta profiling of selected miRNAs (https://www.biosino.org/dbDEMC/help#help00) (35).

Cell lines and transfections

We have used three cell lines in the current study: H1299 (lung adenocarcinoma cell line lacking p53/Δ40p53), HCT116 p53+/+ cells (colon carcinoma cell line harboring wild-type p53; hereafter called HCT116+/+); and HCT116 p53-/- cells (colon carcinoma cell line harboring only Δ40p53; hereafter called HCT116-/-). HCT116-/- is a commercially available cell line, with a deletion in the first ATG region of p53 mRNA, therefore the only protein getting expressed from the mRNA is Δ40p53 (being the only translational isoform) from the second ATG. These cells were maintained in DMEM (Sigma) with 10% Foetal Bovine Serum (GIBCO, Invitrogen). 70%-80% confluent monolayer of cells was transfected with various plasmid constructs using Lipofectamine 2000 (Invitrogen) and Turbofectamine in Opti-MEM (Invitrogen). The medium was replaced with DMEM (with antibiotic) and 10% FBS 4 h later. The cells were harvested and processed as required at the desired time point. For the stress-induced experiments, the cells were treated with doxorubicin (2 µM) for 16 h, Thapsigargin (0.1 µM, 1 µM) for 16 h, DMEM minus Glucose medium for 8 h. For stability experiments cells were transfected with siRNA directed to Δ40p53, post 40 h of transfection cells were treated with Actinomycin D (1 mg/ml) and cells were harvested at 0 h, 4 h and 8 h post treatment.

Plasmids and constructs

pGFP-hp-p53- 5'UTR-cDNA (14A): It expresses both p53FL and Δ40p53 (a generous gift from Dr. Robin Fahraeus, INSERM, France). pGFP-hp-p53-5'UTR (A135T)-cDNA which expresses only Δ40p53 and pGFP-hp-p53- 5'UTR (A251G/T252 C/G253T)-cDNA which expresses only full-length p53(p53FL) (17). Two constructs were used for Tagged RNA Affinity Purification: Plasmid pMS2 and pMS2-GST. (Both the constructs were a generous gift from Je-Hyun Yoon and Myriam Gorospe Laboratory of Genetics and Genomics, National Institutes of Health, Baltimore, MD 21224, USA) (36,37). LINC00176 (3495–4302) construct obtained in the study (a generous gift from Dr. T Tamura Institut fuer Biochemie, Hannover, Germany) has been used to generate the overexpression construct. LINC00176 overexpression construct was generated by cloning the insert into pMS2 plasmid between HindIII and EcoRI sites. 5’CCGGTGTCGTCTTGCAAACATTAAACTCGAGTTTAATGTTTGCAAGACGAC ATTTTTG-3’ sequence of shRNA was used to target LINC00176.

siRNA transfections

HCT116+/+ and HCT116-/- cells were transfected with 30 nM si p53 RNA (IDT). 5'-AACCUCUUGGUGAACCUUAGUACCU-3' is the p53 siRNA sequence directed against the 3'UTR of p53; therefore, it targets both p53 as well as Δ40p53. A nonspecific siRNA (si Nsp) was used in the experiments as control (Dharmacon). si-LINC00176 (5'-CUCGUUCUGUAGACUUGUU-3') was used for partial silencing of LINC00176 (a generous gift from Dr. T Tamura, Institut fuer Biochemie, Hannover, Germany) (28).

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 (Bio-Rad). Samples were then analyzed by Western blotting using rabbit-raised anti-p53 polyclonal antibody (CM1, 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-β-actin antibody (Sigma) was used as a control for equal loading of total cell extracts. 1801 antibody (Santa Cruz Biotechnology, Cat. No. SC-98) was used for pulldown of p53 and Δ40p53 in the Immunoprecipitation experiments. E-cadherin antibody (Santa Cruz Biotechnology, Cat. No. SC-8426), Slug antibody (Santa Cruz Biotechnology, Cat. No. SC-166476), Bip antibody (Abclonal, Cat. No. A4908) and GST Antibody (B-14) (Santa Cruz Biotechnology, Cat. No. SC-138) was used to detect E-cadherin, Slug, Bip and GST proteins respectively.

RNA isolation and Real time PCR

Total RNA was isolated from cells with TRI Reagent ™ (Sigma), according to manufacturer's protocol. The isolated RNA was treated with 10 units of DNase I (Promega), extracted with acidic phenol-chloroform, and precipitated with 3 M sodium acetate (pH 5.2) and 2.5 volumes of absoluteethanol. RNA amount was quantified in Nano-spectrophotometer and cDNAs were synthesized using specific reverse primers and MMLV RT (Revertaid ™ Thermo Scientific) at 42°C for 1 h, according to standard protocol. While 2-5 µg of total RNA has been used for checking the expression of mRNAs and lncRNAs, 50 ng of total RNA has been used to check the expression of miRNAs.

SYBR green Assay System was used for mRNA, lncRNA and miRNA detection and quantification. We have used actin as endogenous control for mRNA, lncRNA and 5SrRNA

as endogenous control for miRNAs. The thermocycling conditions for SYBR green Assay system include 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 30 s and 72°C for 30 s (Applied Biosystems). $2^{-\Delta\Delta Ct}$ method algorithm was used to analyse the relative expression changes, where actin/5S served as an endogenous control. The fold change was calculated using $2^{-\Delta\Delta Ct}$. ΔCt = Ct (target gene) −Ct (endogenous control) and ΔΔCt = ΔCt target sample) −ΔCt (control sample). Melting curve analysis of every (q)PCR was conducted after each cycle.

Chromatin Immunoprecipitation

ChIP was performed with Human colon carcinoma cell lines (HCT116+/+ and HCT116-/-). Five 60 mm dishes were seeded with 0.399×10^6 cells (each) and was allowed to grow for 48 h. The cells were harvested for the processing using Sim-pleChIP® Plus Enzymatic Chromatin from Cell Signalling Technologies as per the manufacturer's protocol. Briefly, cells were fixed using formaldehyde for 10 minutes at room temperature. The reaction was stopped using 1X Glycine. The cells were then collected in ice cold 1X PBS (Phosphate Buffer Saline). The cells were lysed in the lysis buffers provided in the kit, digested with Micrococcal Nuclease and processed (sonicated) to acquire nuclear chromatin lysate according to the protocol. The chromatin is then incubated with specific antibodies in the cyclomixer overnight at 4˚C. Pulldown was performed using p53 or Δ40p53 specific antibody. Magnetic protein-G beads supplied in the kit where then added to the antibody bound lysate mixture and incubated in cyclomixer for 2 h at 4˚C. The beads were then separated using magnetic separator and washed with the wash buffer provided in the kit. Before the elution step, 20% of the beads were kept for western blot analysis to confirm equal pulldown of the protein. Elution was carried out in the elution buffer provided in the kit at 55˚C for 30 minutes. This was followed by Proteinase K and 1% SDS treatment for 30minutes-2 h followed by phenol-chloroform based DNA ex-traction. The DNA was precipitated using 1/10th volume of 3M Sodium Acetate (pH 5.2) and 2.5 volume absolute ethanol at -20˚C overnight. The required DNA was used for ChIP sequencing after confirming the pulldown through western.

Protein purification

The recombinant p53 and Δ40p53 proteins were purified from pET28a-p53, pET28aΔ40p53 constructs, respectively. The expression of both the recombinant proteins were induced with 0.6mM isopropyl-1-thio-β-d-galactopyranoside (IPTG) at an optical density of 0.6 at 660 nm and grown for another 16 h at 16˚C. The cells were pelleted, resuspended in lysis

buffer (50mM Tris [pH 8.0], 300 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride [PMSF] and disrupted on ice by sonication. All subsequent steps were carried out at 4°C. The lysates were cleared by centrifugation at 10,000 rpm for 30 min and incubated with a Ni-nitrilotriacetic acid (Ni-NTA)-agarose slurry (Qiagen) with rocking for 4 h at 4°C. The lysate was loaded onto a column, and the flowthrough was discarded. The column was washed with 50 ml of wash buffer (lysis buffer containing 40 mM imidazole). The bound protein was eluted with 500 µl of elution buffer containing 500 mM imidazole. The eluted protein fractions were dialyzed at 4°C for 3 to 5 h in dialysis buffer (50 mM Tris [pH 8.0], 100 mM KCl, 5 mM β-mercaptoethanol [β-ME], and 10% glycerol), aliquoted, and stored at −70°C.

UV Cross linking

α-^{32}P body-labeled RNA probes were mixed with purified recombinant protein for 30 min at 30°C in 1× RNA binding buffer (20 mM HEPES [pH 7.6], 100 mM KCl, 5 mM MgCl2, 10% glycerol, 0.2 mM EDTA, and 2 mM dithiothreitol [DTT]) and then UV irradiated (254 nm) for 20 min at 4°C. The mixture was treated with RNase A at 37°C to digest the unprotected RNAs. The complexes were separated on 10% SDS-PAGE gels and visualized by phosphor-imaging. For the competitive UV cross-linking assay, a molar excess of unlabeled RNAs, where indicated, was added along with the labeled RNA probe.

Tagged RNA Affinity Pull down Assay (TRAP Assay)

The method is based on the addition of MS2 RNA hairpin loops to a target RNA of interest, followed by co-expression of the MS2-tagged RNA together with the protein MS2 (which recognizes the MS2 RNA elements) fused to an affinity tag (37). After purification of the MS2 RNP complex, the miRNAs present in the complex are identified. In this study we have tagged the LINC00176 with MS2 hairpins and have co-expressed it in Human colon carcinoma cells (HCT116-/-) along with the chimeric protein MS2-GST (glutathione S-transferase). After affinity purification using glutathione SH beads, the microRNAs present in the RNP complex

Cell fractionation

Approximately 1×10^6 cells (HCT116-/-) were washed with ice cold PBS, resuspended in 175µl of Cytoplasmic lysis buffer (50mM Tris [pH 8.0], 140 mM NaCl, 1.5 mM $MgCl_2$ and 0.5% NP-40) substituted with RNase Inhibitor (Thermo Fisher) and incubated on ice for 5mins. Cytoplasmic fraction was separated as supernatant by centrifugation at 500g at

4°C/4mins. The remaining nuclear pellet was re-suspended in cold PBS and pelleted with high-speed centrifugation (5000rpm/1min). RNA was extracted from both the fractions using TRI Reagent TM (Sigma), according to manufacturer's protocol. The relative transcript levels were determined in each fraction by normalisation with their total levels.

MTT Assay

HCT116-/- cells were transfected with either LINC00176 o/e or sh-LINC00176 construct, 36 h post transfection these cells were re-seeded in 96 well plate. After 20 h of reseeding 10 µl of MTT (50 mg/ml) reagent was added and cells were incubated for 3-4 h, following which the supernatant was removed and cells were dissolved in 200µl of DMSO. Cell viability was measured as a spectrophotometry reading of the amount of MTT incorporated in the cell at 560 nm. The reading taken on the first day is considered as Day 0. Similarly, the reading was measured on Day 2 and Day 4 post re-seeding to check the effect on cell viability over a period of time.

BrdU cell proliferation assay

BrdU cell proliferation assay was performed as described in BrdU Cell Proliferation ELISA Kit (ab126556). Briefly, HCT116-/- cells were transfected with si-LINC00176. 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 12 h, followed by the protocol as mentioned in the manual.

Statistical analysis

All the experiments have been performed in three independent biological replicates (n=3). The data were expressed as mean ± SD. Statistical significance was determined using two-sided Student's t-test. The criterion for statistical significance was $p \leq 0.05$ (*) or $p \leq 0.01$ (**) or $p \leq 0.001$(***)

RESULTS

Selection and validation of LINC00176 for further studies

We identified LINC00176 based on a preliminary screen (unpublished data from our laboratory) of lncRNA expression in cells expressing different p53 isoforms. As mentioned earlier, distinctive functions of LINC00176 reported in the literature, we investigated its regulation by p53 isoforms. To directly investigate the role of p53 isoforms on LINC00176,

we examined the expression of LINC00176 in three cancer cell lines: H1299 cells with ectopic overexpression of p53, Δ40p53, or both (14A construct); HCT116+/+ and HCT116-/- cells. LINC00176 levels were first validated in H1299 cells after overexpressing p53, Δ40p53, or both p53 and Δ40p53. It was upregulated under all conditions compared to control cells **(Figure 1A, B)**. However, the fold change in LINC00176 levels was higher in cells expressing Δ40p53 alone compared to p53 alone or the combination of the two proteins. Since it would be more physiologically relevant to examine LINC00176 in a cell line with endogenous expression of p53, we also examined the levels of LINC00176 in HCT116+/+ and HCT116-/- cells. Interestingly, we found that the levels of LINC00176 were much higher in HCT116-/- cells than in HCT116+/+ cells **(Figure 1C, D)**. To confirm the ability of p53 to regulate LINC00176, an siRNA targeting to the 3′-UTR of *TP53* was transfected into HCT116+/+ and HCT116-/- cells **(Figure 1F, H)**. The levels of LINC00176 decreased in both cell lines after siRNA transfection **(Figure 1E, G)**. However, the decrease was not noteworthy because p53/Δ40p53 are not the only regulators of LINC00176. Nevertheless, silencing Δ40p53 in HCT116-/- cells **(Figure 1G)** led to a greater reduction in LINC00176 levels than silencing p53 in HCT116+/+ cells **(Figure 1E)**. To further examine whether both isoforms could modulate the levels of LINC00176, p53 was overexpressed in HCT116-/- cells **(Figure 1J)**, which reduced LINC00176 levels similar to those of 14A construct (expressing both p53 and Δ40p53) observed earlier **(Figure 1I)**. These preliminary results suggest that both p53 translational isoforms regulate LINC00176, but the levels differ with respect to isoform abundance. When p53 or Δ40p53 is present alone, either protein can upregulate LINC00176; however, there is higher expression of LINC00176 when only Δ40p53 is present.

To correlate the above findings with cancer scenario, we assessed the expression of LINC00176 in lung and colon cancer conditions via GEPIA (Gene Expression Profile Interactive Analysis) database. Box plot analysis suggested that the expression of LINC00176 was lower in tumor tissues (LUAD-Lung Adenocarcinoma and COAD-Colon Adenocarcinoma) than in normal tissues (**Figure S1**). We speculate that this observation could be due to the lower levels of functional WT Δ40p53/ WT p53 in cancer conditions.

Regulation of LINC00176 under different stress conditions

p53 and Δ40p53 are translated by an IRES-mediated mechanism under stress conditions, such as DNA damage, ER stress, and glucose starvation (4-6). Cells were treated with doxorubicin and thapsigargin for 16 h to induce DNA damage and ER stress, respectively, and cultured in

glucose-deprived DMEM for 8 h to induce glucose starvation **(Figure 2A)**. In HCT116-/- cells, Δ40p53 levels increased under all stress conditions **(Figure 2C**, **E, G).** LINC00176 levels also increased, with the highest increase observed in cells treated with thapsigargin **(Figure 2B, D, F)**. In HCT116+/+ cells, although p53 levels increased under all the stress conditions, LINC00176 levels were inconsistent and could not be correlated with p53 levels. It was slightly upregulated in the doxorubicin-treated and glucose-deprived cells; however, there was no significant change in cells treated with thapsigargin **(Figure S2 A-F)**. Moreover, it is unclear whether p53 or Δ40p53 was responsible for the increase in LINC00176 in HCT116+/+ cells as clearly visible in case of Doxorubicin induction (**Figure S2 B**) the levels of both the proteins increased. However, in line with our earlier observation of dominant regulation of LINC00176 by Δ40p53, the results in HCT116-/- cells were more significant and consistent.

Next, we wanted to confirm whether Δ40p53 alone was involved in regulating LINC00176 levels under stress or whether another factor could also regulate LINC00176. We selected ER stress as it maximally induced Δ40p53 in this model, and we observed significant upregulation of LINC00176 in cells treated with thapsigargin **(Figure 2D)**. We knocked down Δ40p53 in HCT116-/- cells, followed by induction of ER stress **(Figure 2H)**. Compared to control cells that were untreated and not transfected with siΔ40p53, there was no change in LINC00176 levels in the cells transfected with siΔ40p53 and treated with thapsigargin **(Figure 2I-J).** This indicated that in the absence of Δ40p53, LINC00176 levels are not induced in the background of ER stress, confirming Δ40p53 to be a major regulator of LINC00176. These results suggest that the expression of LINC00176 is closely linked to Δ40p53 levels. However, we cannot exclude the possibility that other factors might also be involved in regulating LINC00176 expression. We have also confirmed the ER stress induction by checking the ER chaperone Bip levels (38),which regulates the stress (**Figure S2 G**).

Mechanism of LINC00176 regulation by Δ40p53/p53

Next, we investigated whether LINC00176 is directly regulated by Δ40p53/p53 **(Figure 3A)**. Using PROMO ALGGEN, we found several Δ40p53/p53 binding sites in the *LINC00176* gene, which suggests that Δ40p53/p53 may regulate LINC00176 at the transcriptional level. An earlier study on LINC00176 revealed that it has three putative promoter regions in different cell types (28,30). We used ChIP experiments to examine the association of Δ40p53/p53 with these three probable promoter regions, one located at the proximal region of

LINC00176 exon 1 (62665399–62666196) and two located at the proximal region of E2 (62667411–62667655 and 62667984–62668233). We immunoprecipitated HCT116-/- and HCT116+/+ crosslinked lysates with 1801 antibody which can detect both Δ40p53 and p53 proteins **(Figure S3A, B).** The results showed an association between Δ40p53 and the *LINC00176* promoters in HCT116-/- cells. However, no such association was found for p53 in HCT116+/+ cells **(Figure 3B, C)**. Because we observed a decrease in LINC00176 levels upon p53 overexpression in HCT116-/- cells, we also examined the effect of ectopic p53 on the binding of Δ40p53 to *LINC00176* promoters in HCT116-/- cells. Interestingly, we found that the binding of Δ40p53 to all three *LINC00176* promoters decreased with p53 overexpression in HCT116-/- cells **(Figure 3D, E)**. This observation follows the reported literature that p53 and Δ40p53 regulate each other's activity by forming hetero-oligomers (4). Hence, these results show that Δ40p53 controls LINC00176 regulation more than p53.

We also wanted to assess the ability of Δ40p53/p53 to regulate LINC00176 at the RNA level directly. p53 is known to directly affect specific RNAs through its C-terminal domain (39) which is shared with Δ40p53. Therefore, we aimed to determine whether Δ40p53 could also regulate LINC00176 RNA directly or indirectly. Firstly, we checked whether Δ40p53 could affect LINC00176 RNA stability. We treated HCT116-/- cells with actinomycin D after transfection with siΔ40p53 to assess LINC00176 stability **(Figure 3F).** The results indicated that knockdown of Δ40p53 decreased LINC00176 levels after treatment with actinomycin D, implying that the stability of LINC00176 is increased by Δ40p53 **(Figure 3G, H)**. Based on this observation we speculated if Δ40p53 could directly bind to LINC00176 RNA. Moreover, since Δ40p53 shares its protein domains with p53, we also wanted to check if p53 could bind directly to LINC00176. CATRAPID and RPI-Seq software was used to identify potential interactions between mature LINC00176 and Δ40p53 and p53 **(Figure 3I-J, S3 C-D)**. Both the databases predicted probable binding regions and positive interaction score for LINC00176 and proteins.

We used a UV-crosslinking approach to validate the probable binding between LINC00176 and Δ40p53/p53. In this experiment, recombinant proteins were incubated with α-^{32}P labeled LINC00176, followed by UV crosslinking and RNase A treatment. Results reveal that both Δ40p53 and p53 can interact with LINC00176 RNA. We have also performed competition UV-crosslinking to confirm specific binding. Recombinant Δ40p53/p53 was incubated with α-^{32}P labeled LINC00176 and competed with self and non-specific non-labelled RNA. Self RNA competition with Δ40p53/p53 proteins suggested that the binding is specific **(Figure 3 K-L)**.

Interestingly, we observe more competition of LINC00176 RNA in binding to Δ40p53 than binding to p53. The results also reveal that p53 can regulate LINC00176 at the RNA level, which could be the possible reason for LINC00176 upregulation upon p53 overexpression in H1299 cells **(Figure 1A).** Moreover, we also performed cell fractionation in HCT116-/- to determine the endogenous abundance of LINC00176 and observed that 60% of the transcript localized to the nucleus and 40% localized to the cytoplasm **(Figure S3 E).**

Deciphering the mechanism of action of LINC00176

Thus far, the results obtained in this study indicate that Δ40p53 can regulate long non-coding RNAs in addition to miRNAs (17). Therefore, it is crucial to understand how LINC00176 functions in cells and what could be its mechanism of action **(Figure 4A)**. Long non-coding RNAs have recently gained attraction as competing endogenous RNAs (ceRNAs) known to interact with miRNAs, acting as a decoy or promoting their degradation, thereby liberating the mRNAs which directly impact different cellular processes in cancer (cell proliferation, cell growth, metastasis etc.) (40-43). Therefore, miRNA-interacting partners of LINC00176 were predicted using the Starbase V.2.0 database **(Figure 4B),** and their levels were measured in HCT116+/+ and HCT116-/- cells **(Figure 4C)**. The expressions of these miRNAs (miR-761, miR-184, miR-503-5p, miR-15b-5p, miR-138, and miR-9-5p) varied. Since most had lower expression in HCT116-/- cells than in HCT116+/+ cells, they could be targets of LINC00176, as LINC00176 levels are very high in HCT116-/- cells. In particular, miR-9-5p, a known target of LINC00176 (28), was downregulated in HCT116-/- cells compared to HCT116+/+ cells. To assess the direct effect of LINC00176 on these miRNAs, LINC00176 was silenced in HCT116-/- cells, and the corresponding changes in miRNA levels were assessed. Upon silencing LINC00176, the levels of LINC00176 were reduced by 60% compared to that in control cells **(Figure 4D)**. The levels of the miRNAs increased from 30% to 80% **(Figure 4E)**. To investigate whether LINC00176 sequesters target miRNAs, tagged RNA affinity pulldown assay was performed **(Figure 4G).** miRNA levels were quantified in the pulldown fractions for both MS2-control and MS2-LINC00176 and normalized to their respective input levels. Further, the normalized miRNA levels in MS2-LINC00176 were compared to MS2-Control to calculate the fold enrichment miR-184, miR-138, miR-503, miR-15b-5p, and miR-9-5p were significantly enriched in the pulldown fraction, indicating that they are direct targets of LINC00176 **(Figure 4F).** However, miR-761 did not show any enrichment in the pulldown fraction, implying that it might indirectly interact with LINC00176 or could be degraded in a complex. These results indicate that LINC00176 can titrate miRNA levels in cells, which may affect cellular processes.

To determine the direct downstream effect of LINC00176-miRNA axis on cellular processes, we have validated mRNA targets of the miRNAs. We used miRTarBase to identify some unique and common validated miRNAs' targets **(Figure 4H)**. For further investigation, we selected known mRNA targets of three miRNAs (miR-9-5p, miR-15b-5p, miR-503-5p) which had known implications in several cancer conditions **(Figure S4 A).** To assess the direct effect of LINC00176 on these miRNA targets, LINC00176 was silenced in HCT116-/- cells, and the corresponding changes in mRNA levels were assessed. Upon silencing, we obtained differential levels of the targets with maximum decrease in the levels of BCL2 mRNA **(Figure 4I)**. We also wanted to correlate the results in the background of silencing of Δ40p53 being the major regulator of the axis. Upon silencing of Δ40p53 in HCT116-/-, we observed that all the target levels changed variably, with a consistent decrease in SIRT1 and BCL2 levels **(Figure 4 J-K)** corroborating the results obtained in siLINC00176 background. While we observed an increase in the miRNA levels upon silencing of LINC00176 **(Figure 4E)**, the concomitant decreases in some of the mRNA targets unravelled the proximate players of Δ40p53-LINC00176-miRNA axis on the cellular processes.

The impact of the Δ40p53-LINC00176 axis on cellular processes

LncRNAs regulate a wide variety of cellular processes (44). The ceRNA network involving lncRNA/miRNA/mRNA synergistically regulate tumor hallmark processes across pan cancers (45). An lncRNA can have multiple networks in cancer (22), therefore, lncRNAs being the master regulator it is crucial to know their ultimate impact on cells (primarily on cell growth, expression of EMT markers, viability etc.). To understand the direct effect of LINC00176 on cellular behavior, we overexpressed or knocked down LINC00176 in HCT116-/- cells and examined the effects **(Figure 5A-C)**. First, we examined the effect of LINC00176 on EMT marker expression. LINC00176 overexpression increased the levels of E-cadherin (epithelial marker) and decreased the levels of Slug and vimentin (mesenchymal markers) **(Figure 5D, E)**. Interestingly, LINC00176 overexpression also increased Δ40p53 levels **(Figure 5 E, S4 B)**. By contrast, knocked down LINC00176 decreased the mRNA levels of E-cadherin but increased those of vimentin and Slug **(Figure 5F);** however, we did not observe the same trend for the protein levels of E-cadherin and Slug **(Figure 5G)**. We further analyzed cell viability after LINC00176 overexpression or knockdown and observed that LINC00176 overexpression decreased cell viability **(Figure 5H)**, whereas LINC00176 knockdown increased cell viability **(Figure 5I)**. However, these effects on cell viability may not be very prominent, because LINC00176 might contribute to only a small percentage of the vast majority of cellular processes. We also found that LINC00176 knockdown increased

proliferation using the BrdU assay (**Figure 5J).** Together, these results indicate that LINC00176 promotes the epithelial phenotype and suppresses the mesenchymal phenotype, cell growth, viability, and proliferation, all of which suggest that this lncRNA contributes to tumor suppression in cells.

DISCUSSION

In cancer, lncRNAs are key players in the p53-dependent transcriptional pathway, wherein lncRNAs and p53 can regulate each other (46). Therefore, to identify critical cancer signaling pathways, it is vital to recognize novel lncRNAs that are regulated through the p53 pathway. *TP53* is the most frequently mutated gene in human cancer (47); however, these mutations are not found in all cancers, indicating that other factors could interfere with WT p53 function (48). These factors have complicated our understanding of p53 signaling and include more recently discovered p53 isoforms (49). Owing to the lack of detailed functional characterization, little information is available regarding p53 isoforms. However, one isoform that has recently gained attention in cancer research is Δ40p53, the only translational isoform of p53 (50). Although a few reports have shown the transactivation capacity of Δ40p53, there are no studies on the regulation of lncRNAs by Δ40p53. We selected LINC00176 for our current study because of its variable regulation by different transcription factors and differential expression in cancer.

Significant changes in the levels of LINC00176 were observed in H1299 cells expressing p53, Δ40p53, and both isoforms. However, the maximum upregulation was observed in cells expressing only Δ40p53. The upregulation of LINC00176 by Δ40p53 was also observed in other cell line expressing only Δ40p53 (HCT116-/-) as compared to cells expressing both isoforms (HCT116+/+). Furthermore, LINC00176 was upregulated under different stress conditions, and its expression correlated with the abundance of Δ40p53. However, we cannot rule out the possibility that other factors could be involved in regulating LINC00176 because of certain observations. First, we did not observe a prominent decrease in LINC00176 levels upon knockdown of Δ40p53 in HCT116-/-. Second, upon induction of ER stress in cells with Δ40p53 knockdown, LINC00176 levels did not change with respect to control cells. This indicates that although Δ40p53 could be a major regulator of LINC00176, other factors regulate LINC00176 abundance. This regulation could be at the transcriptional level or post transcriptional; therefore, we investigated any direct binding between the molecules.

Δ40p53 was previously thought to be devoid of transactivation capacity because it lacks TAD1 (51); however, subsequent studies reported that it can regulate some p53 target genes (13,52). It has also been reported that Δ40p53 is sufficient to induce cell death independent of p53 through a specific subset of genes (Fas, Dr5, Api1, and Pig3) (15). More recently, a study suggested an FLp53-independent, pro-tumoral role for Δ40p53 because of its ability to transactivate the transcription of antiapoptotic proteins, such as netrin-1 (53). Therefore, we examined the association of Δ40p53 and p53 with LINC00176. We observed binding of Δ40p53 to *LINC00176* promoters; surprisingly, p53 did not bind to *LINC00176* promoters. However, overexpression of p53 in HCT116-/- cells decreased the association of Δ40p53 with *LINC00176* promoters, suggesting the formation of hetero-oligomers between p53 and Δ40p53 interfered with the transactivation function of Δ40p53, is consistent with the results of earlier reports (8). Besides the role of p53 as a typical transcription factor, there are reports of p53 binding to RNA through a nucleic acid binding domain, which is independent of its core DNA binding domain(54,55). Recently it has been found that p53 cand bind to the lncRNA MEG3(39); however, there are no such reports on other p53 isoforms. The RNA binding property could be true for Δ40p53 as well, which shares majority of the protein domains with p53. Therefore, we speculated if Δ40p53 could also regulate LINC00176 at the RNA level.

With Actinomycin D experiment we got a clue that Δ40p53 could regulate the stability of LINC00176 RNA, which was further confirmed through UV-Cross linking experiments. Results show that not only Δ40p53 but p53 can also specifically bind to LINC00176 RNA, however we observed more competition of LINC00176 for binding onto Δ40p53 than p53. Since Δ40p53, lacks the first 40 amino acids, it may become structurally more favorable in binding to LINC00176 RNA than p53, however the exact mechanisms remain unknown and requires further investigation. Nevertheless, we observe binding for p53 also, which unravels its possible mechanism behind LINC00176 regulation. From here we infer that, the enhanced levels of LINC00176 in the presence of Δ40p53 compared to p53 could be due to its multistep regulation at the level of transcription and RNA binding and stability. Additionally, we find a 60:40 Nuclear: Cytoplasmic ratio of LINC00176 in HCT116-/-, which confirms its levels in both the compartments, which could be a possible consequence of the multistep regulation.

The significance of LINC00176 upregulation by Δ40p53 was determined by examining different miRNA targets. We identified different miRNA targets from StarBase V2.0 for LINC00176, from which we selected a few targets (miR-761, miR-138, miR-15b-5p, miR-

503-5p, miR-184 and miR-9-5p) based on the number of binding sites and their functions in different cancers **(Figure S4 A)**. Most of the selected miRNAs are differentially expressed in cancer owing to their diverse regulatory pathways (56-72). Their levels were mostly decreased in HCT116-/- cells compared to those in HCT116+/+ cells, and their expressions negatively correlated with LINC00176 levels. Upon further analyzing their levels after knockdown of LINC00176 and TRAP assays, we found that most of these miRNAs (miR-138, miR-15b-5p, miR-503-5p, miR-184 and miR-9-5p) were enriched in the pulldown complex of LINC00176. However, although the level of miR-761 increased upon LINC00176 knockdown, it was not sequestered by LINC00176. The results suggest LINC00176 to be an important ceRNA titrating the levels of multiple miRNAs, thereby attenuating the possible miRNA-mRNA interactions. To elucidate the effect of LINC00176 on the downstream mRNAs, we have validated mRNA targets of the miRNAs. We used miRTarBase to identify some unique and common validated targets (CDH1, SIRT1, ONECUT2, BCL2, PIK3R1, FGF2, CCND1, CCNE1) of selected miRNAs (miR-9-5p, miR-503-5p and miR-15b-5p). We knocked down the two upstream regulators, Δ40p53 and LINC00176 in HCT116-/- and checked the corresponding changes in the mRNA levels. SIRT1 and BCL2 levels consistently decreased upon silencing both upstream regulators, indicating their potential involvement in the Δ40p53/LINC00176/miRNA axis. However, detailed investigation is necessary to correlate the direct effect of mRNA targets on the downstream processes.

Finally, we examined the direct effects of LINC00176 on cellular behavior through ectopic overexpression or silencing of LINC00176. Based on the different hallmark processes of cancer (73) we examined EMT marker expression, cell proliferation, cell viability. LINC00176 overexpression enhanced the epithelial phenotype, decreased cell viability and also upregulated Δ40p53 protein levels **(Figure S4 B)**, suggesting a possible feed-forward loop between Δ40p53 and LINC00176 through which they might regulate each other's levels. LINC00176 knockdown led to increase in cell viability and proliferation, however it did not give consistent results with the EMT markers which also decreased upon LINC00176 knockdown. We speculate that the decrease in the protein levels of EMT markers could be due to a decrease in Δ40p53, which might also directly regulate EMT markers. Therefore, the effect of LINC00176 knockdown is inconclusive. Δ40p53 function is known to be cell context-specific, and Δ40p53 can have differential effects when overexpressed or silenced (48). This strengthens our hypothesis that, since there is a possible feed-forward loop between LINC00176 and Δ40p53, many of the lncRNA effects will be dependent on the

levels of Δ40p53 and therefore will vary. The mechanistic details of the possible feed-forward loop is an interesting axis which we would like to decipher in future.

In conclusion, our results indicated that LINC00176 contributes to the tumor-suppressive function of Δ40p53, independent of p53FL function **(Figure 6: Graphical Abstract).** However, it would be interesting to determine whether the function of the Δ40p53-LINC00176 axis is universal or cell type-specific. Taken together, this study opens up avenues for mechanistic insights into how Δ40p53 governs cellular processes through lncRNAs.

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AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Selection and validation of LINC00176 for further studies

(A) Quantitative PCR for validation of LINC00176 in H1299 cells expressing control, p53 only, Δ40p53 only and 14A construct. (B) Western blot analysis of cell extracts from H1299 cells expressing control, p53 only, Δ40p53 only and 14A construct, probed with CM1 after 48 h. (C) Quantitative PCR for validations of LINC00176 in HCT116+/+ and HCT116-/- cells. (D) Western blot analysis of cell extracts from HCT116+/+ and HCT116-/-cells, probed with CM1. (E) Quantitative PCR of LINC00176 in HCT116+/+ cells transfected with si p53 (30nM) and non-specific si (si Nsp). (F) Western blot analysis of cell extracts from HCT116+/+ cells transfected with either si p53 (30nM) and nonspecific si (si Nsp), probed with CM1. (G) Quantitative PCR of LINC00176 in HCT116-/- cells transfected with si Δ40p53 (30nM) and non-specific si (si Nsp). (H) Western blot analysis of cell extracts from HCT116-/- cells transfected with siΔ40p53 (30nM) and nonspecific si (si Nsp), probed with

CM1. (I) Quantitative PCR of LINC00176 in HCT116-/- cells transfected with control and p53 only. (J) Western blot analysis of cell extracts from HCT116-/- cells transfected with control and p53 only, probed with CM1. The criterion for statistical significance was $p \leq 0.05$ (*) or $p \leq 0.01$ (**) or $p \leq 0.001$(***)

Figure 2. Regulation of LINC00176 under different stress conditions

(A) Schematic of different stress induction in HCT116-/- cells. (B) Quantitative PCR of LINC00176 in HCT116-/- treated with Doxorubicin (2μM) for 16 h. (C) Western blot analysis of cell extracts from HCT116-/- treated with Doxorubicin (2μM) for 16 h, probed with CM1. (D) Quantitative PCR of LINC00176 in HCT116-/- treated with Thapsigargin (0.1μM and 1μM) for 16 h. (E) Western blot analysis of cell extracts from HCT116-/- treated with Thapsigargin (0.1μM and 1μM) for 16 h, probed with CM1. (F) Quantitative PCR of LINC00176 in HCT116-/- deprived of Glucose in medium for 8 h. (G) Western blot analysis of cell extracts from HCT116-/- deprived of Glucose in medium for 8 h, probed with CM1. (H) Schematic of experiment in HCT116-/- transfected with siΔ40p53 followed by Thapsigargin (0.1μM) treatment. (I) Quantitative PCR of LINC00176 in HCT116-/- transfected with siΔ40p53 followed by Thapsigargin (0.1μM) treatment. (J) Western blot analysis of cell extracts from HCT116-/- transfected with siΔ40p53 followed by Thapsigargin (0.1μM) treatment, probed with CM1. The criterion for statistical significance was $p \leq 0.05$ (*) or $p \leq 0.01$ (**) or $p \leq 0.001$(***)

Figure 3. Mechanistic basis of LINC00176 regulation by Δ40p53/p53

(A) Schematic of possible routes of LINC00176 regulation. (B) Quantitative PCR of LINC00176 promoters in HCT116-/- cells processed for ChIP Assay 48 h post seeding. Δ40p53 was pulled down with 1801 Ab (Santa Cruz) and associated LINC00176 promoters were probed using qPCR. (C) Quantitative PCR of LINC00176 promoters in HCT116+/+ cells processed for ChIP Assay 48 h post seeding. p53 was pulled down with 1801 Ab (Santa Cruz) and associated LINC00176 promoters were probed using qPCR. (D) Quantitative PCR of LINC00176 promoters in HCT116-/- cells transfected with p53 only and control, which has been processed for ChIP Assay 48 h post transfection. Δ40p53/p53 was pulled down with 1801 Ab (Santa Cruz) and associated LINC00176 promoters were probed using qPCR. (E) Western blot analysis of cell extracts from HCT116-/- transfected with p53 only and control, probed with CM1. (F) Schematic of experiment to determine Δ40p53 mediated regulation on LINC00176 RNA stability. (G) Quantitative PCR of LINC00176 in HCT116-/- transfected with siΔ40p53 followed by Actinomycin D treatment for 0 h, 4 h and 8 h. (H) Western blot

analysis of cell extracts from HCT116-/- transfected with si∆40p53 followed by Actinomycin D treatment, probed with CM1. (I) Predicted interaction profile of LINC00176 and ∆40p53 obtained from catRAPID software. (J) Predicted interaction profile of LINC00176 and p53 obtained from catRAPID software. (K) UV cross-linking assay of recombinant ∆40p53 with α^{32}P-labeled LINC00176 RNA alone or in the presence of indicated unlabeled RNA. Lanes 1 and 2 corresponds to no protein and no competition (direct binding) respectively. For the competition, unlabeled LINC00176 RNA (lanes 3 and 4), or nonspecific RNA (lanes 5 and 6) was used as indicated. Unlabeled nonspecific RNA of equivalent length was derived from the multiple-cloning site (MCS) region of the pcDNA 3.0 vector. (L) UV cross-linking assay of recombinant p53 with α^{32}P-labeled LINC00176 RNA alone or in the presence of indicated unlabeled RNA. Lanes 1 and 2 corresponds to no protein and no competition (direct binding) respectively. For the competition, unlabeled LINC00176 RNA (lanes 3 and 4), or nonspecific RNA (lanes 5 and 6) was used as indicated. Unlabeled nonspecific RNA of equivalent length was derived from the multiple-cloning site (MCS) region of the pcDNA 3.0 vector. The criterion for statistical significance was $p \leq 0.05$ (*) or $p \leq 0.01$ (**) or $p \leq 0.001$(***)

Figure 4. Deciphering the mechanism of action of LINC00176

(A) Schematic for the probable downstream regulation by LINC00176. (B) Table for the selected miRNA targets of LINC00176 obtained from Starbase V2.0, as mentioned in methods section. (C) Quantitative PCR of selected miRNAs in HCT116+/+ and HCT116-/- cells. (D) Quantitative PCR of LINC00176 in HCT116-/- cells transfected with siRNA (30nM) directed to LINC00176 and nonspecific si (si Nsp). (E) Quantitative PCR of selected miRNAs in HCT116-/- cells transfected with siRNA (30nM) directed to LINC00176 and nonspecific si (si Nsp). (F) Quantitative PCR of selected miRNAs in GST pulldown lysates of HCT116-/-cells (co-transfected with LINC00176 overexpression construct and pMS2-GST construct) normalised to Input values. (G) Western blot analysis of cell extracts from Input and Pulldown fraction of HCT116-/- cells (co-transfected with LINC00176 overexpression construct and pMS2-GST construct), probed with CM1 and GST Antibody. (H) Table for the selected mRNA targets of miRNAs obtained from miRTarBase, as mentioned in methods section. (I) Quantitative PCR of selected mRNAs in HCT116-/- cells transfected with siRNA (30nM) directed to LINC00176 and nonspecific si (si Nsp). (J) Western blot analysis of cell extracts from HCT116-/- cells transfected with si∆40p53 (30nM) and nonspecific si (si Nsp). (K) Quantitative PCR of selected mRNAs in HCT116-/- cells transfected with si∆40p53 (30nM) and nonspecific si (si Nsp). The criterion for statistical significance was $p \leq 0.05$ (*) or $p \leq 0.01$ (**) or $p \leq 0.001$(***).

Figure 5: The impact of Δ40p53-LINC00176 axis on cellular processes

(A)Schematic of the different processes that has been checked for any probable effect by LINC00176 over-expression or partial silencing. (B) Quantitative PCR of LINC00176 in HCT116-/- cells transfected with LINC00176 overexpression construct. (C) Quantitative PCR of LINC00176 in HCT116-/- cells transfected with LINC00176 shRNA construct. (D) Quantitative PCR of EMT markers (E-cadherin, Vimentin, Slug) in HCT116-/- cells transfected with LINC00176 overexpression construct. (E) Western blot analysis of cell extracts from HCT116-/-cells transfected with LINC00176 overexpression construct, probed with E-cadherin Antibody, Slug Antibody and CM1. (F) Quantitative PCR of EMT markers (E-cadherin, Vimentin, Slug) in HCT116-/- cells transfected with LINC00176 shRNA construct. (G) Western blot analysis of cell extracts from HCT116-/-cells transfected with LINC00176 shRNA construct, probed with E-cadherin Antibody, Slug Antibody and CM1. (H) HCT116-/- cells were transfected with LINC00176 overexpression construct, at 36 h post transfection cells were reseeded on 96 well plate for MTT Assay. MTT incorporation was calculated at Day 0, Day 2 and Day 4 post seeding. (I) HCT116-/- cells were transfected with LINC00176 shRNA construct, at 36 h post transfection cells were reseeded on 96 well plate for MTT Assay. MTT incorporation was calculated at Day 0, Day 2 and Day 4 post seeding. (J) HCT116-/- cells were transfected with 30nM si-LINC00176, 36 h post transfection cells were reseeded for BrdU assay. After 12 h of seeding BrdU was added to the cells and incubated for 12 h. Following which, percentage of cell proliferation was checked by calculating BrdU incorporation. The criterion for statistical significance was $p \leq 0.05$ (*) or $p \leq 0.01$ (**) or $p \leq 0.001$(***).

Graphical Abstract: Model depicting the regulation of Δ40p53-LINC00176-miRNA axis

(A) In absence of Δ40p53, LINC00176 levels do not increase which leads to increased pool of free miRNAs which probably binds to several mRNA targets preventing their function, ultimately resulting in increased cell proliferation. (B) However, when Δ40p53 is present it upregulates LINC00176 levels both at the level of transcription and post-transcription, which sequesters oncogenic miRNAs preventing it to bind to their respective mRNA targets, which ultimately results in decreased cell proliferation.

Figure: 1

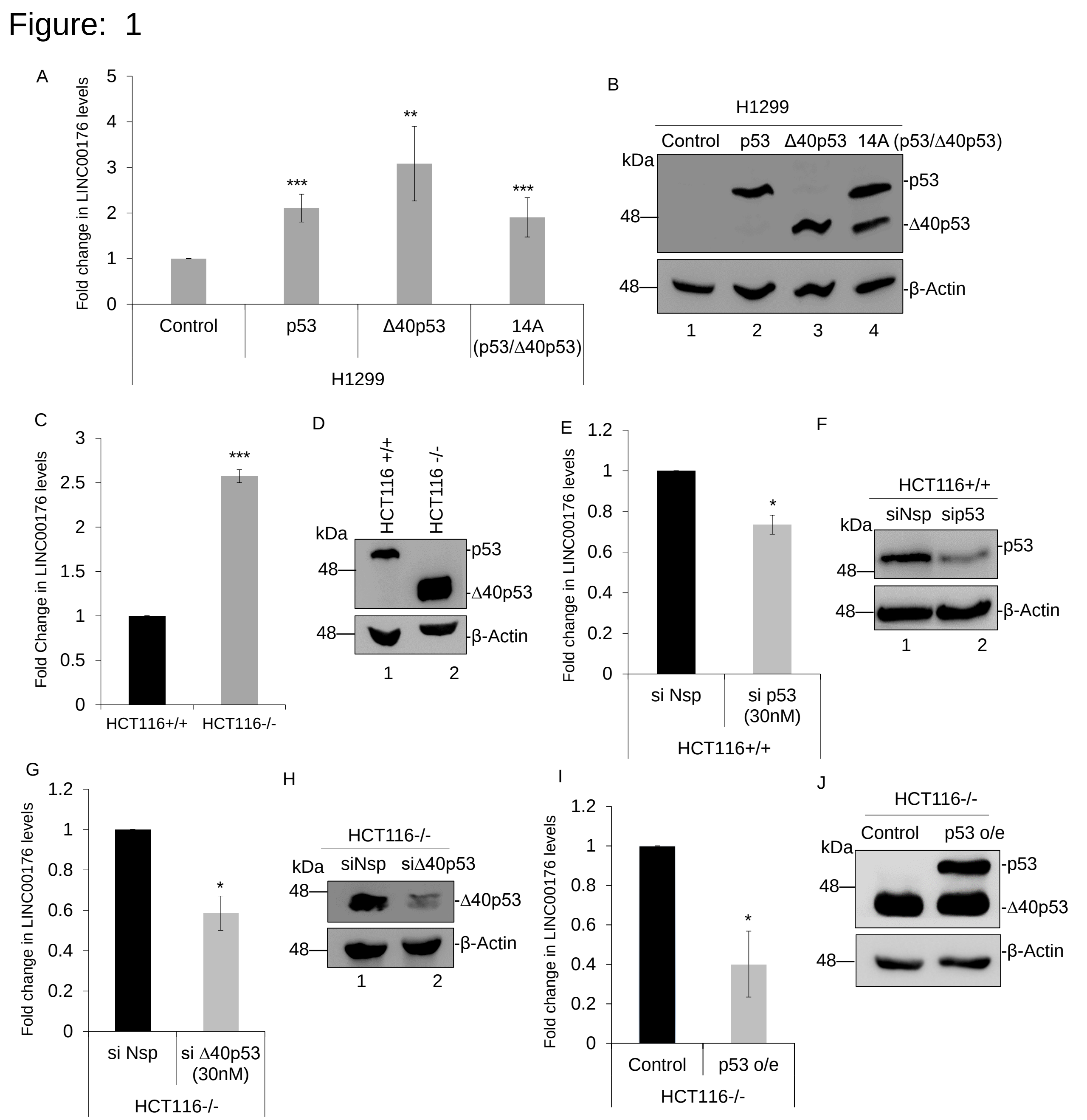

A
Fold change in LINC00176 levels
5
4
3
2
1
0
***
**
***
Control
p53
Δ40p53
14A (p53/Δ40p53)
H1299
B
H1299
Control
p53
Δ40p53
14A (p53/Δ40p53)
kDa
48
-p53
-Δ40p53
48
-β-Actin
1
2
3
4
C
Fold Change in LINC00176 levels
3
2.5
2
1.5
1
0.5
0
***
HCT116+/+
HCT116-/-
D
HCT116 +/+
HCT116 -/-
kDa
48
-p53
-Δ40p53
48
-β-Actin
1
2
E
Fold change in LINC00176 levels
1.2
1
0.8
0.6
0.4
0.2
0
*
si Nsp
si p53 (30nM)
HCT116+/+
F
HCT116+/+
siNsp
sip53
kDa
48
-p53
48
-β-Actin
1
2
G
Fold change in LINC00176 levels
1.2
1
0.8
0.6
0.4
0.2
0
*
si Nsp
si Δ40p53 (30nM)
HCT116-/-
H
HCT116-/-
kDa
siNsp
siΔ40p53
48
-Δ40p53
48
-β-Actin
1
2
I
Fold change in LINC00176 levels
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
p53 o/e
HCT116-/-
J
HCT116-/-
Control
p53 o/e
kDa
48
-p53
-Δ40p53
48
-β-Actin

Figure: 2

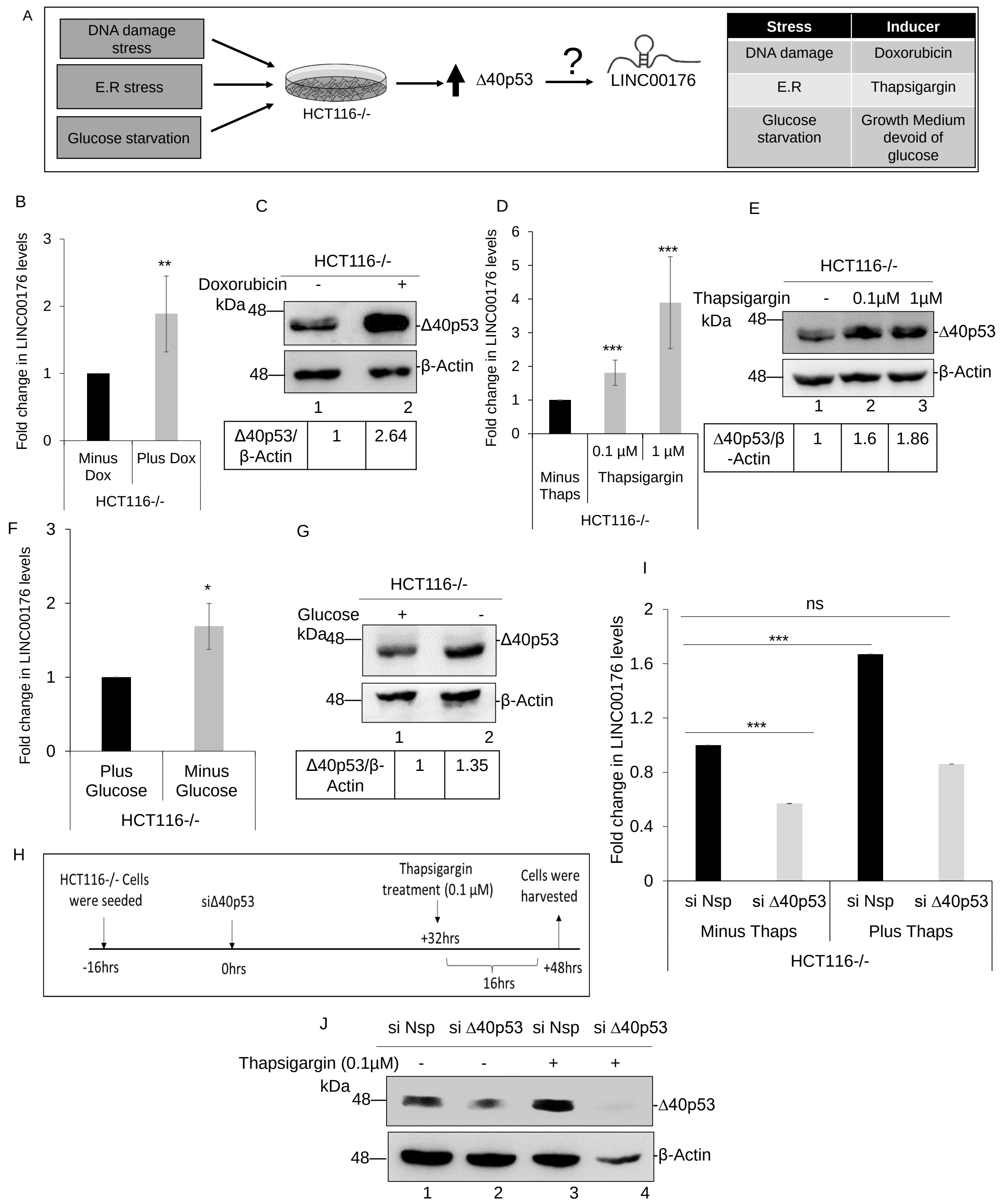
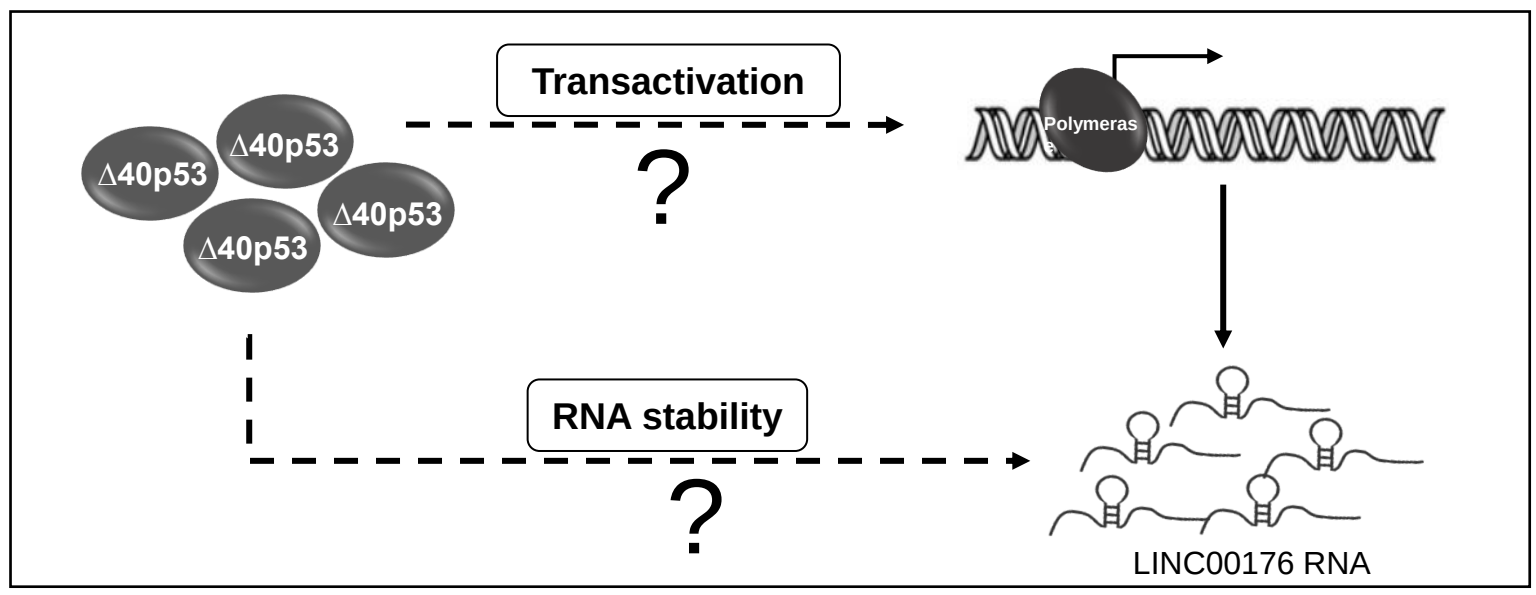

A
DNA damage stress
E.R stress
Glucose starvation
HCT116-/-
Δ40p53
?
LINC00176
Stress
Inducer
DNA damage
Doxorubicin
E.R
Thapsigargin
Glucose starvation
Growth Medium devoid of glucose
B
Fold change in LINC00176 levels
Minus Dox
Plus Dox
HCT116-/-
C
HCT116-/-
Doxorubicin
kDa
Δ40p53
β-Actin
Δ40p53/ β-Actin
1
2.64
D
Fold change in LINC00176 levels
Minus Thaps
0.1 μM
1 μM
Thapsigargin
HCT116-/-
E
HCT116-/-
Thapsigargin
0.1μM
1μM
Δ40p53/β -Actin
1.6
1.86
F
Fold change in LINC00176 levels
Plus Glucose
Minus Glucose
HCT116-/-
G
HCT116-/-
Glucose
Δ40p53/β-Actin
1.35
H
HCT116-/- Cells were seeded
siΔ40p53
Thapsigargin treatment (0.1 μM)
Cells were harvested
-16hrs
0hrs
+32hrs
16hrs
+48hrs
I
ns
Fold change in LINC00176 levels
si Nsp
si Δ40p53
Minus Thaps
Plus Thaps
HCT116-/-
J
si Nsp
si Δ40p53
Thapsigargin (0.1μM)
kDa
Δ40p53
β-Actin

Figure: 3

A



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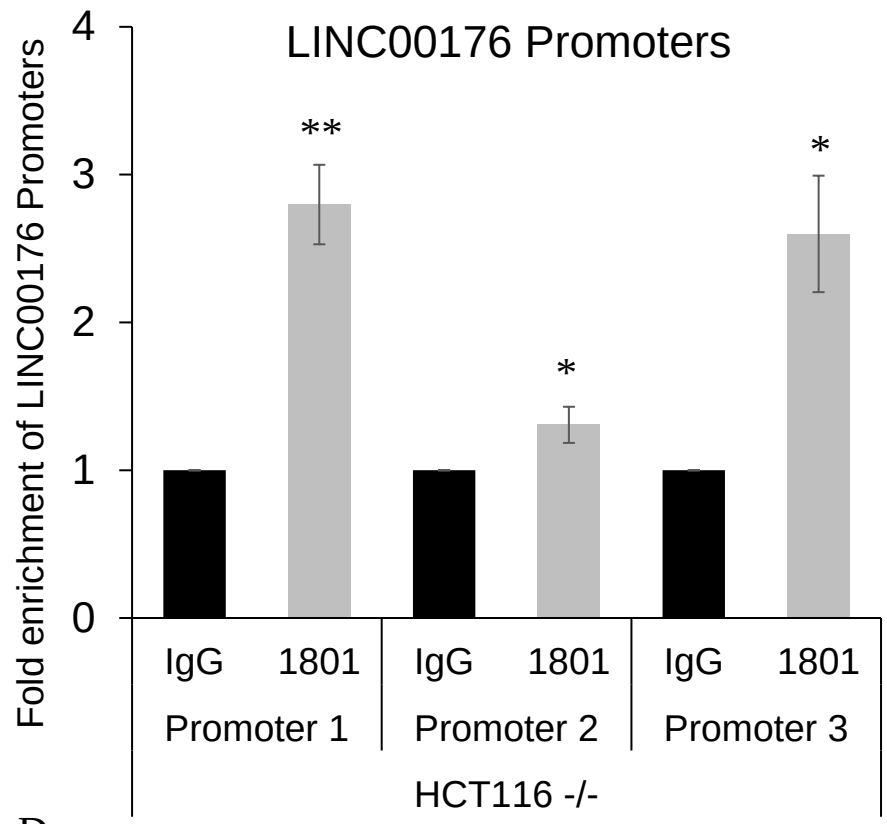

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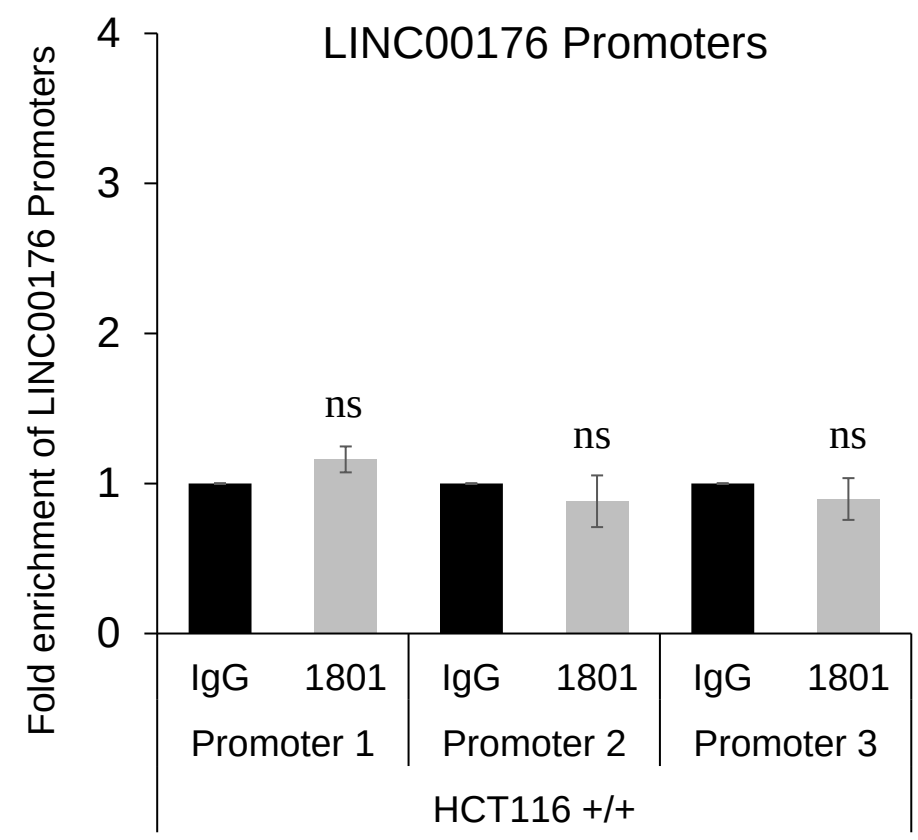

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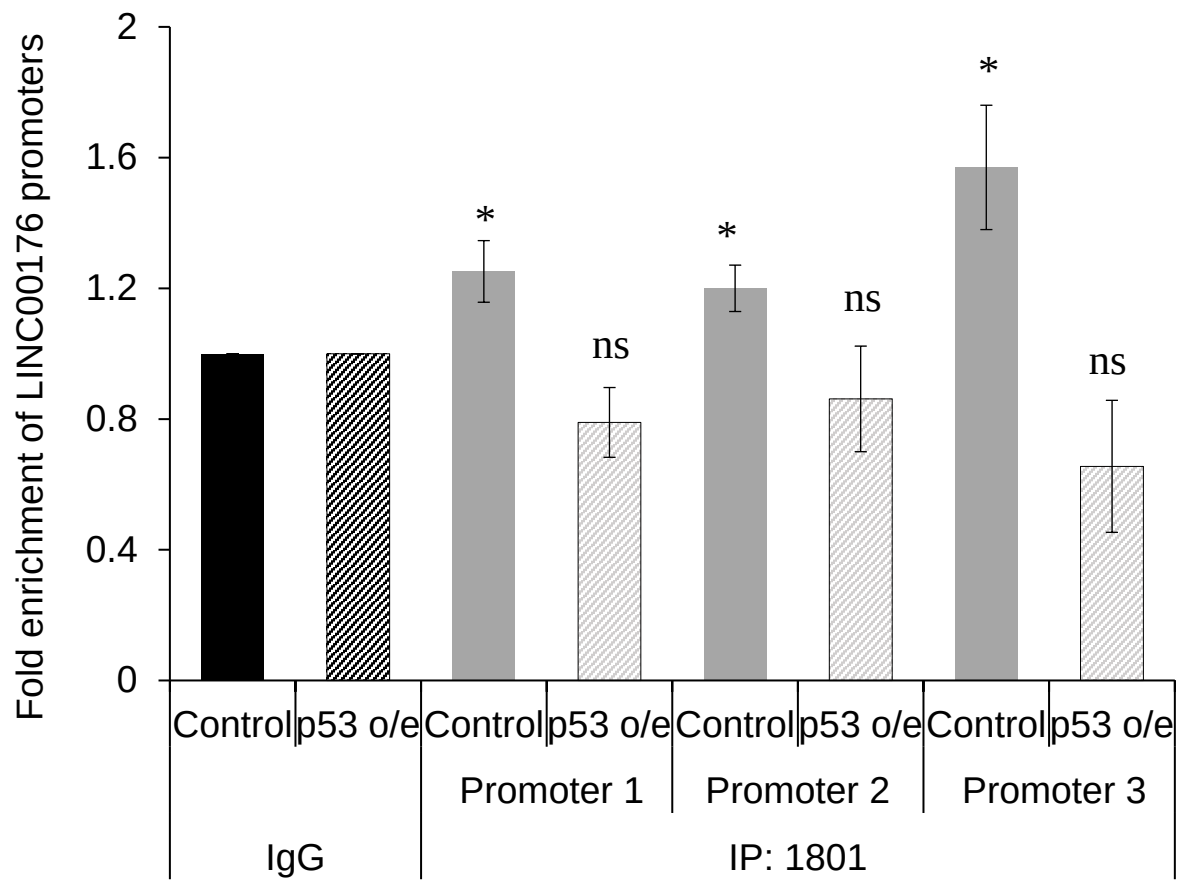

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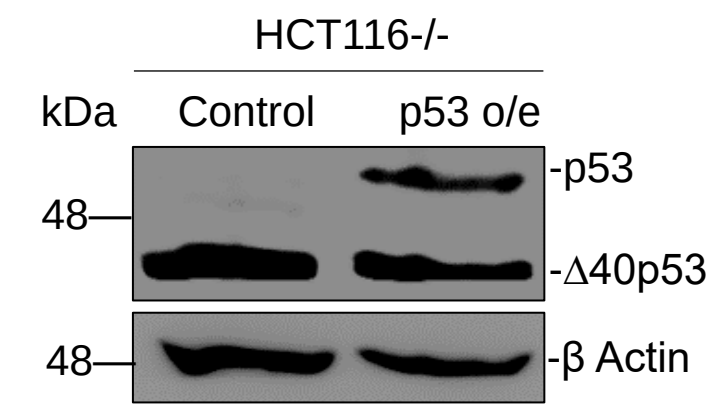

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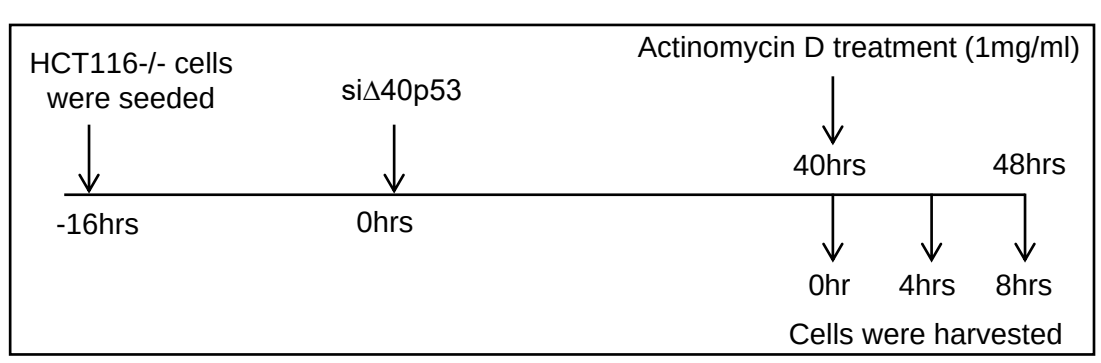

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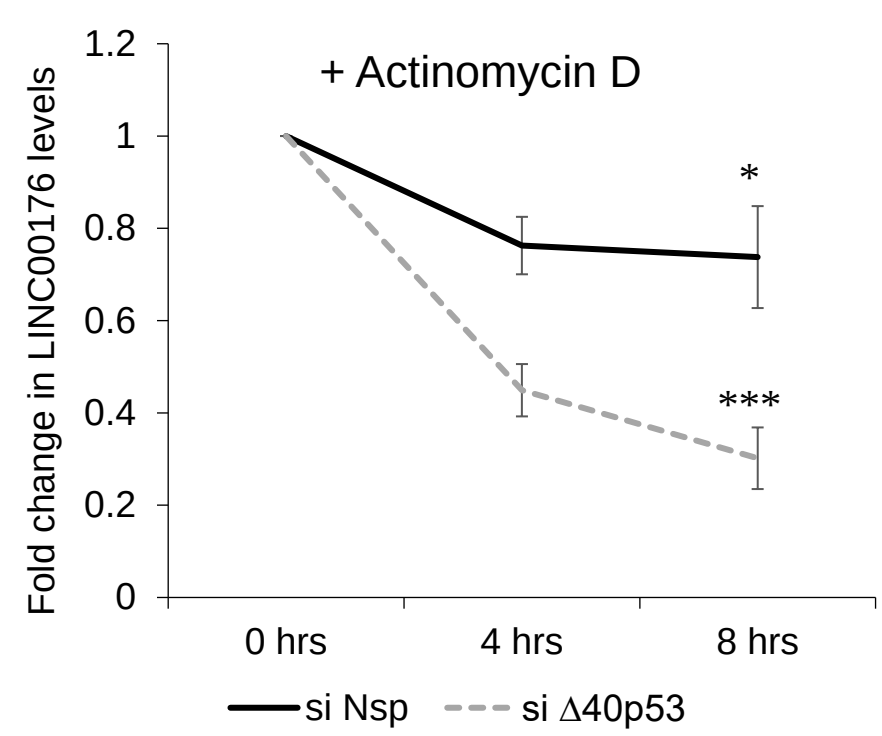

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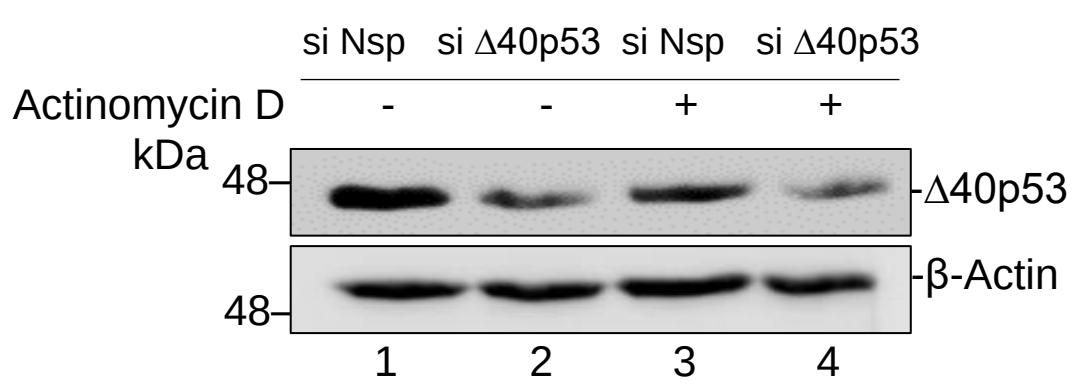
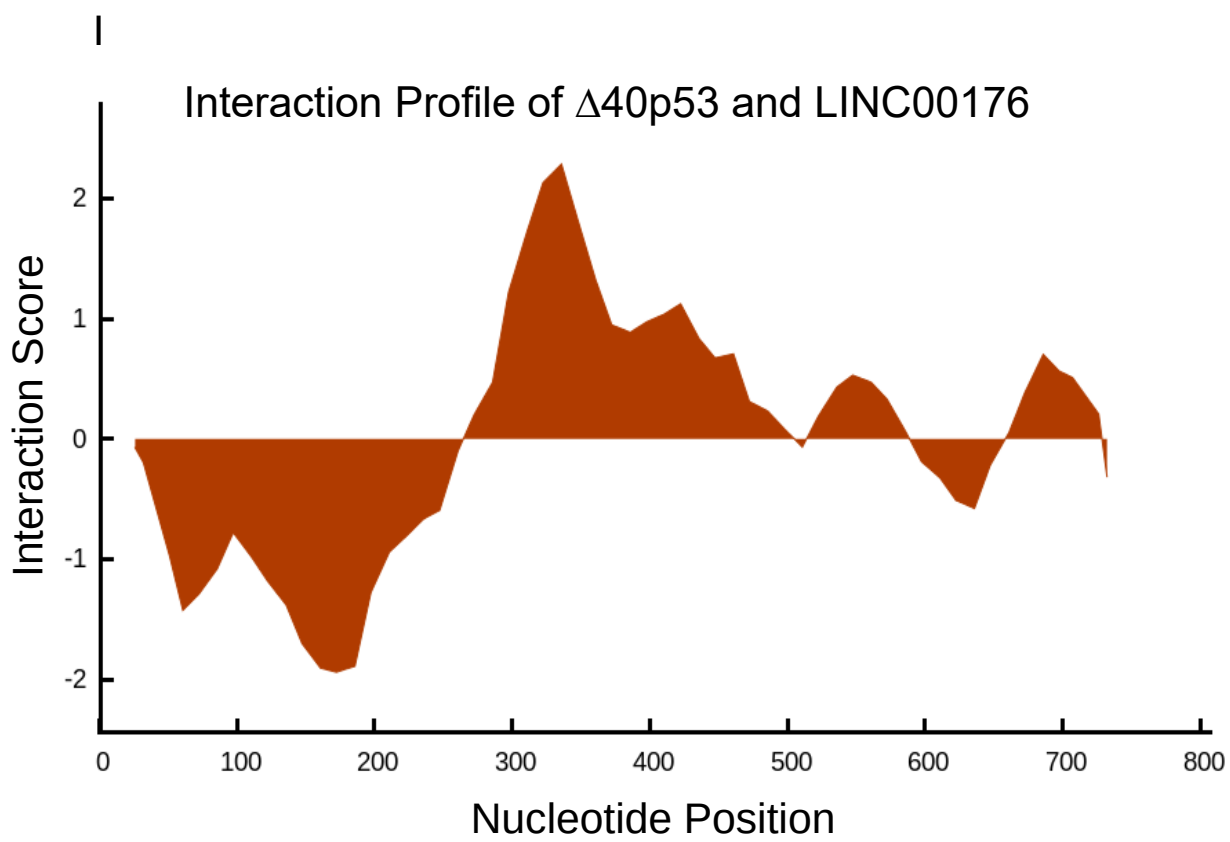


Figure: 3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Interaction Profile of Δ40p53 and LINC00176
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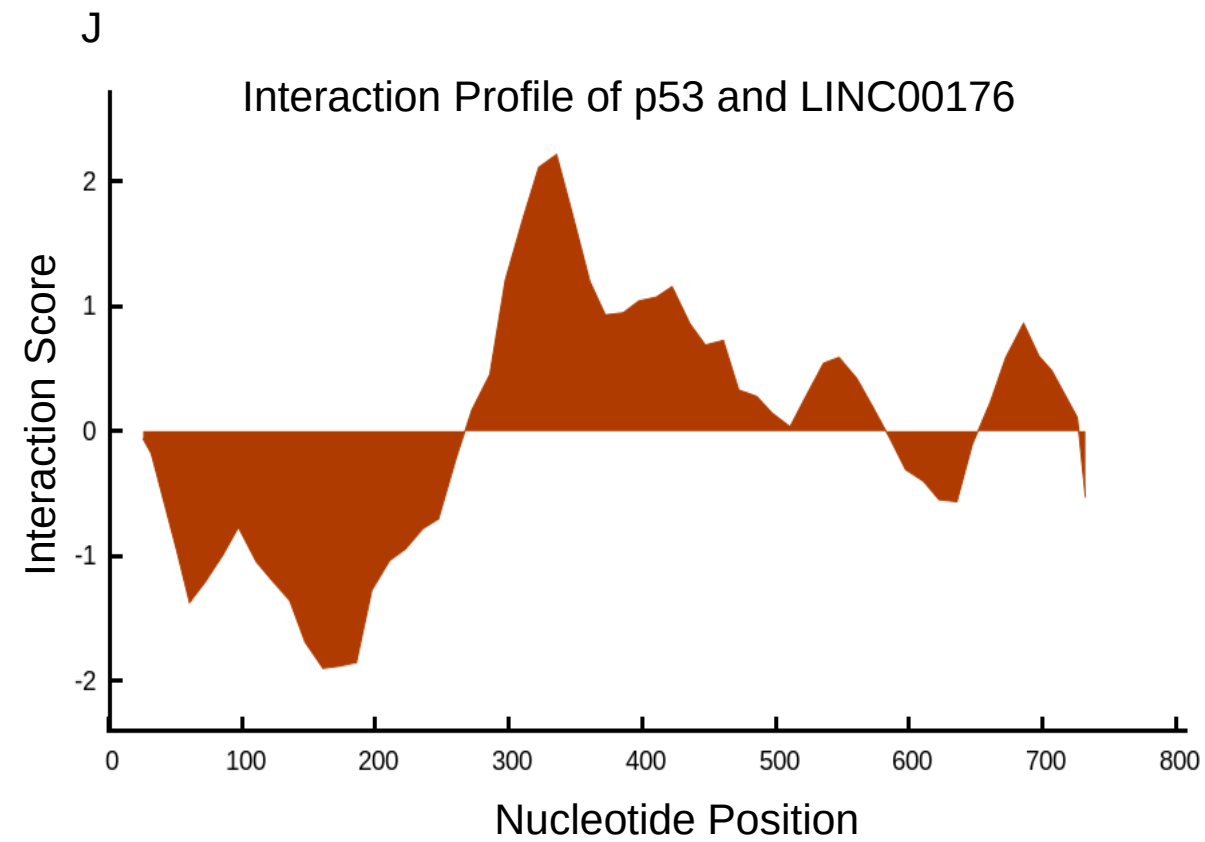
-1
-2
0
100
200
300
400
500
600
700
800
Nucleotide Position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-1
-2
0
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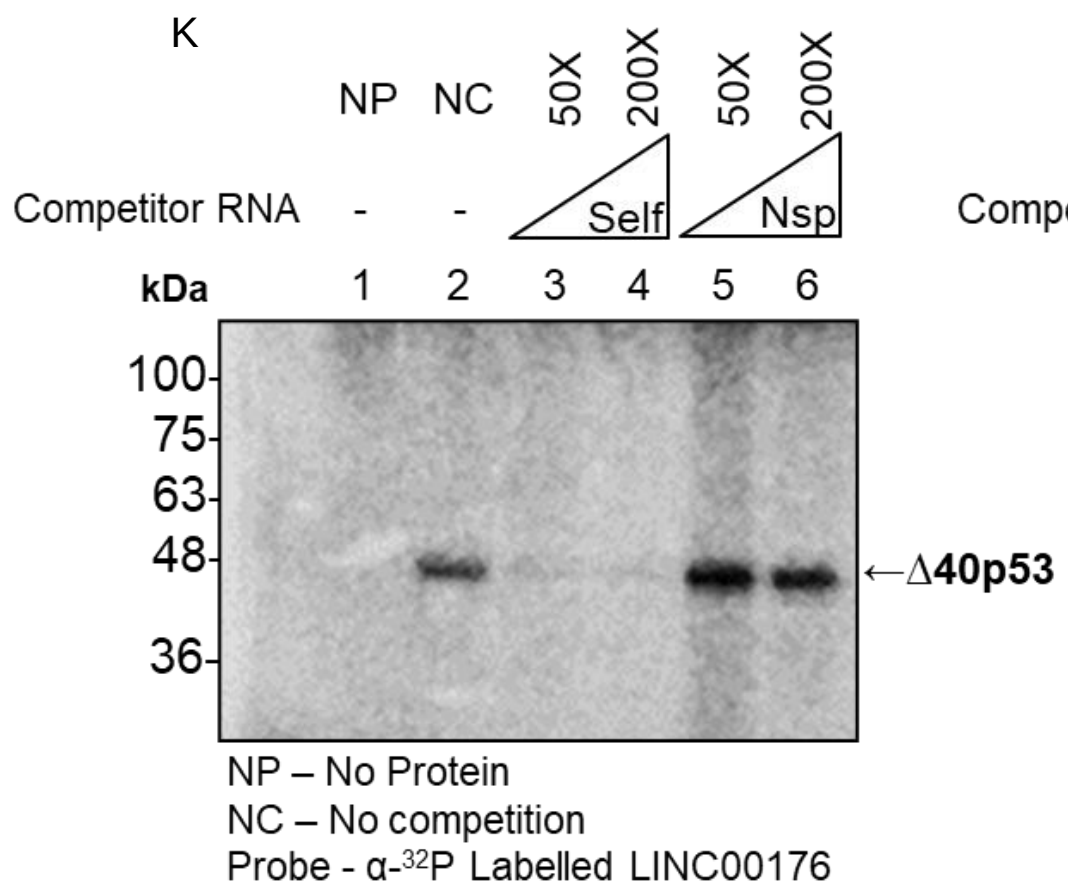
600
700
800
Nucleotide Position


K
NP
NC
50X
200X
50X
200X
Competitor RNA
-
-
Self
Nsp
kDa
1
2
3
4
5
6
100
75
63
48
36
←Δ40p53
NP – No Protein
NC – No competition
Probe - α-32P Labelled LINC00176

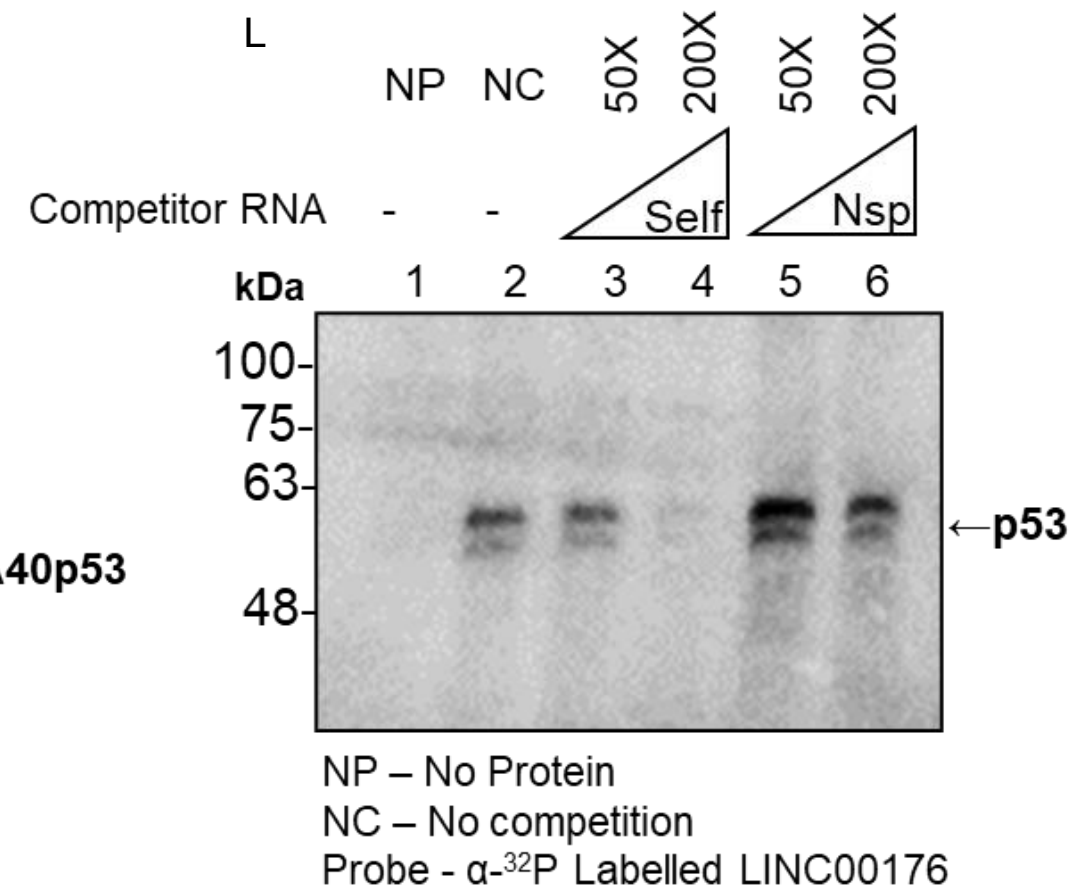

L
NP
NC
50X
200X
50X
200X
Competitor RNA
-
-
Self
Nsp
kDa
1
2
3
4
5
6
100
75
63
48
←p53
NP – No Protein
NC – No competition
Probe - α-32P Labelled LINC00176

Figure: 4

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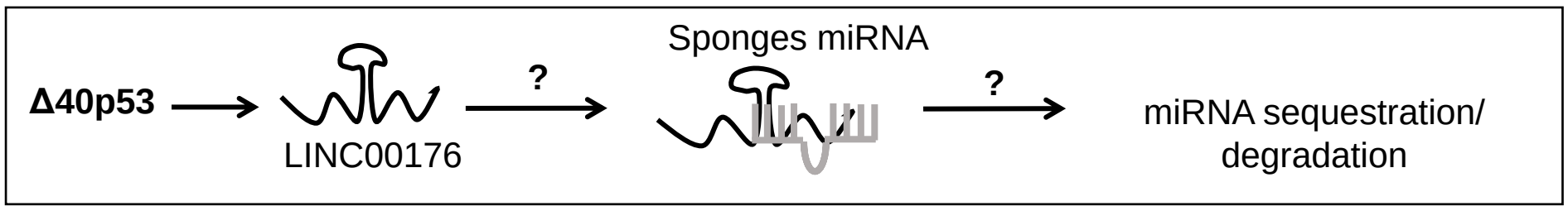
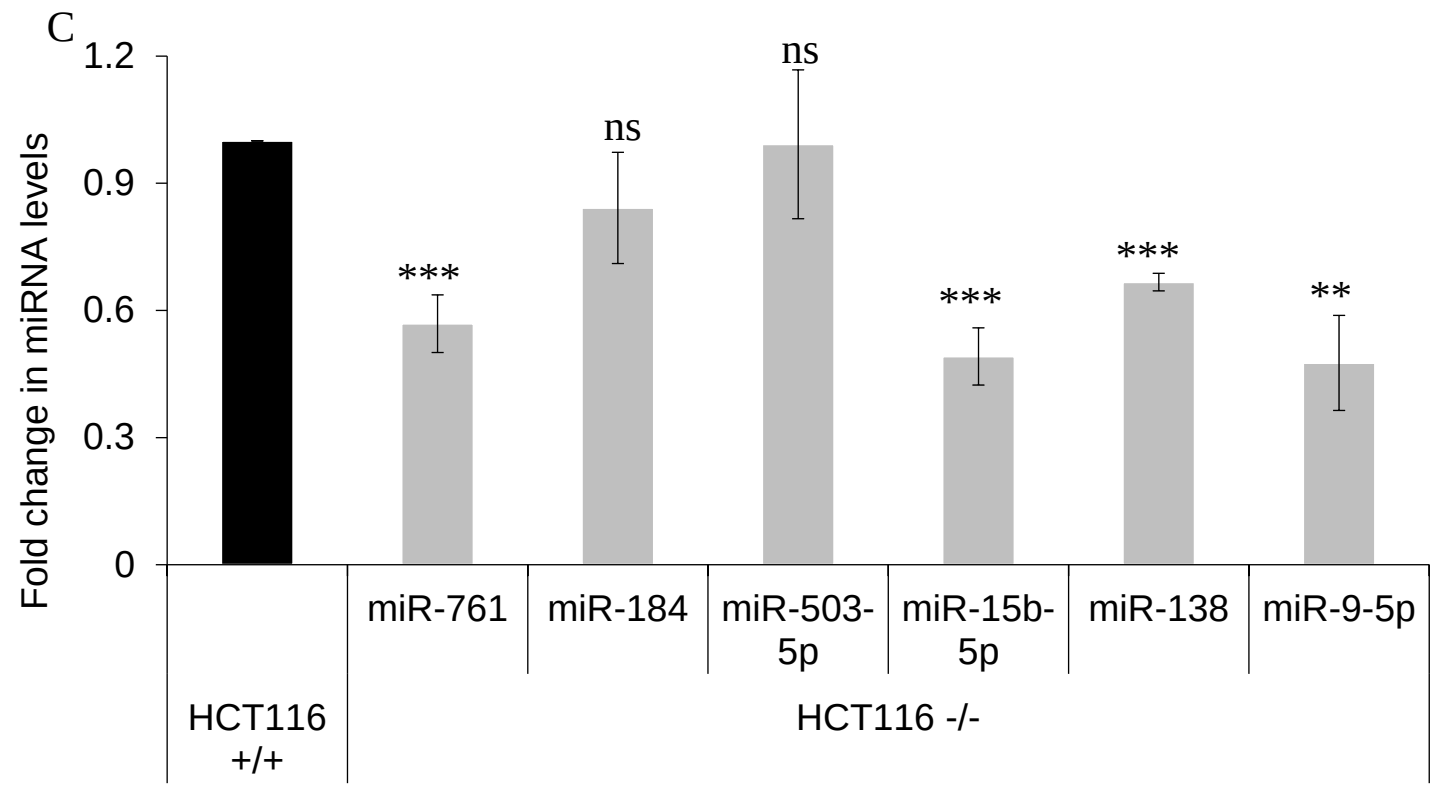


B

LncRNA	Predicted miRNA Targets	General Functions
LINC00176	miR-761	Proliferation
	miR-184	Cell cycle regulation
	miR-503-5p	Apoptosis
	miR-138	Senescence
	miR-15b	Transcription Regulation
	miR-9-5p	Autophagy

C



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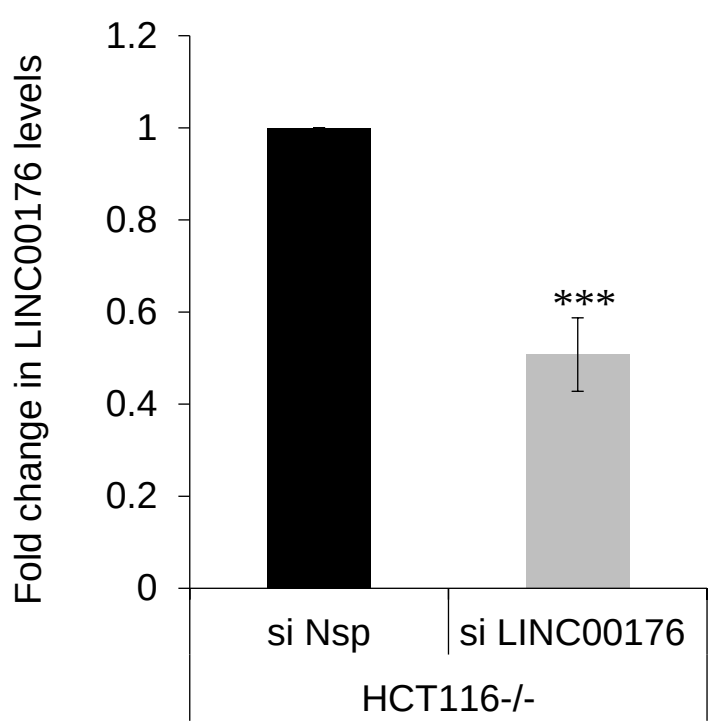

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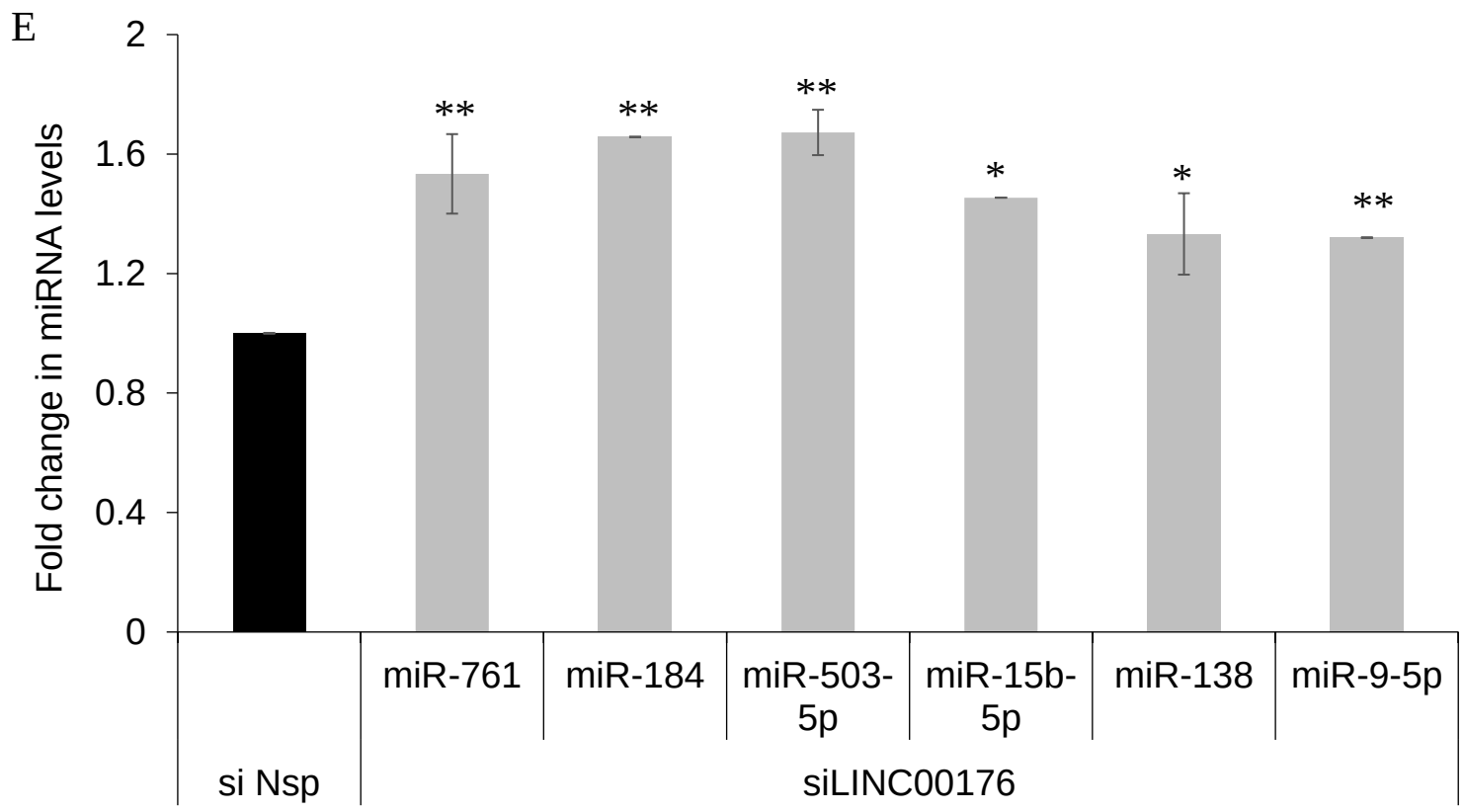

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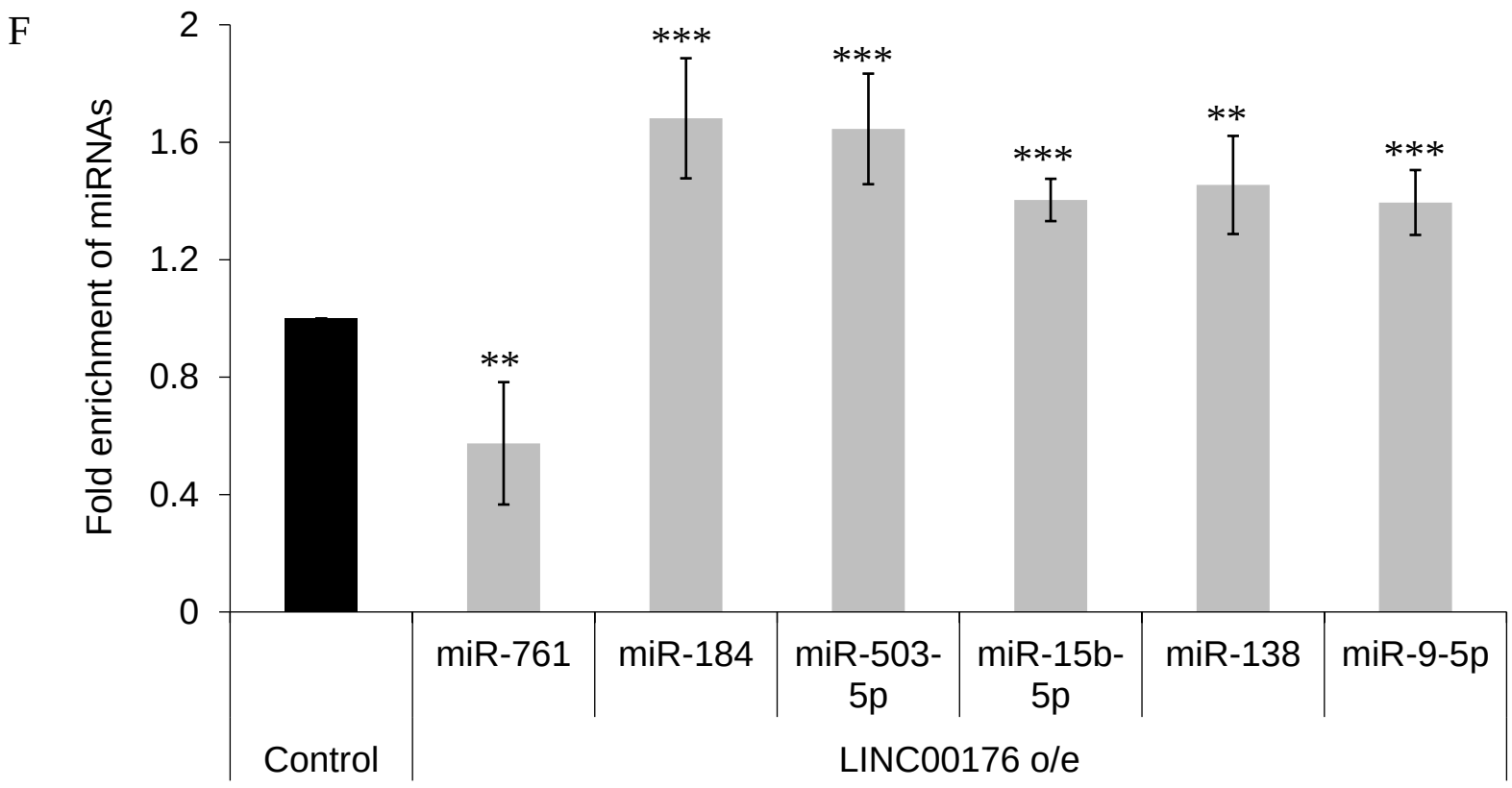

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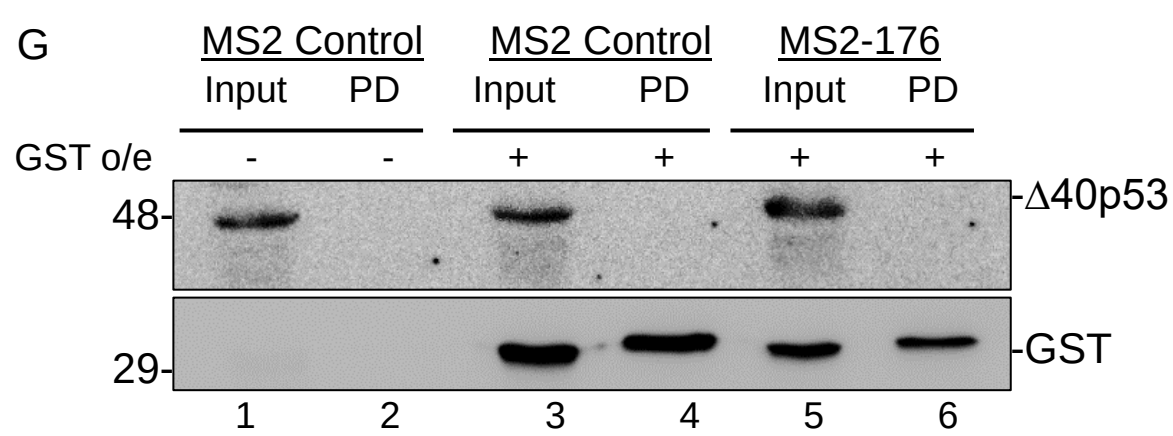
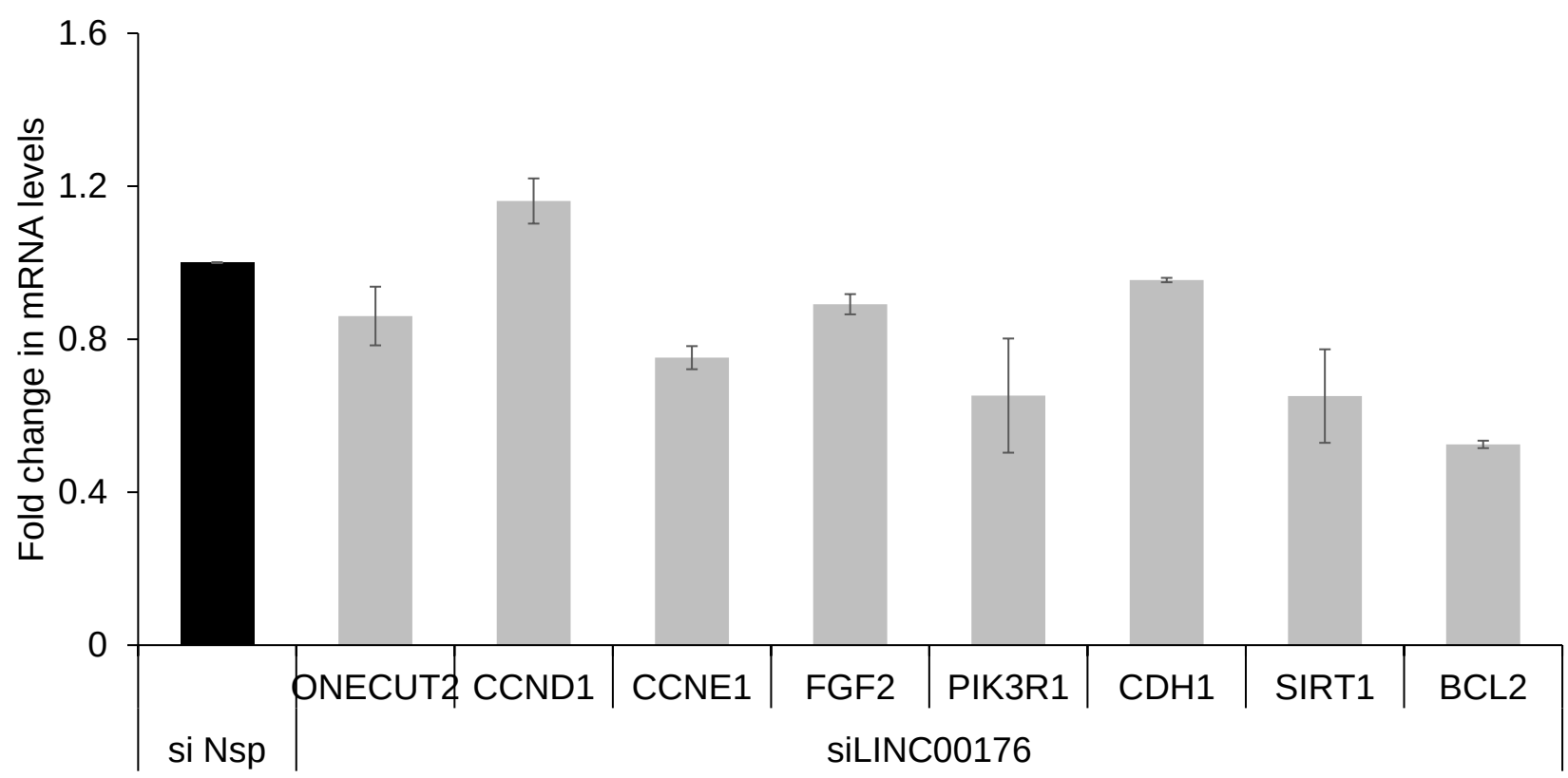


H

miRNAs	Validated Targets (miRTarBase)		Major Functions
miR-9-5p	CDH1	Cadherin 1	Proliferation Cell cycle regulation Apoptosis Senescence Transcription Regulation Autophagy Cell Growth Epithelial to Mesenchymal Transition
	SIRT1	NAD-dependent deacetylase sirtuin-1	
	ONECUT2	One Cut Homeobox 2	
miR-15b-5p	CCND1	Cyclin D1	
	CCNE1	Cyclin E1	
	BCL2	BCL2 Apoptosis Regulator	
miR-503-5p	CCND1	Cyclin D1	
	BCL2	BCL2 Apoptosis Regulator	
	FGF2	Fibroblast Growth Factor 2	
	PIK3R1	Phosphoinositide-3-Kinase Regulatory Subunit 1	

I



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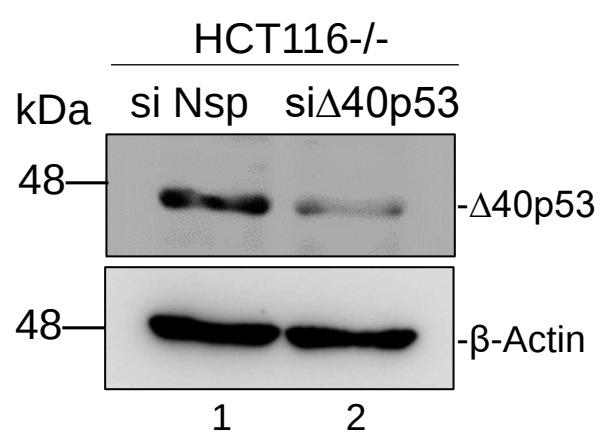

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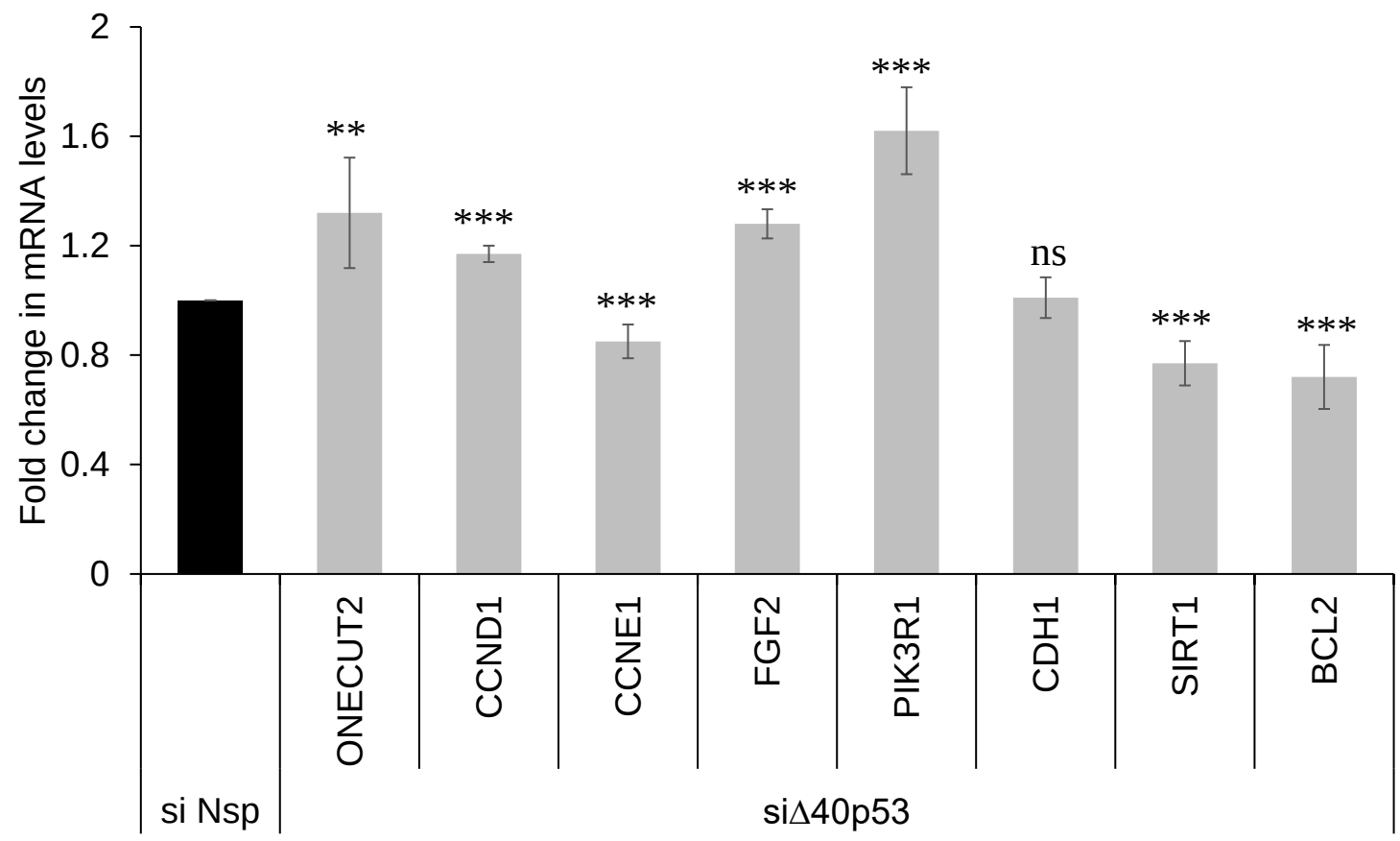
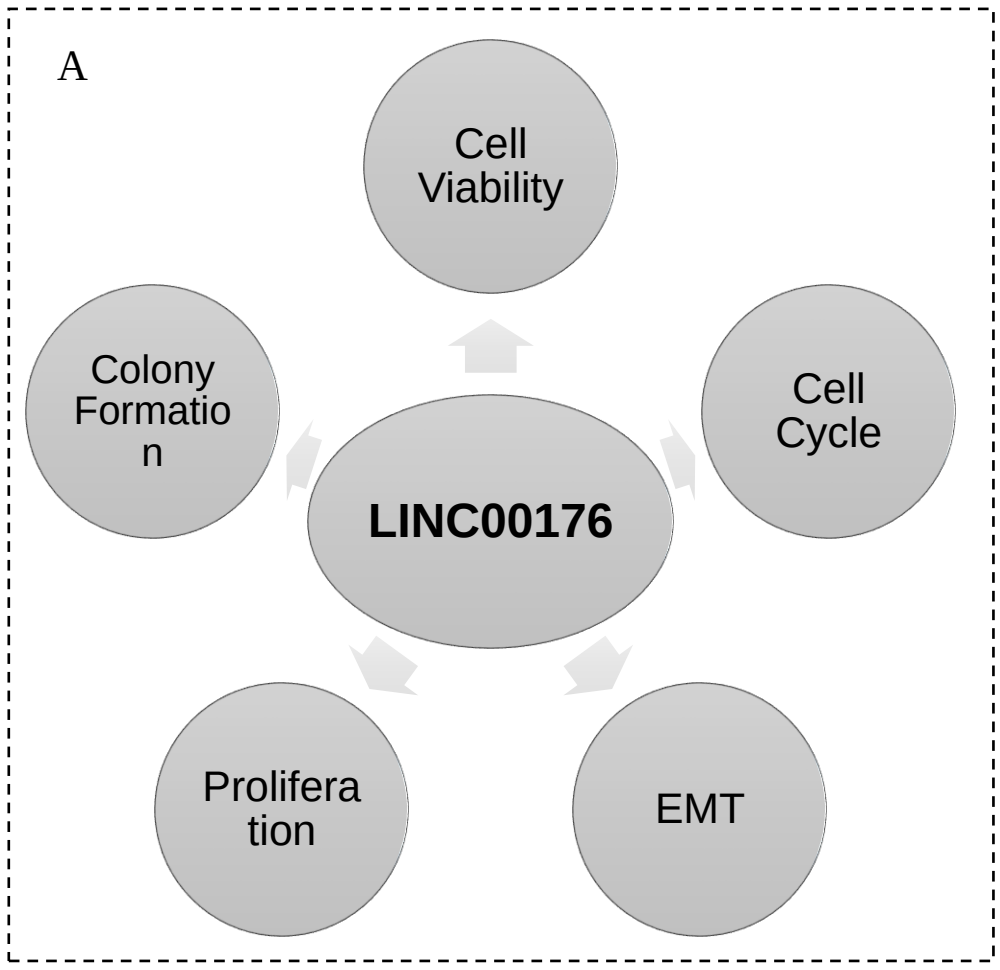
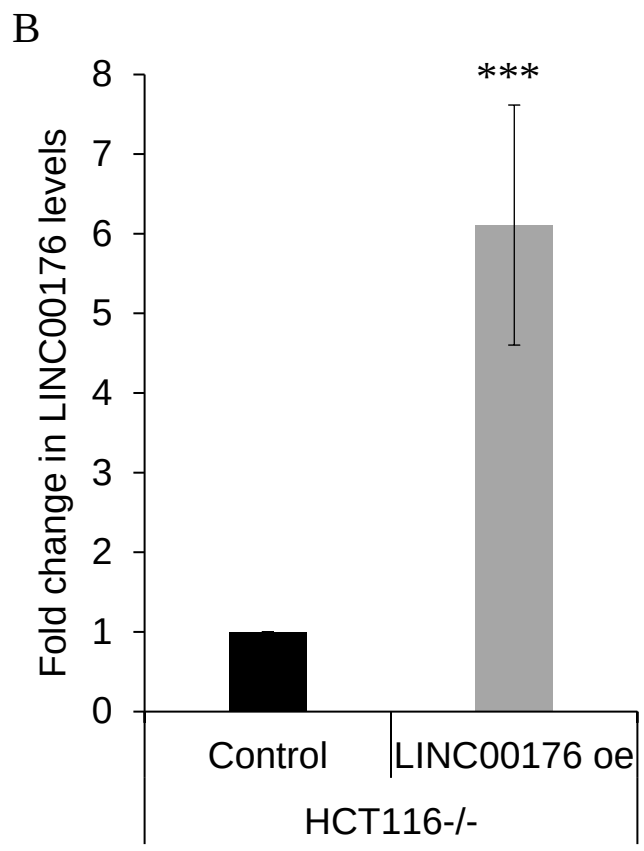
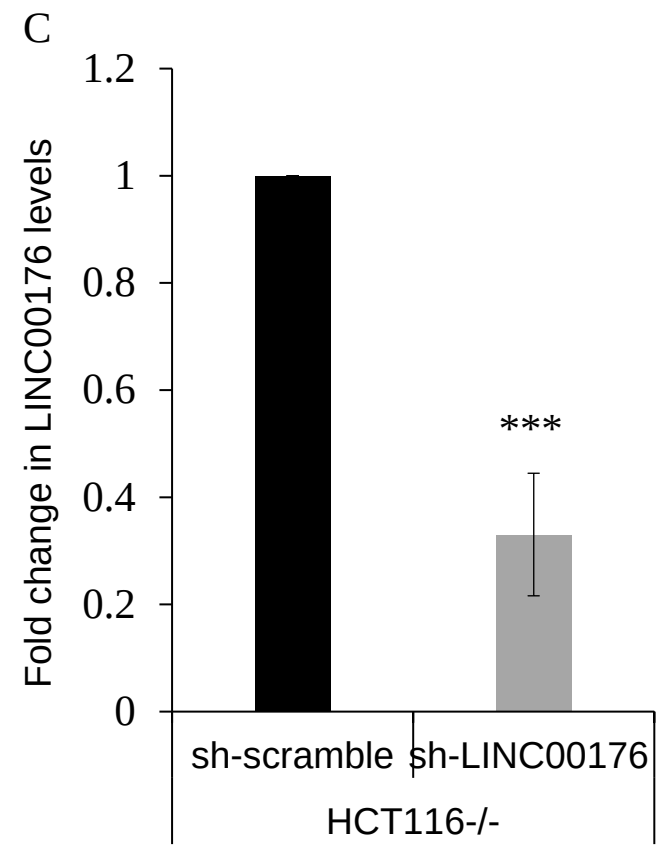
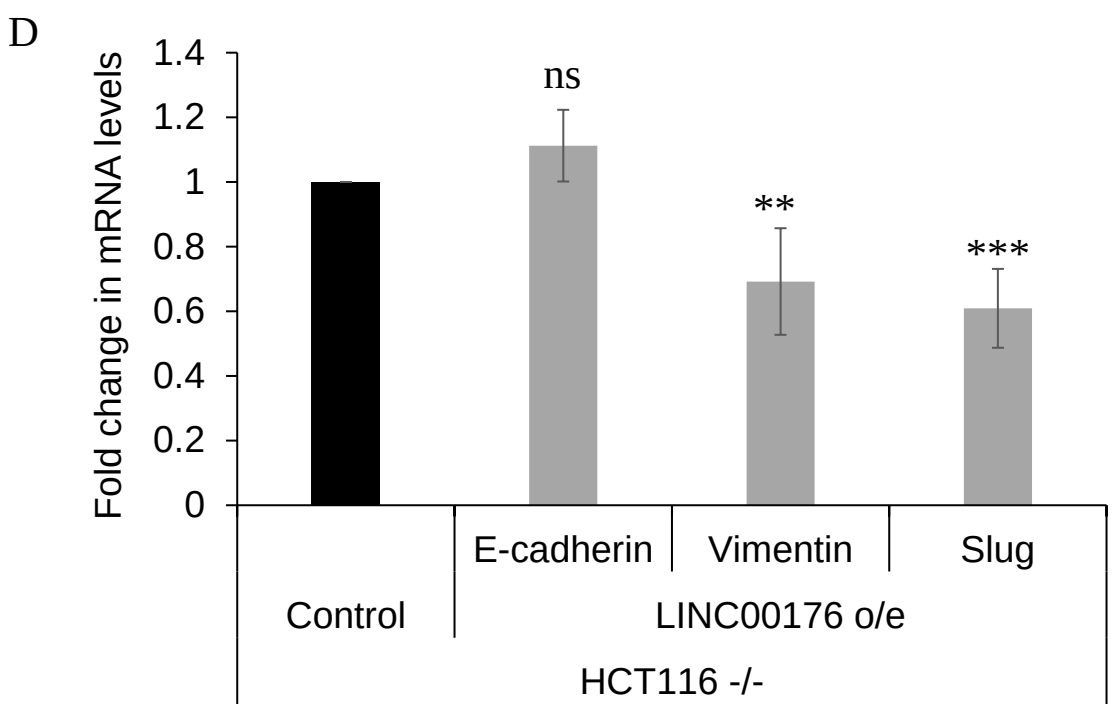


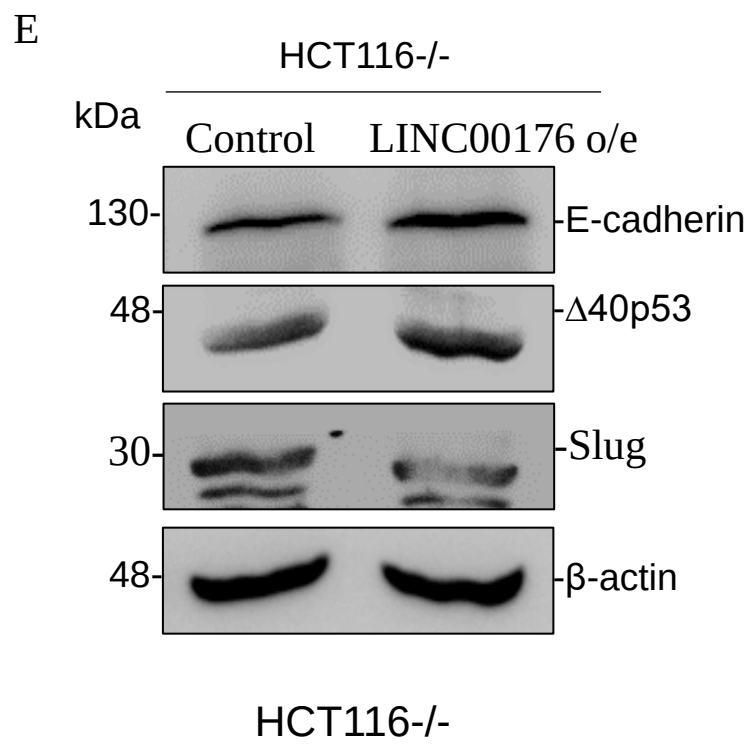
Figure: 5

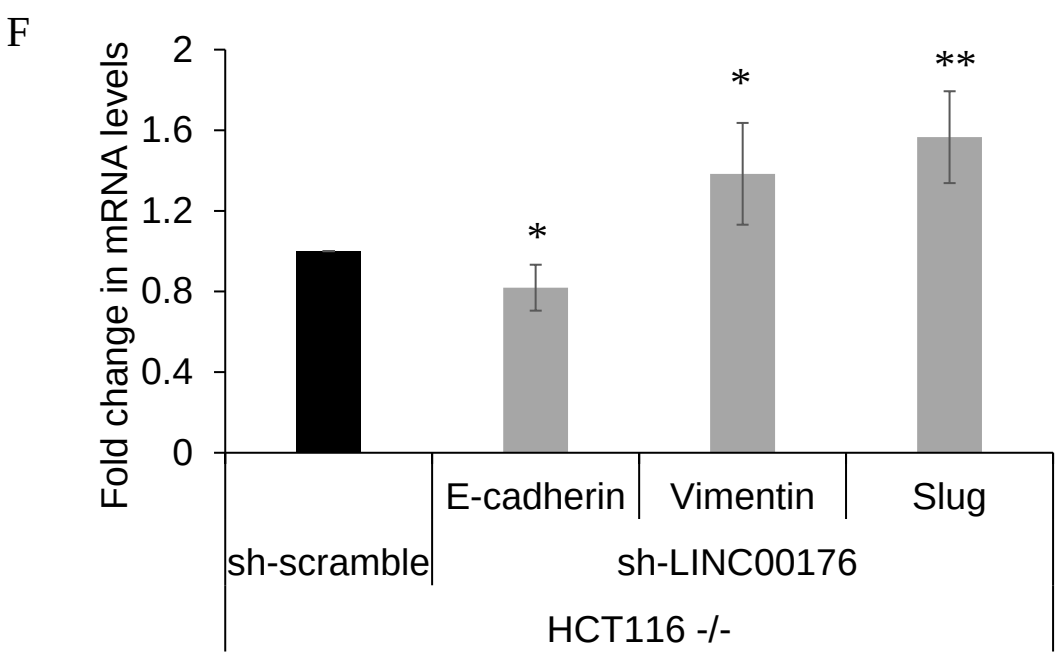

A
Cell Viability
Colony Formatio n
Cell Cycle
LINC00176
Prolifera tion
EMT

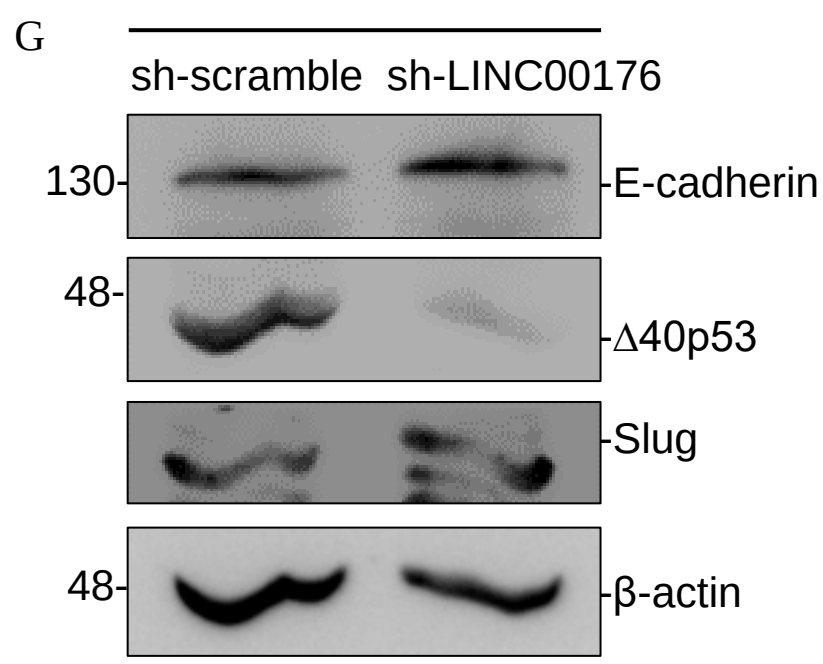

B
Fold change in LINC00176 levels
8
7
6
5
4
3
2
1
0
***
Control
LINC00176 oe
HCT116-/-

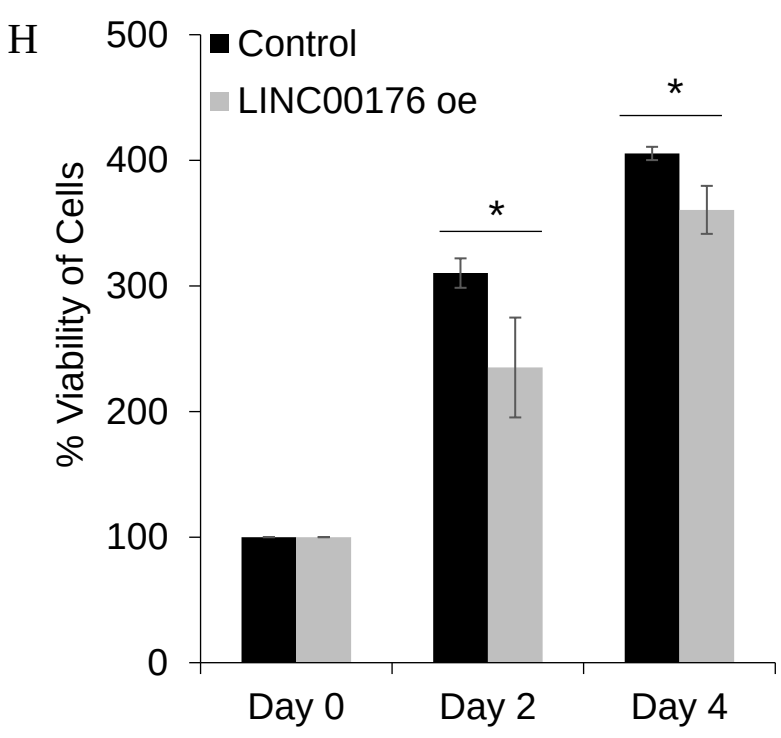

C
Fold change in LINC00176 levels
1.2
1
0.8
0.6
0.4
0.2
0
***
sh-scramble
sh-LINC00176
HCT116-/-

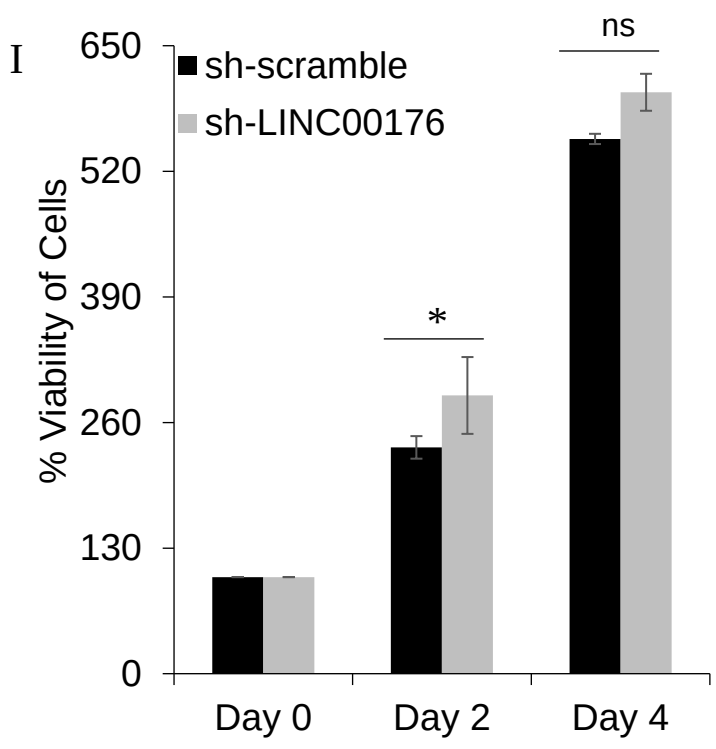

D
Fold change in mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ns
**
***
E-cadherin
Vimentin
Slug
Control
LINC00176 o/e
HCT116 -/-


E
HCT116-/-
kDa
Control
LINC00176 o/e
130-
E-cadherin
48-
Δ40p53
30-
Slug
48-
β-actin


F
Fold change in mRNA levels
2
1.6
1.2
0.8
0.4
0
*
*
**
E-cadherin
Vimentin
Slug
sh-scramble
sh-LINC00176
HCT116 -/-


G
HCT116-/-
sh-scramble
sh-LINC00176
130-
E-cadherin
48-
Δ40p53
Slug
48-
β-actin


H
% Viability of Cells
500
400
300
200
100
0
Control
LINC00176 oe
*
*
Day 0
Day 2
Day 4


I
% Viability of Cells
650
520
390
260
130
0
sh-scramble
sh-LINC00176
ns
*
Day 0
Day 2
Day 4

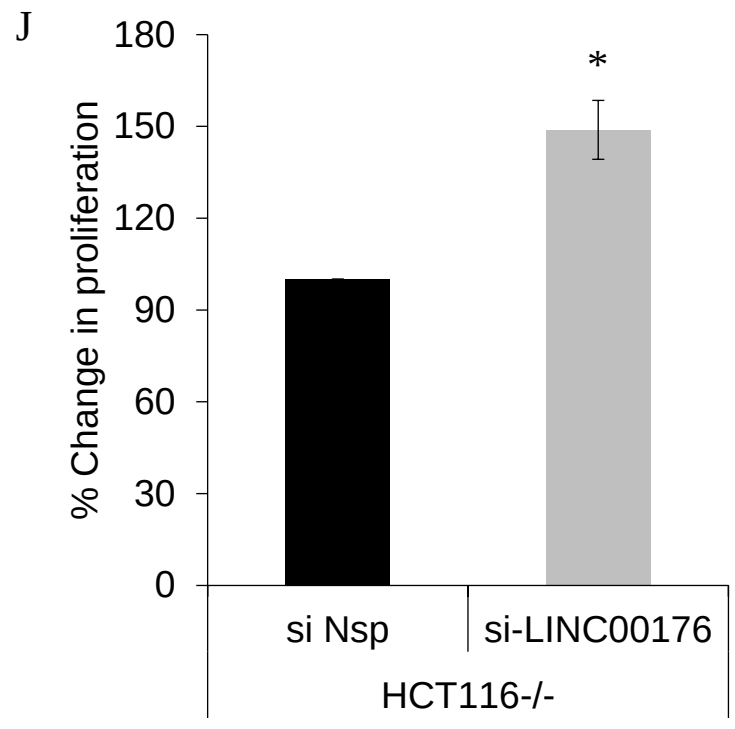
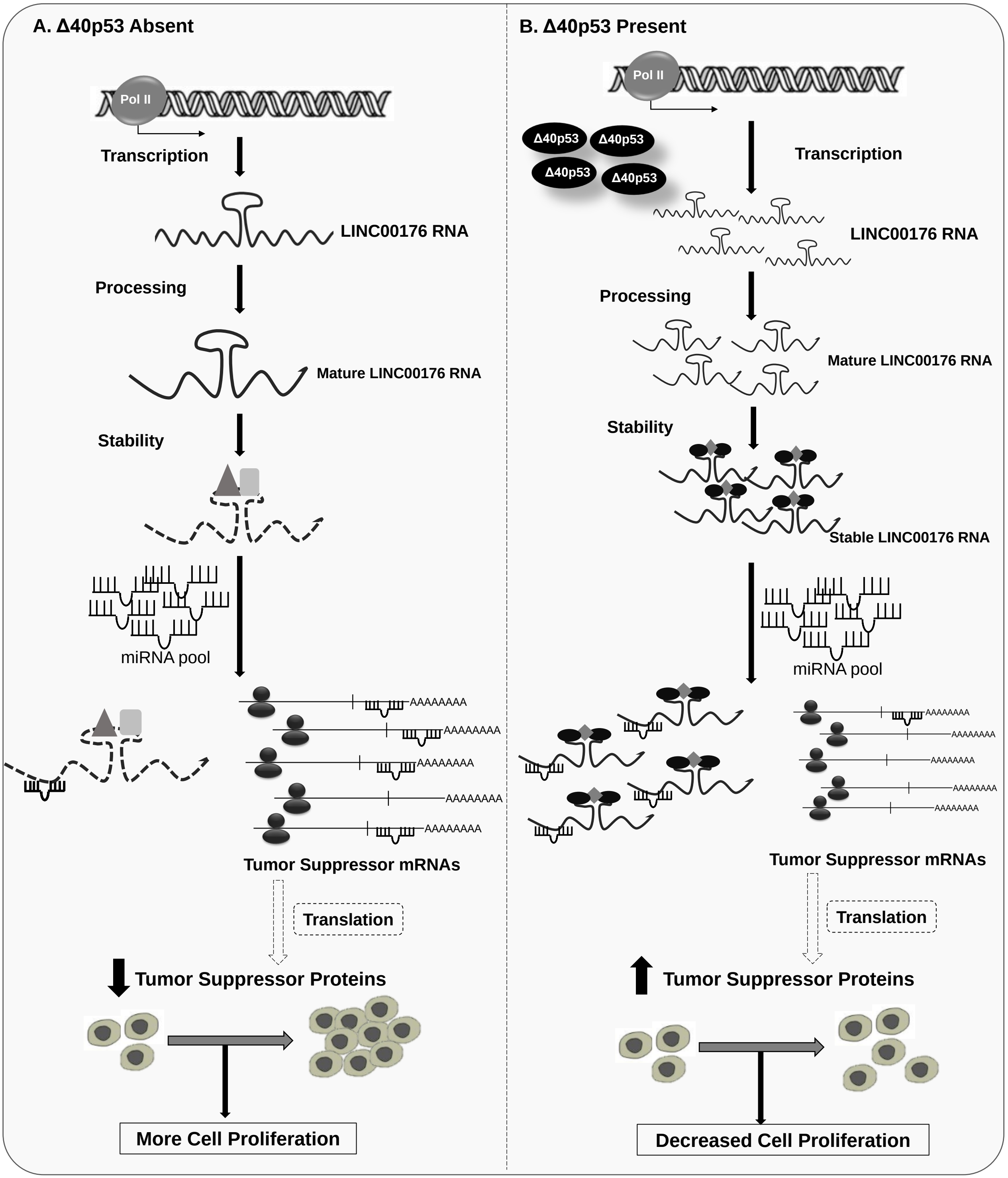

J
% Change in proliferation
180
150
120
90
60
30
0
*
si Nsp
si-LINC00176
HCT116-/-

Figure: 6


A. Δ40p53 Absent
Pol II
Transcription
LINC00176 RNA
Processing
Mature LINC00176 RNA
Stability
miRNA pool
AAAAAAAA
Tumor Suppressor mRNAs
Translation
Tumor Suppressor Proteins
More Cell Proliferation
B. Δ40p53 Present
Pol II
Δ40p53
Transcription
LINC00176 RNA
Processing
Mature LINC00176 RNA
Stability
Stable LINC00176 RNA
miRNA pool
Tumor Suppressor mRNAs
Translation
Tumor Suppressor Proteins
Decreased Cell Proliferation