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

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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 axis. Based on early research from our laboratory, we selected LINC00176 for further exploration. LINC00176 levels were affected by the overexpression and knockdown of Δ40p53. Further, under DNA damage, ER stress, and glucose deprivation, LINC00176 was upregulated in HCT116 p53-/- cells (harboring only Δ40p53) compared to HCT116 p53+/+ cells. Additionally, ChIP and RNA stability assays revealed that Δ40p53 transactivates LINC00176 transcriptionally and stabilizes it post-transcriptionally. We ectopically overexpressed and knocked down LINC00176 in HCT116 p53-/- cells, which affected proliferation, cell viability, and the expression of epithelial markers. Finally, RNA pulldown revealed that LINC00176 sequesters several putative miRNA targets. These results provide important insights into the pivotal role of Δ40p53 in regulating the lncRNA-miRNA axis.

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 are capable of modulating 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 they 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 (11). It also regulates proliferation and glucose homeostasis in mice (12). Moreover, it uniquely regulates genes involved in apoptosis (13) and 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). One of our latest studies showed that Δ40p53 can specifically regulate miR-186-5p (17). However, although 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 have been shown to be 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 noncoding 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). Therefore, we were interested in exploring the regulation of long noncoding RNAs by Δ40p53.

To understand the potential role of Δ40p53-mediated regulation of lncRNAs, we investigated a cellular lncRNA profile (unpublished observation) in our laboratory in H1299 cells (a p53-null cell line) with overexpression of full-length p53, Δ40p53, or both isoforms. Preliminary results suggested that several lncRNAs are differentially regulated under these conditions. Interestingly, LINC00176 was predominantly regulated by Δ40p53. LINC00176 is a long noncoding RNA that is differentially expressed in cancer and normal tissues and may have a tumor-suppressive role. Recent studies have demonstrated that LINC00176 regulates the expression of microRNAs in hepatocellular carcinoma (22) and that it promotes ovarian cancer progression by downregulating ceruloplasmin (23). By contrast, a report on genome-scale CRISPR screening has emphasized its possible role as a tumor suppressor lncRNA (24). LINC00176 is regulated through several pathways involving different upstream regulators and downstream targets. Based on the results obtained from our preliminary experiments, we decided to focus on LINC00176 and its differential regulation by p53 isoforms. We found

that LINC00176 is predominantly regulated by Δ40p53. Therefore, in this study, we explored the role of the Δ40p53/p53-LINC00176 axis in microRNA-mediated regulation of 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 (25). starBase v2.0 database (https://starbase.sysu.edu.cn/starbase2/browseNcRNA.php) was used to identify potential miRNA targets of LINC00176 (26). 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). 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). dbDEMC2.0 has been used to perform a meta profiling of selected miRNAs (https://www.biosino.org/dbDEMC/help#help00) (27).

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-/-). 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 four hours 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 (2μM) 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 onlyΔ40p53 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) (28,29). 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 sequence for p53 siRNA, directed against the 3’UTR of p53; therefore, it targets both p53 as well as Δ40p53. A non-specific siRNA (Dharmacon) was used in the partial silencing of p53/Δ40p53 in the experiments as control. si-LINC00176 (5'-CUCGUUCUGUAGACUUGUU-3') was used for partial silencing of LINC00176 (a generous gift from Dr. T Tamura, Institut fuer Biochemie, Hannover, Germany) (22).

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 to detect p53 and Δ40p53. 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 absolute alcohol. RNA amount was quantified with 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 2-5µg of total RNA has been used for checking the expression of mRNAs and lncRNAs, 50ng 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 changes in expressions, 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 $\Delta\Delta Ct$ = ΔCt target sample) −ΔCt (control sample). Melting curve analysis of every (q)PCR was conducted after each cycle.

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 (29). 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 were identified by reverse transcription (RT) and real-time, quantitative (q)PCR.

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 amount of MTT incorporated in the cell at 560 nm wavelength. The reading taken on the first day is considered as Day 0. Similarly, the reading was measured on Day 2 and Day 4 and Day 6 post re-seeding to check the effect on cell viability over a period of time.

Cell cycle Analysis

HCT116-/- cells were transfected with either LINC00176 o/e or sh-LINC00176 construct, 48 h post transfection the cells were harvested and fixed with methanol. The fixed cells were treated with RNase A (10 mg/ml) for 1 h at 37°C, following which PI (Propidium iodide-1mg/ml) was added to the cells and taken for Flow cytometric analysis.

Colony formation Assay

HCT116-/- cells were transfected with either LINC00176 o/e or sh-LINC00176 construct. 36 h post transfection 1000 cells from each condition were reseeded on 6 well plate and was incubated for 10-14 days to observe the number of colonies obtained. Post 10-14 days, cells were harvested by directly adding methanol to the plates for cell fixation, followed by which the cells were stained with Crystal Violet solution. After the cells got stained, the excess colour was washed with water 2-3 times, following which the colonies were quantified for any differences in number.

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

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, LINC00176 is known to have multiple regulators that govern important cancer-driven processes. We assessed the expression of LINC00176 using the GEPIA database. LINC00176 was differentially expressed in different cancer types **(Figure S1A).** Box plot analysis suggested that the expression of LINC00176 was lower in tumor tissues (lung and colon) than in normal tissues, which is analogous to WT p53 and Δ40p53 levels in cancer **(Figure S1B)**. To draw a direct association with p53, we assessed the correlation between p53 and LINC00176 expression and found that in normal tissues, LINC00176 was positively correlated with p53 levels **(Figure S1C, E, G).** However, in tumor tissues, they were negatively correlated **(Figure S1D, F, H)**. This observation suggests that LINC00176 may be positively regulated by WT p53 in normal conditions and negatively regulated by mutant p53 in tumor conditions. Since Δ40p53 was not annotated in the GEPIA database, we could not analyze the correlation between Δ40p53 and LINC00176. However, we hypothesize that Δ40p53 is one of the most important isoforms of p53 and could be a potential regulator of LINC00176.

Given the 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 was highest in cells with Δ40p53 overexpression, suggesting that Δ40p53 might be a more important regulator than p53. 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, we did not observe a significant decrease in LINC00176 in either cell line, which may be 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 LINC00176 is regulated by both p53 translational isoforms, 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.

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, LINC00176 was upregulated in the doxorubicin-treated and glucose-deprived cells; however, in cells treated with thapsigargin, there was no significant change **(Figure S2 A-F)**. It is unclear whether p53 or Δ40p53 was responsible for the increase in LINC00176 in HCT116+/+ cells as both were found increased upon induction of stress. 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-K),** indicating that Δ40p53 is 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.

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 that there are 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 (22,24). 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 is in accordance with the reported literature that p53 and Δ40p53 regulate each other's activity through the formation of hetero-oligomers (4). Hence, these results strengthen the observation that Δ40p53 controls LINC00176 regulation more than p53.

We also wanted to further assess the ability of Δ40p53/p53 to directly regulate LINC00176 at the RNA level. p53 is known to directly affect certain RNAs through its C-terminal domain (30) which is shared with Δ40p53. We aimed to determine whether Δ40p53 could regulate LINC00176 RNA directly or indirectly. Analysis using CATRAPID and RPI-Seq software was used to identify potential interactions between mature LINC00176 and Δ40p53 **(Figure S3 C-F)**. Both the databases predicted probable binding regions and positive interaction score for LINC00176 and the p53/Δ40p53. Because the protein domains of Δ40p53, which might

bind to LINC00176, are common with p53, we cannot exclude the possibility that there could be an interaction between LINC00176 and p53. However, based on our initial results, we focused on the Δ40p53-LINC00176 interaction. Based on the probable RNA binding, we treated cells with actinomycin D after transfection with siΔ40p53 to assess LINC00176 stability **(Figure 3F).** The results indicate that knockdown of Δ40p53 decreased LINC00176 levels after treatment with actinomycin D, indicating that the stability of LINC00176 is increased by Δ40p53 **(Figure 3G, H)**. Therefore, the increased LINC00176 levels observed in the presence of Δ40p53 in comparison to p53 could be due to multistep regulation at both the transcriptional and RNA stability levels.

Deciphering the mechanism of action of LINC00176

Thus far, the results obtained in this study indicate that Δ40p53 can regulate long noncoding RNAs in addition to miRNAs (17). Therefore, it is important to understand how LINC00176 functions in cells and what its mechanism of action could be **(Figure 4A)**. Long noncoding RNAs are known to interact with miRNAs, acting as a decoy or promoting their degradation, thereby impacting cellular processes (31). 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 possible targets of LINC00176, as LINC00176 levels are very high in HCT116-/- cells. In particular, miR-9-5p, which is a known target of LINC00176, 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 assays were performed **(Figure 4G).** miRNA levels were quantified in the pulldown fraction and normalized to input levels (**Figure 4F**). 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. However, miR-761 did not show any enrichment in the pulldown fraction, implying that it might have an indirect interaction with LINC00176

or could be degraded in a complex. These results indicate that LINC00176 is capable of titrating miRNA levels in cells, which may affect cellular processes.

The impact of the Δ40p53-LINC00176 axis on cellular behavior

LncRNAs regulate a wide variety of cellular processes (32). To understand the 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. 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 S5A)**. We further analyzed cell viability after LINC00176 overexpression or knockdown and observed that LINC00176 overexpression decreased cell viability **(Figure 5G, S5 B, C)**, whereas LINC00176 knockdown increased cell viability **(Figure 5H, S5 D, E)**. However, these effects on cell viability may not be very significant, 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 5I)**. Δ40p53 abundance is closely linked with cell cycle phases (3); therefore, we also assessed the effect of LINC00176 on cell cycle progression. We observed a slight increase in the proportion of cells in G1 phase and a slight decrease in the proportion of cells in S phase with LINC00176 overexpression **(Figure S5F)**. Furthermore, to check for any effect on cell growth, we performed a colony formation assay, wherein we found a decrease in the number of colonies after LINC00176 overexpression **(Figure S5G)** and an increase in the number of colonies after LINC00176 knockdown **(Figure S5H)**. 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 (33). Therefore, to identify important cancer signaling pathways, it is extremely important to identify novel lncRNAs that are regulated through the p53 pathway. *TP53* is the most frequently mutated gene in human cancer (34);

however, these mutations are not found in all cancers, indicating that there could be other factors interfering with WT p53 function (35). These factors, which have complicated our understanding of p53 signaling, include more recently discovered p53 isoforms (36). 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 (37). 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 lines 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 there could be other factors involved in the regulation of LINC00176 because of certain observations. First, we did not observe a significant decrease in LINC00176 levels upon knockdown of Δ40p53. 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, there are other factors that regulate LINC00176 abundance. This regulation could be at the transcriptional or processing stages; therefore, we investigated any direct binding between the molecules.

Δ40p53 was previously thought to be devoid of transactivation capacity because it lacks TAD1 (38); however, subsequent studies reported that it can regulate some p53 target genes (13,39). 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 (40). 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 that the formation of hetero-oligomers between p53 and Δ40p53 interfered with the transactivation function of Δ40p53, which is consistent with

the results of earlier reports (8). We also observed that Δ40p53 regulates LINC00176 not only at the transcriptional level but also post-transcriptionally, since the stability of LINC00176 also decreased after Δ40p53 knockdown. p53 is known to bind to the lncRNA MEG3 (28); however, there are no previous reports of Δ40p53 binding to RNA. Whether Δ40p53 can directly or indirectly bind to LINC00176 RNA remains unknown and requires further investigation. Although we also observed LINC00176 upregulation with p53 expression, it is possible that p53 might regulate it through an unrelated mechanism.

The significance of LINC00176 upregulation by Δ40p53 was determined by examining different miRNA targets. We identified different miRNA targets for LINC00176, from which we selected a few targets based on the number of binding sites and their functions in different cancers **(Figure S4)**. The majority of the selected miRNAs are differentially expressed in cancer owing to their diverse regulatory pathways. 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 were sequestered by LINC00176. However, although the level of miR-761 increased upon LINC00176 knockdown, it was not sequestered by LINC00176. As lncRNAs are known to serve as miRNA decoys, LINC00176 may be degrading these miRNAs; however, the mechanism of miRNA regulation by LINC00176 needs to be further examined.

Finally, we examined the direct effects of LINC00176 on cellular behavior through ectopic overexpression or silencing. Based on the different hallmark processes of cancer (41) we examined EMT, cell proliferation, cell viability, cell cycle progression, and colony formation. LINC00176 overexpression enhanced the epithelial phenotype and upregulated Δ40p53, suggesting a possible feed-forward loop between Δ40p53 and LINC00176 through which they regulate the other's levels. However, LINC00176 knockdown did not give consistent results, as the expression of EMT markers 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 (35). This strengthens our hypothesis that, since there is a feed-forward loop between LINC00176 and Δ40p53, many of the lncRNA effects will be dependent on the levels of Δ40p53 and therefore will vary. Furthermore, we observed a decrease in cell viability and colony formation and a slight decrease in the proportion of cells in S phase with LINC00176 overexpression.

In conclusion, our results indicated that LINC00176 contributes to the tumor-suppressive function of Δ40p53, independent of p53FL function **(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.

ACKNOWLEDGEMENTS

We are grateful to Prof. J.C. Bourdon, University of Dundee, UK and Dr. Robin Fahraeus, INSERM, France, for providing us the anti-p53 antibody. We would like to thank Dr. Je-Hyun Yoon and Dr. Myriam Gorospe, National Institutes of Health, USA, for providing us with the TRAP constructs. We are grateful to Dr. T Tamura, Institut fuer Biochemie, Germany, for providing us with LINC00176 overexpression construct and siRNA. We would like to thank Prof. Sathees C. Raghavan for his valuable suggestions. We also thank the present and past SD lab-members for helpful discussion of the work. This work is supported by a research grant to SD from the Department of Biotechnology (DBT), Govt. of India. SD also acknowledges J.C. Bose fellowship for research support. This study was also supported by the DBT-IISc partnership program. DST Fund for Improvement of Science and Technology Infrastructure (FIST) level II infrastructure and University Grants Commission Centre of Advanced Studies support is acknowledged. We would like to thank Editage (www.editage.com) for English language editing.

AUTHOR CONTRIBUTIONS

AP and SD: Conception and design of studies, analysis and interpretation, and writing the article. AP 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 (Nsp si). (F) Western blot analysis of cell extracts from HCT116+/+ cells transfected with either si p53 (30nM) and non-specific si (Nsp si), probed with CM1. (G) Quantitative PCR of LINC00176 in HCT116-/- cells transfected with si Δ40p53 (30nM) and non-specific si (Nsp si). (H) Western blot analysis of cell extracts from HCT116-/- cells transfected with siΔ40p53 (30nM) and non-specific si (Nsp si), 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.

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. (K) Western blot analysis of cell extracts from HCT116-/- transfected with siΔ40p53 followed by Thapsigargin (0.1μM) treatment, probed with Bip Antibody.

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 siΔ40p53 followed by Actinomycin D treatment, probed with CM1.

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-/- transfected with siRNA (30nM) directed to LINC00176 and non-specific si (Nsp si). (E) Quantitative PCR of selected miRNAs in HCT116-/-cells transfected with siRNA (30nM) directed to LINC00176 and non-specific si (Nsp si). (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.

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

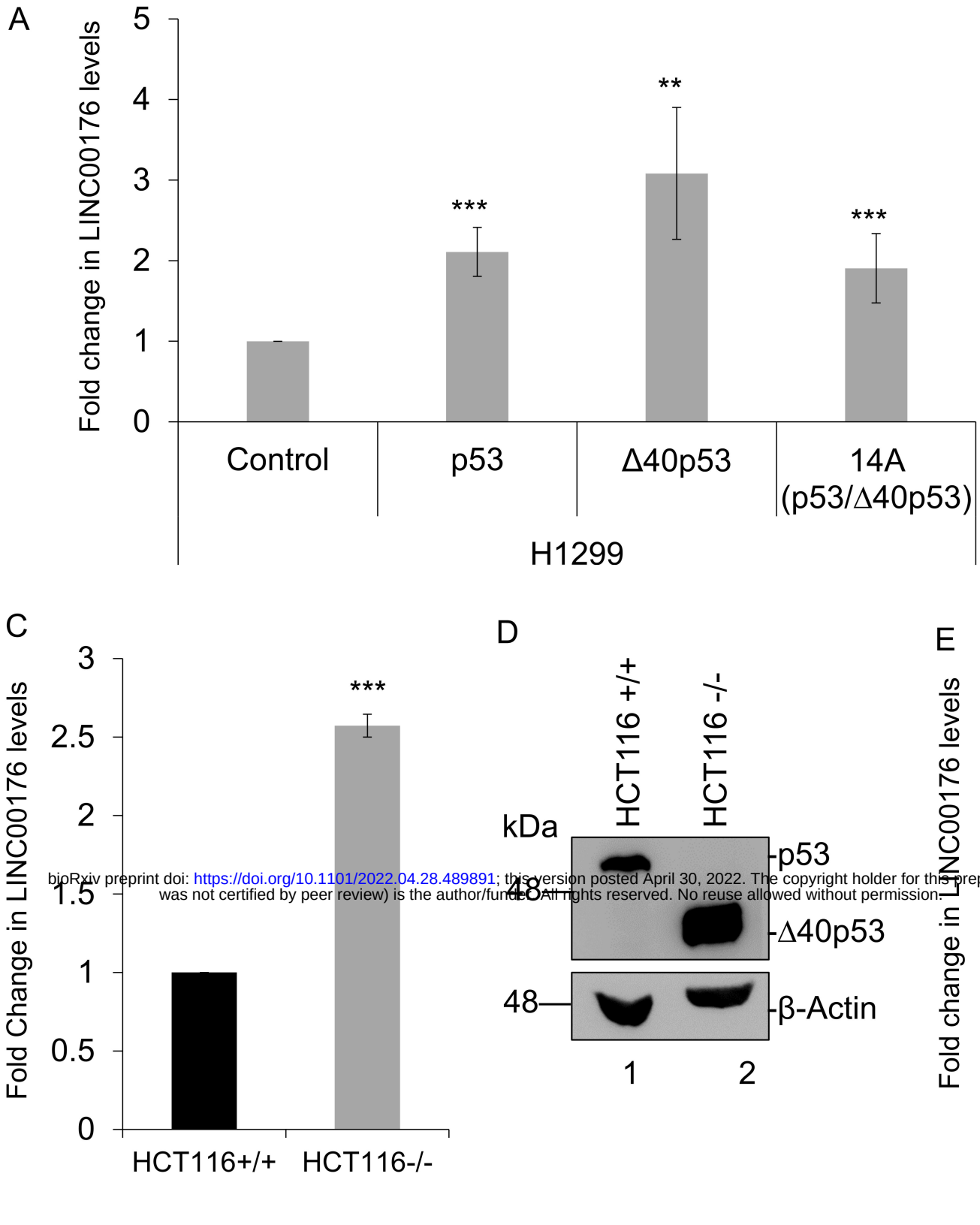
(A)Schematic of the different processes that has been checked for any probable effect by LINC00176 over-expression or partial silencing. (B) Quantitative PCR of LINC00176 in HCT116-/- cells transfected with LINC00176 overexpression construct. (C) Quantitative

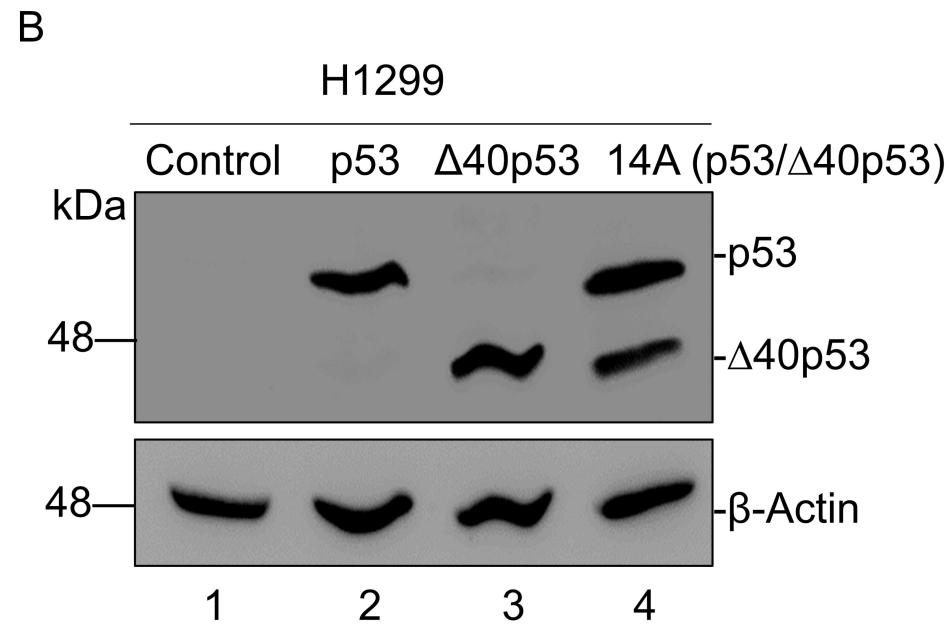
PCR of LINC00176 in HCT116-/- cells transfected with LINC00176 shRNA construct. (D) Quantitative PCR of EMT markers (E-cadherin, Vimentin, Slug) in HCT116-/- cells transfected with LINC00176 overexpression construct. (E) Western blot analysis of cell extracts from HCT116-/-cells transfected with LINC00176 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) 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, Day 4 and Day 6 post seeding (Data represented for Set 1). (H) 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, Day 4 and Day 6 post seeding (Data represented for Set 1). (I) 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.

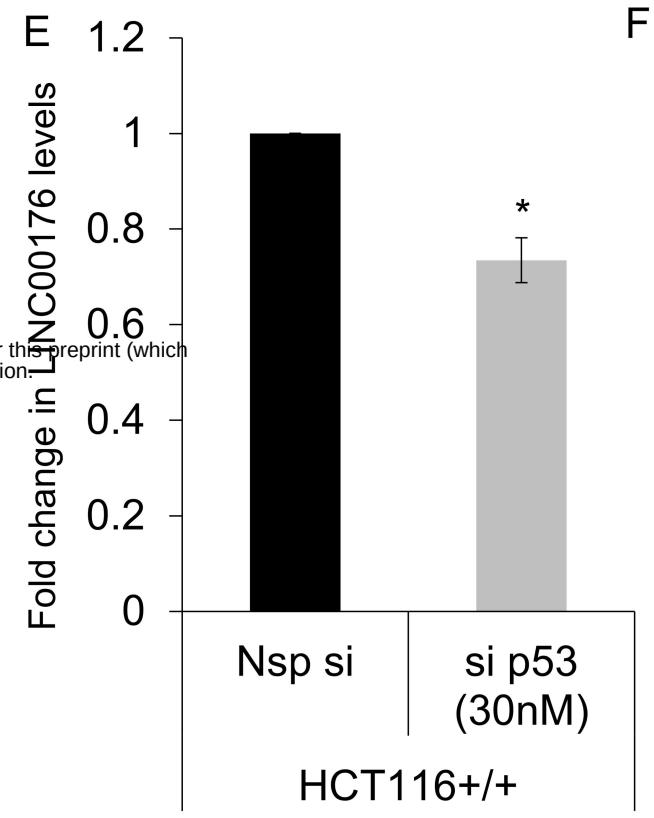
Graphical Abstract: Model depicting the regulation of Δ40p53-LINC00176-miRNA axis

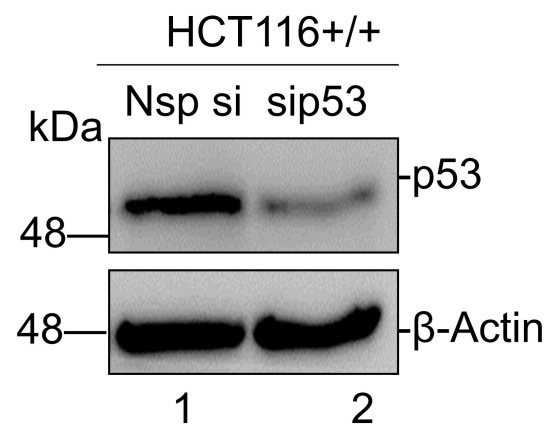
(A) In absence of Δ40p53, LINC00176 levels do not increase which leads to increased pool of free miRNAs which probably binds to several mRNA targets preventing their function, ultimately resulting in increased cell proliferation. (B) However, when Δ40p53 is present it upregulates LINC00176 levels both 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.

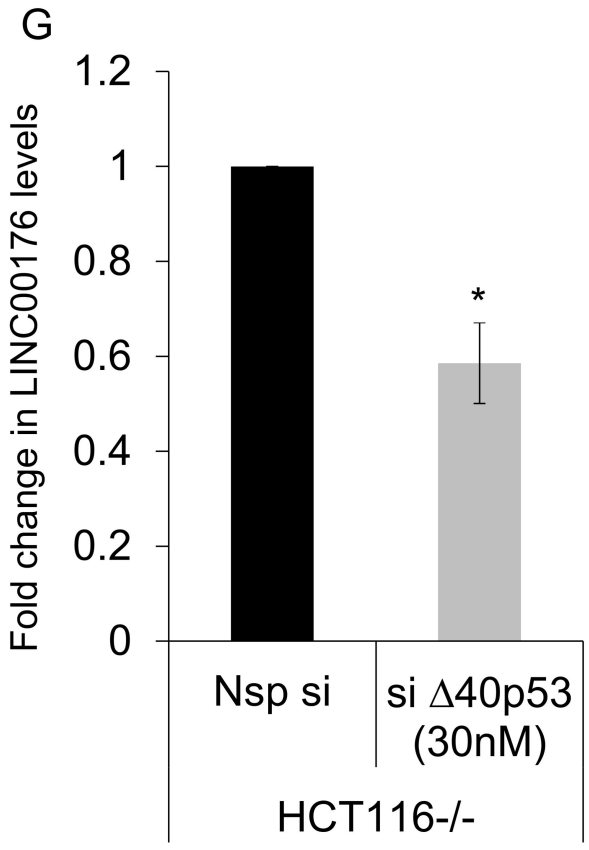
Figure: 1

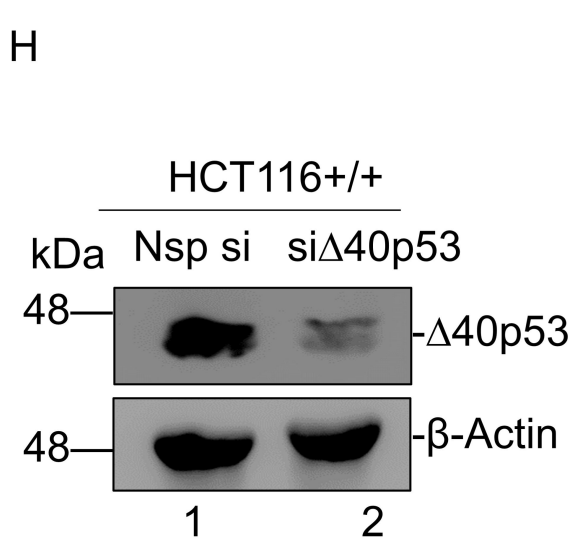


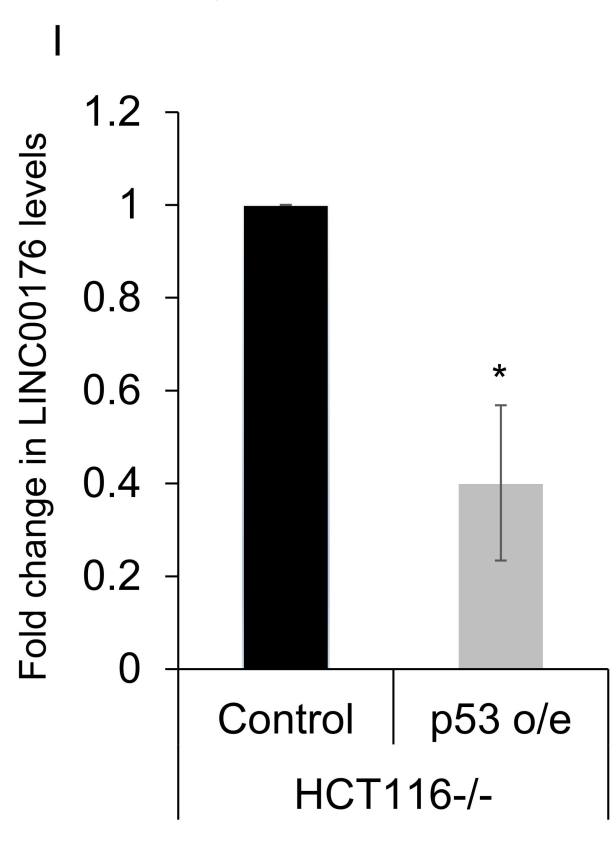












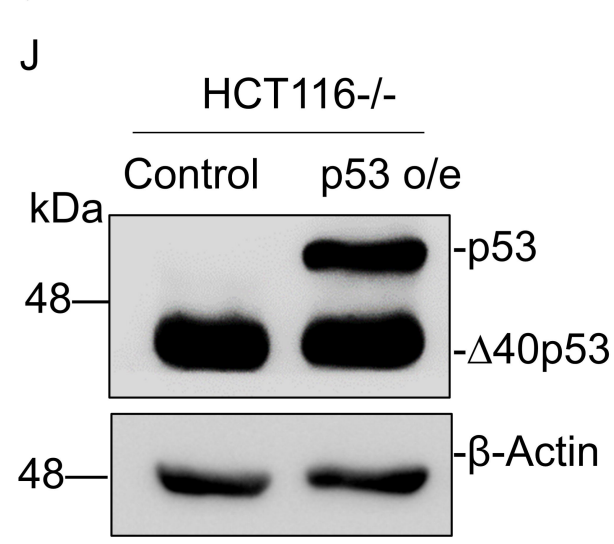
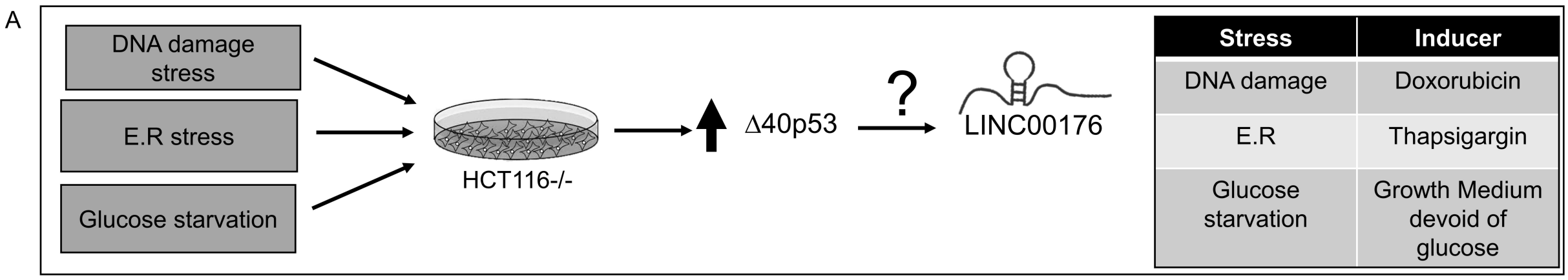
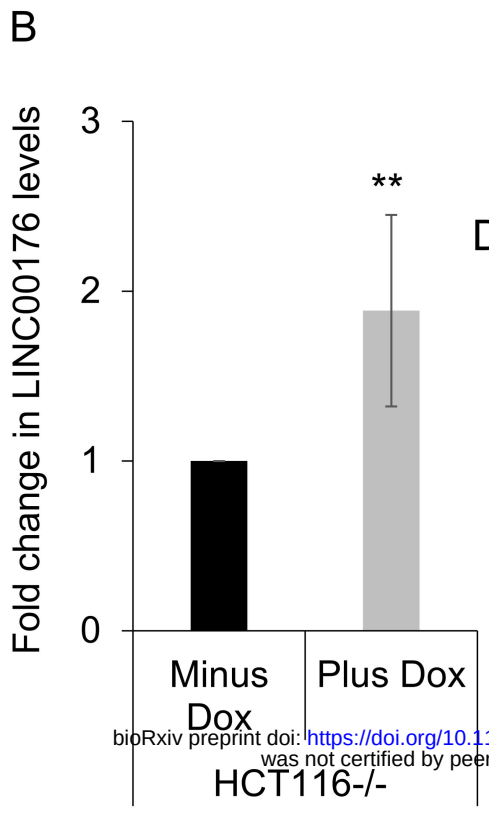
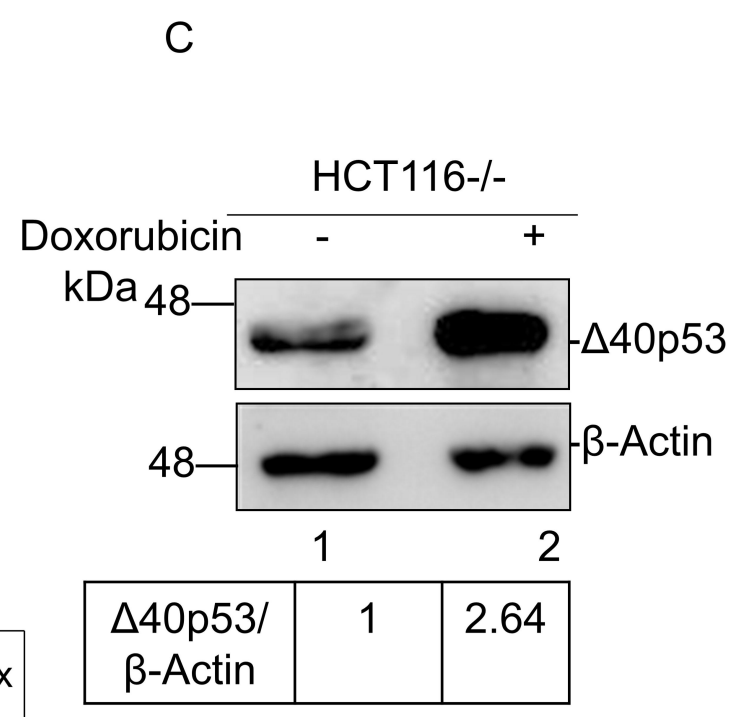


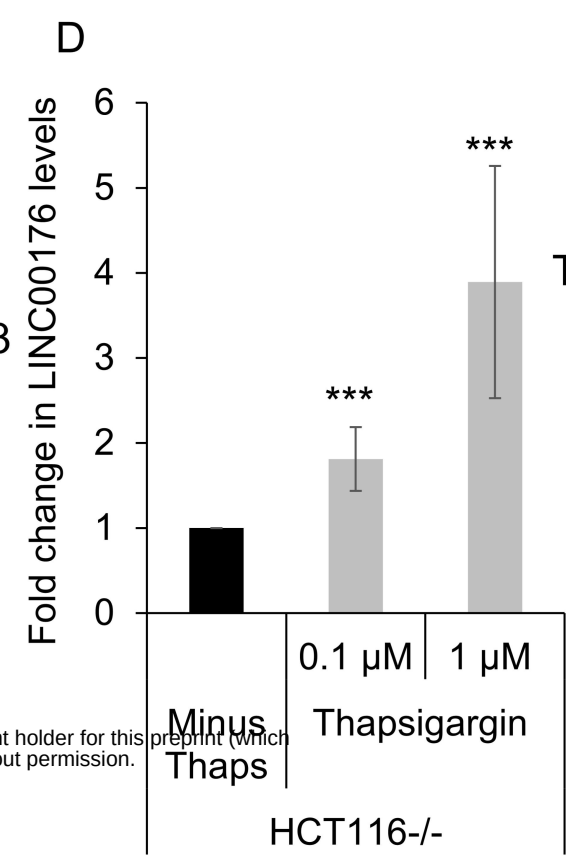
Figure 1. Selection and validation of LINC00176 for further studies

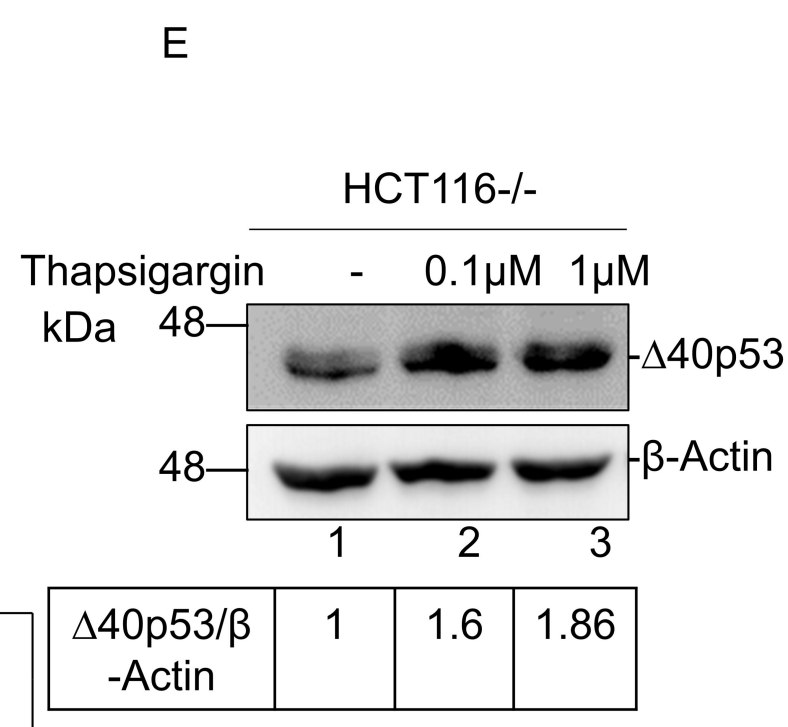
Figure: 2

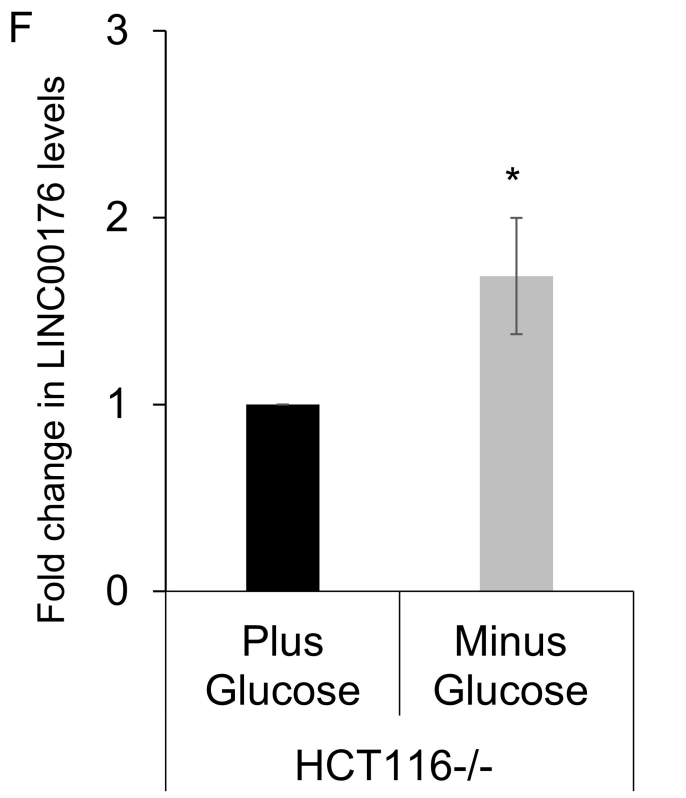


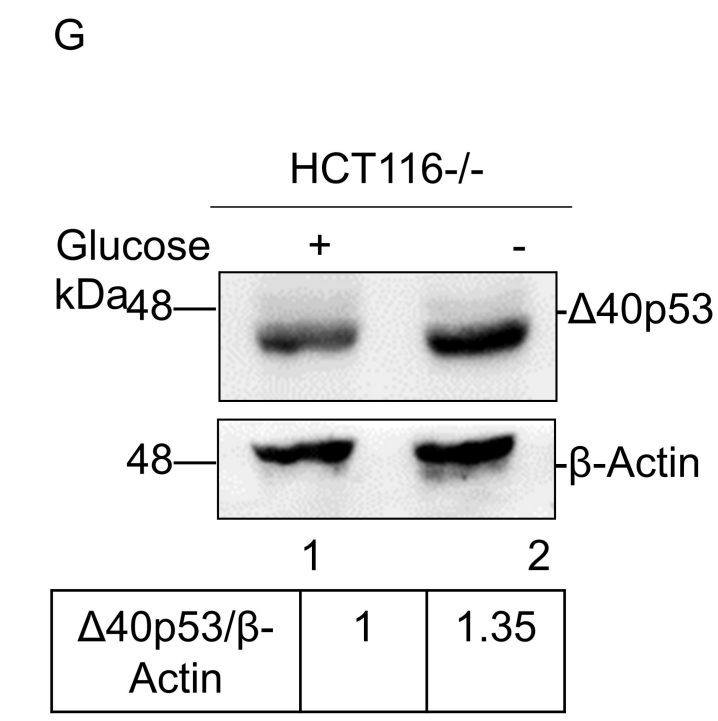


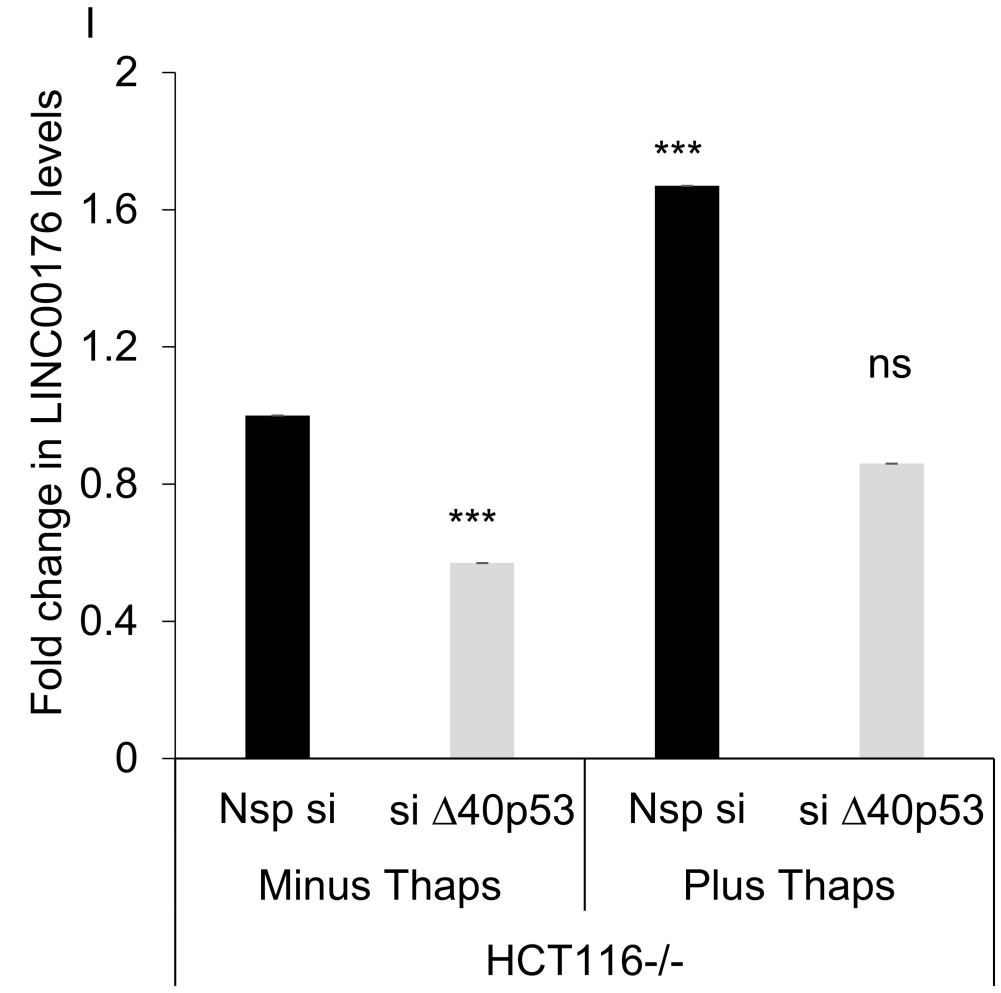


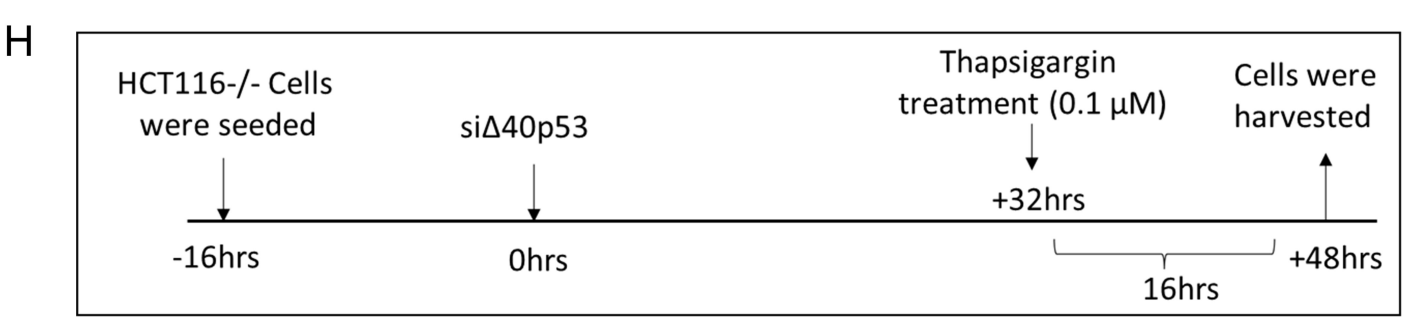


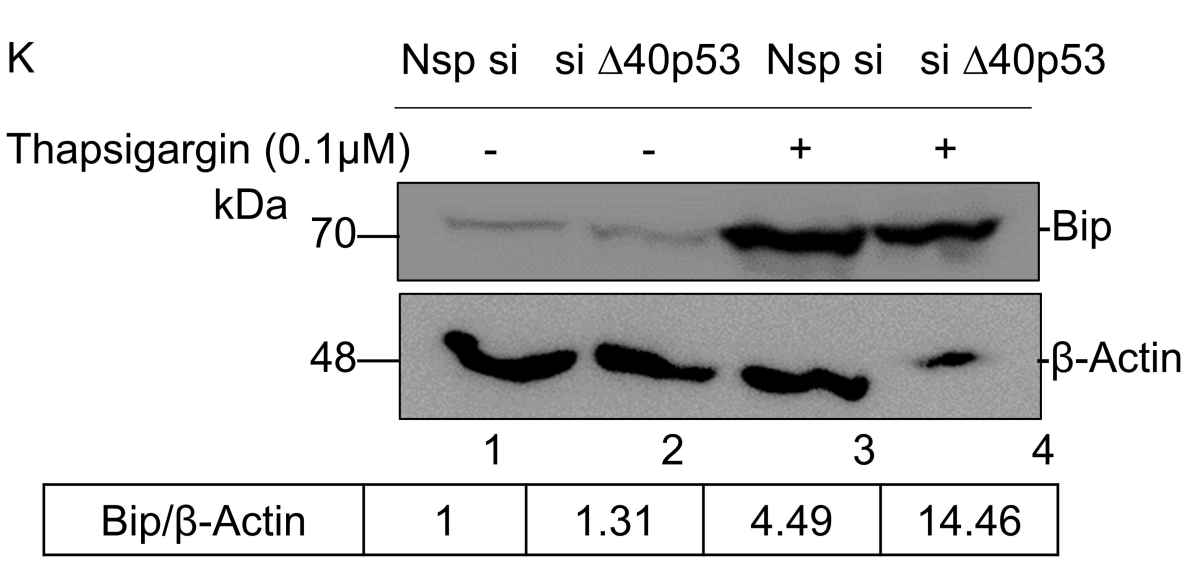












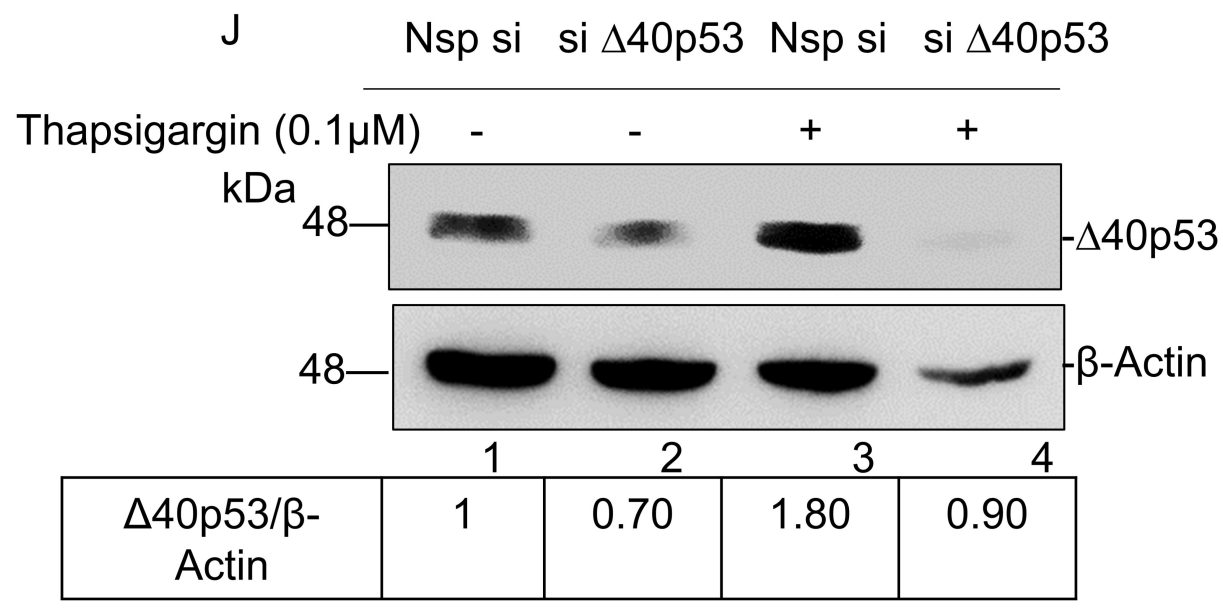
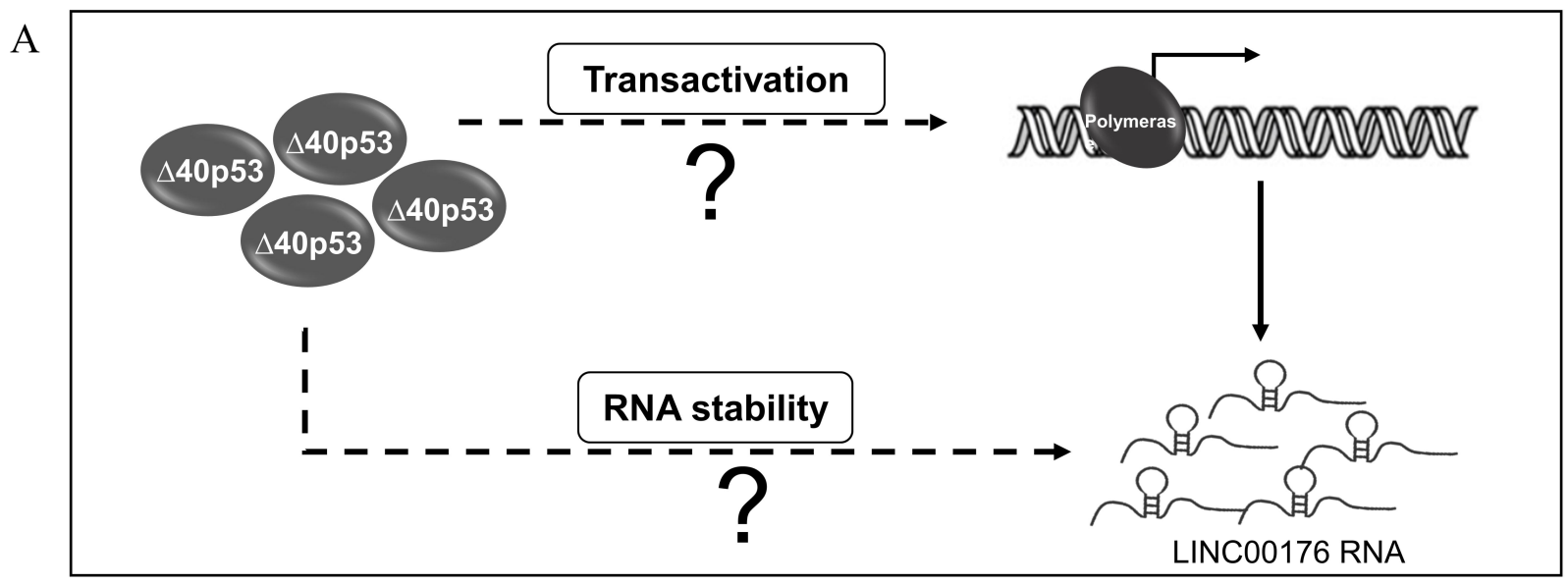
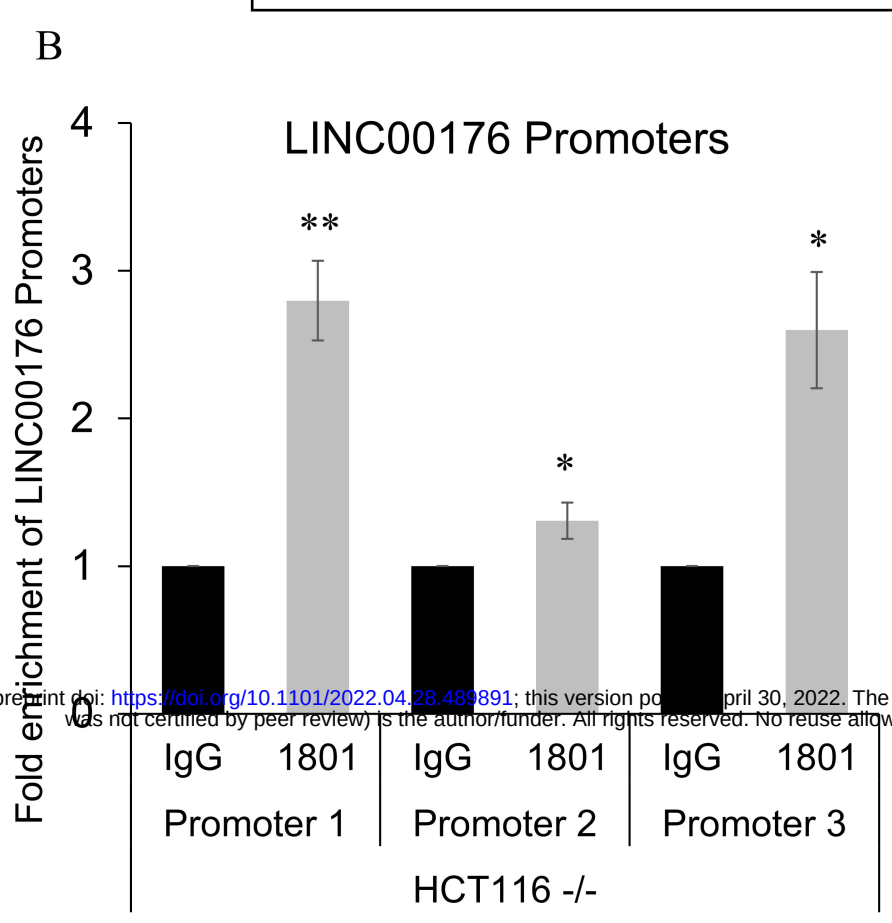
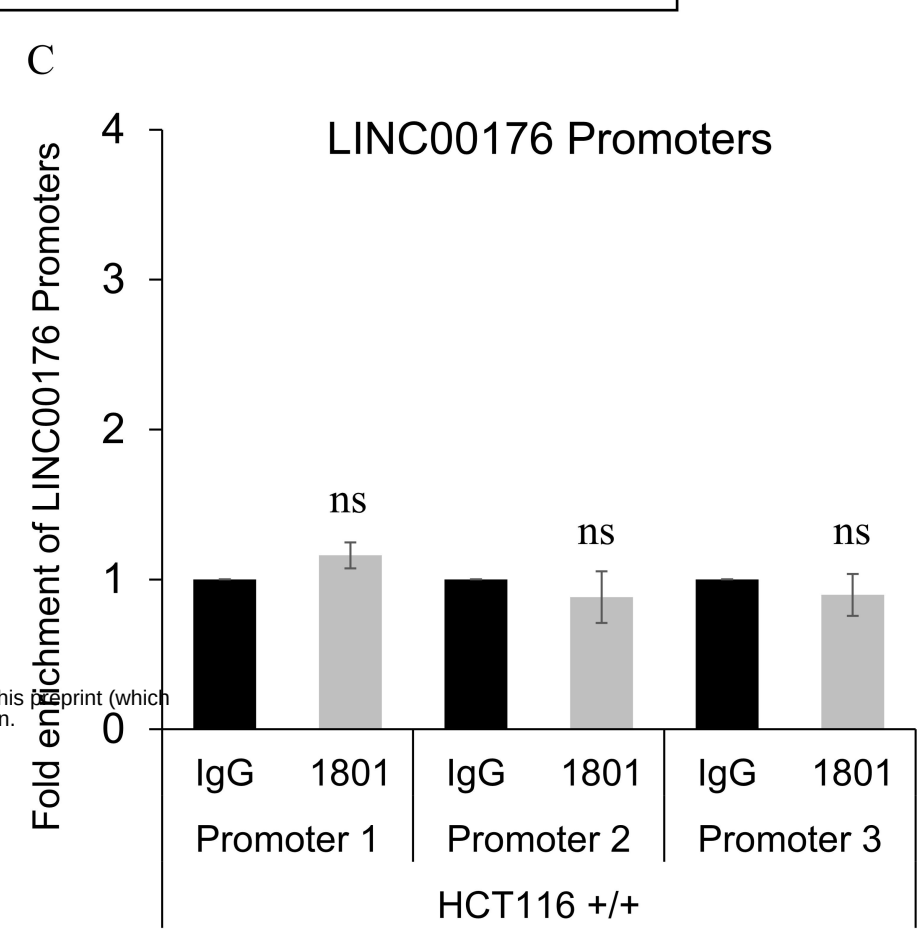


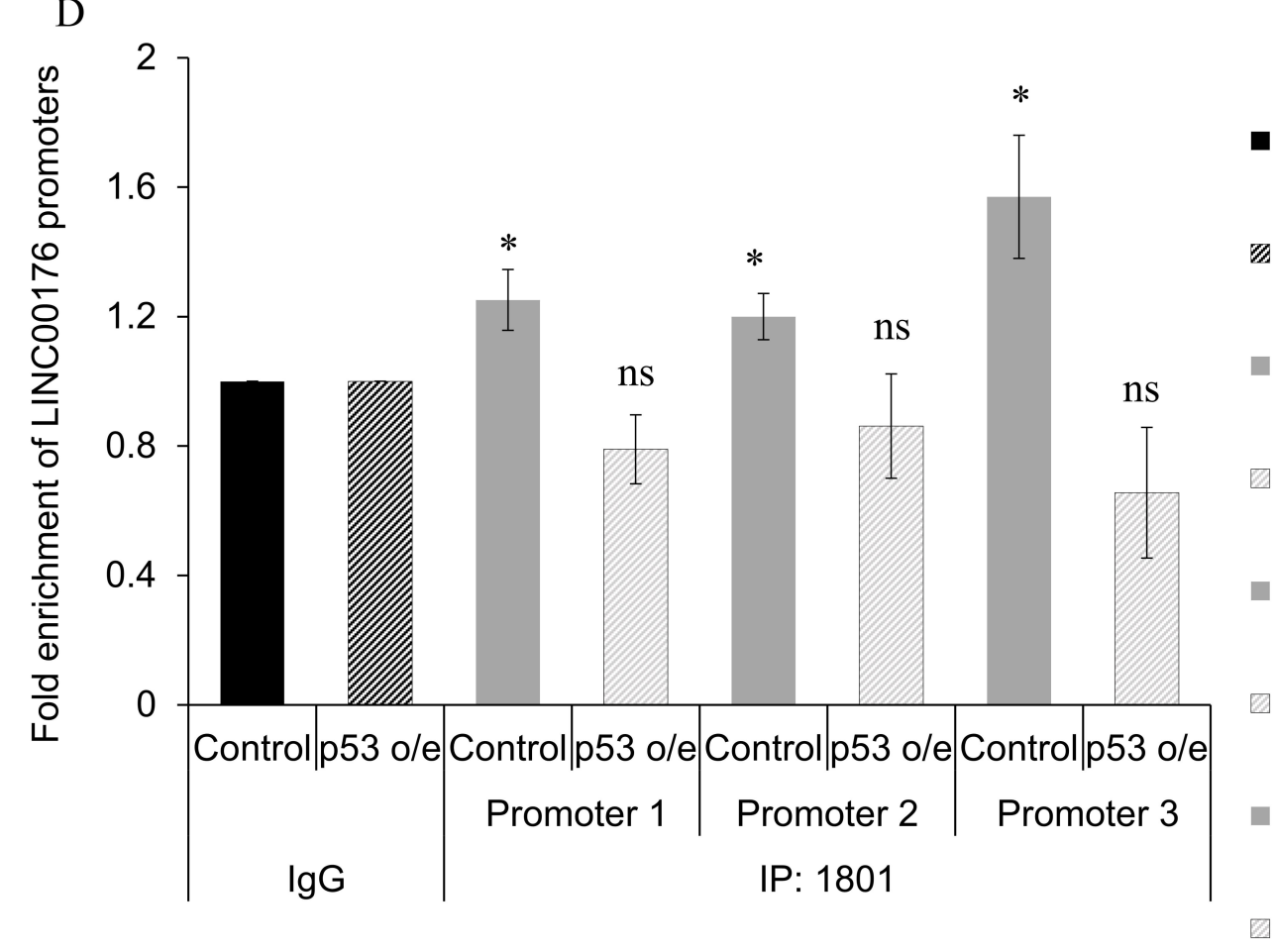
Figure 2. Regulation of LINC00176 under different stress conditions

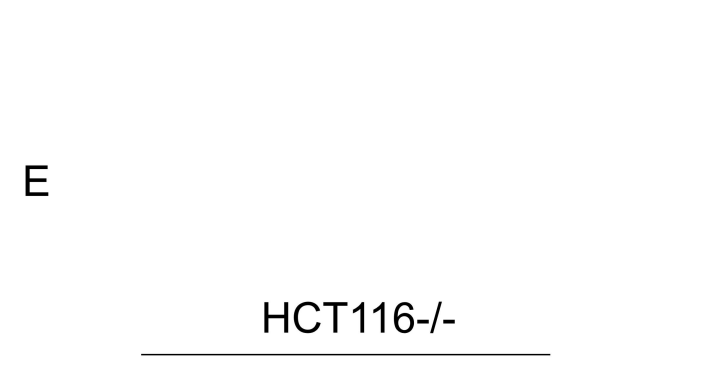
Figure: 3

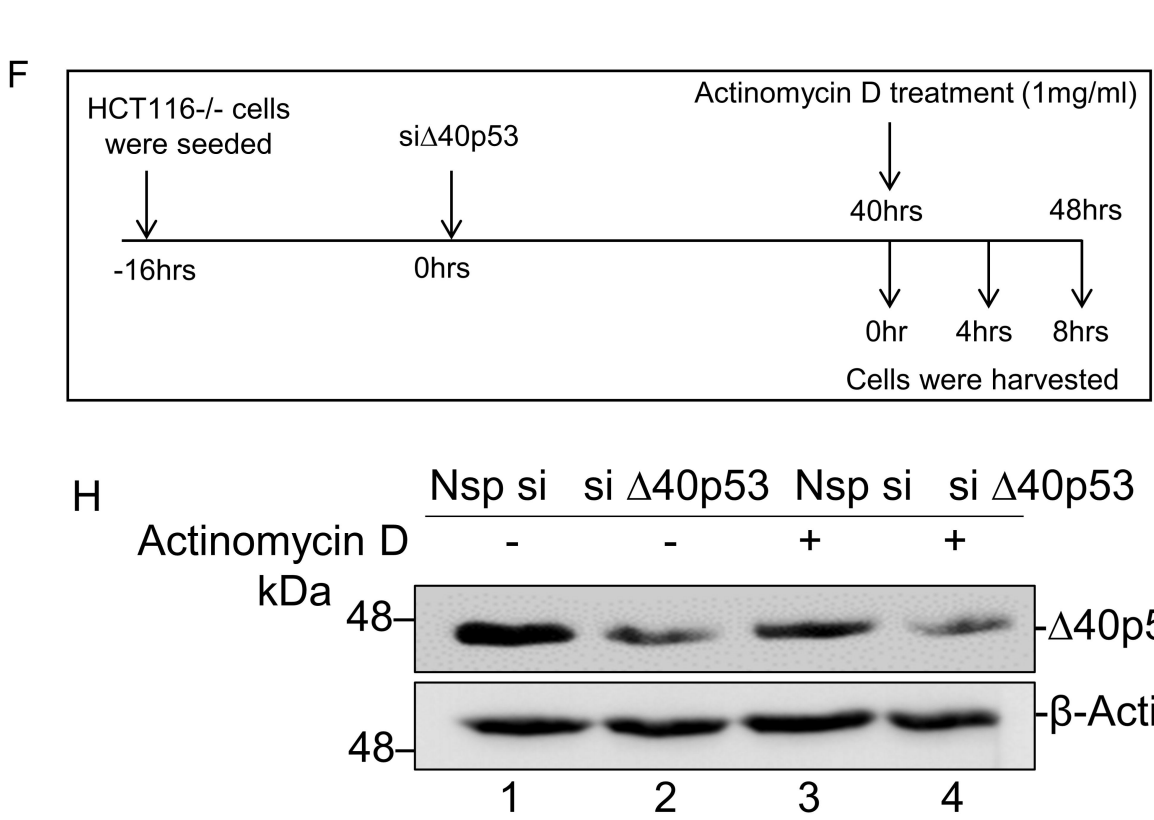












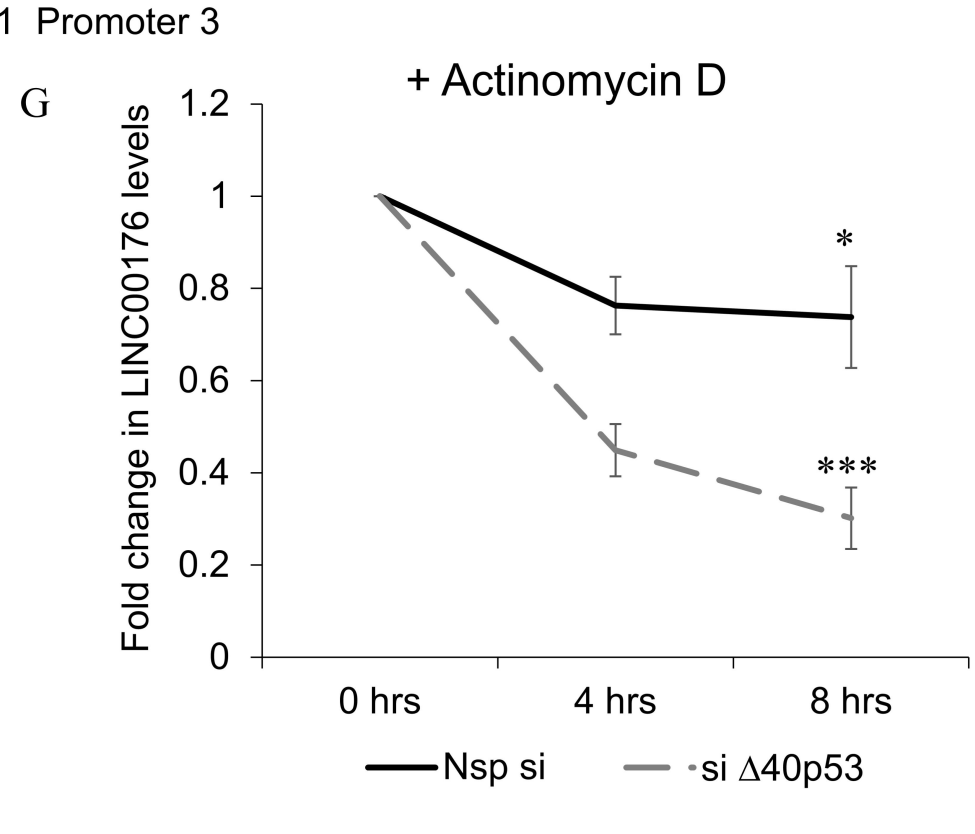
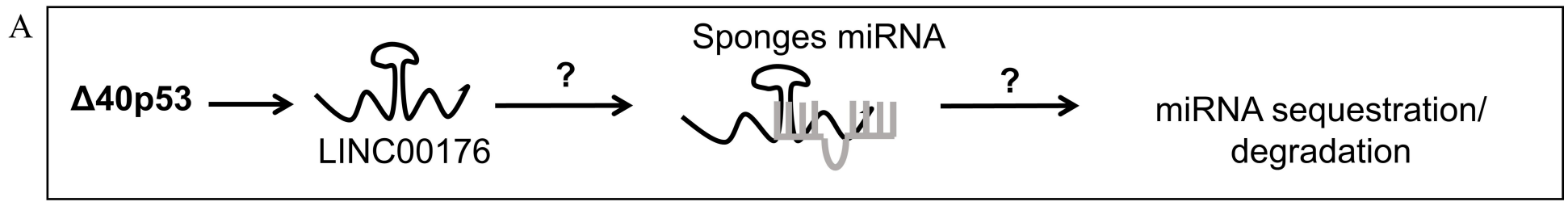


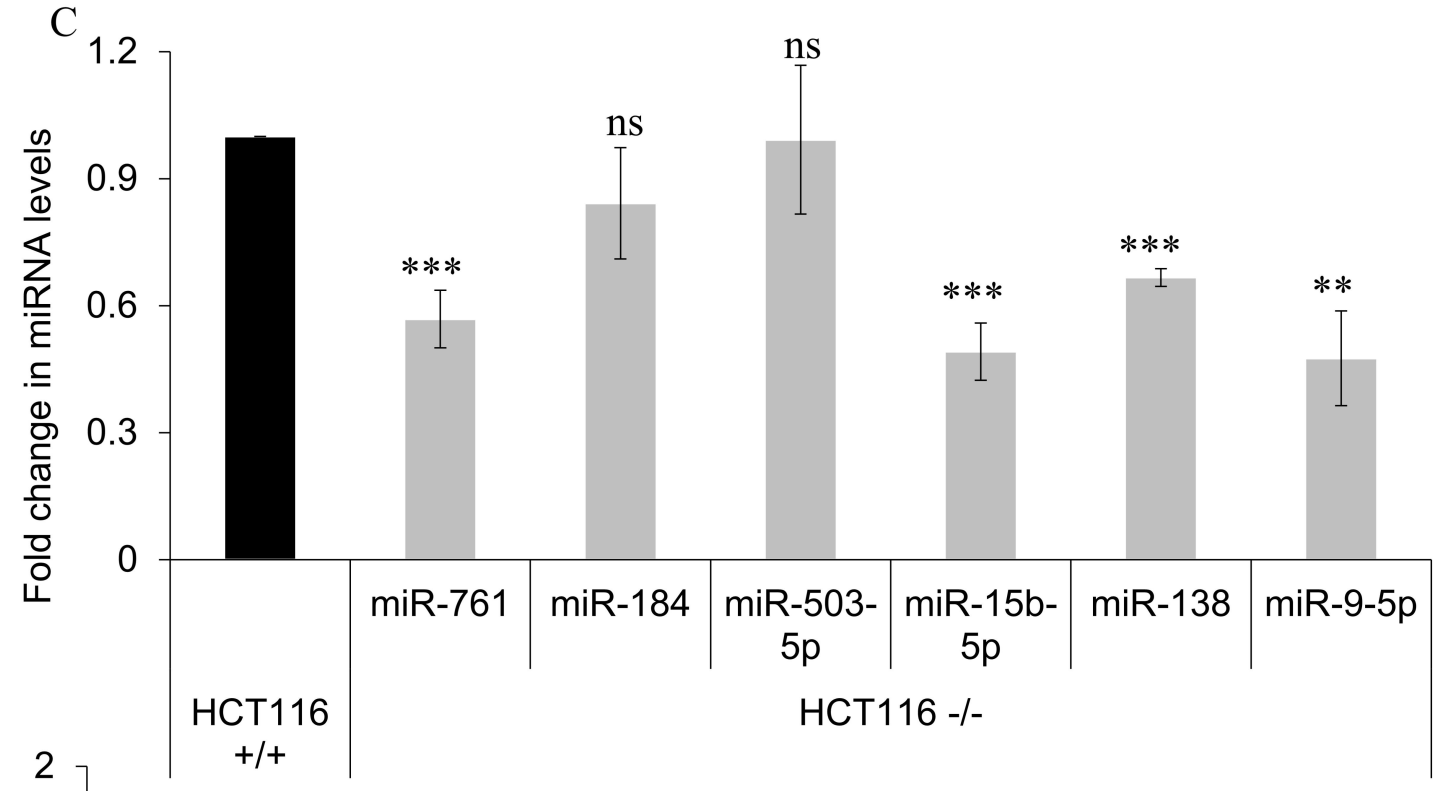
Figure 3. Mechanistic basis of LINC00176 regulation by Δ40p53/p53

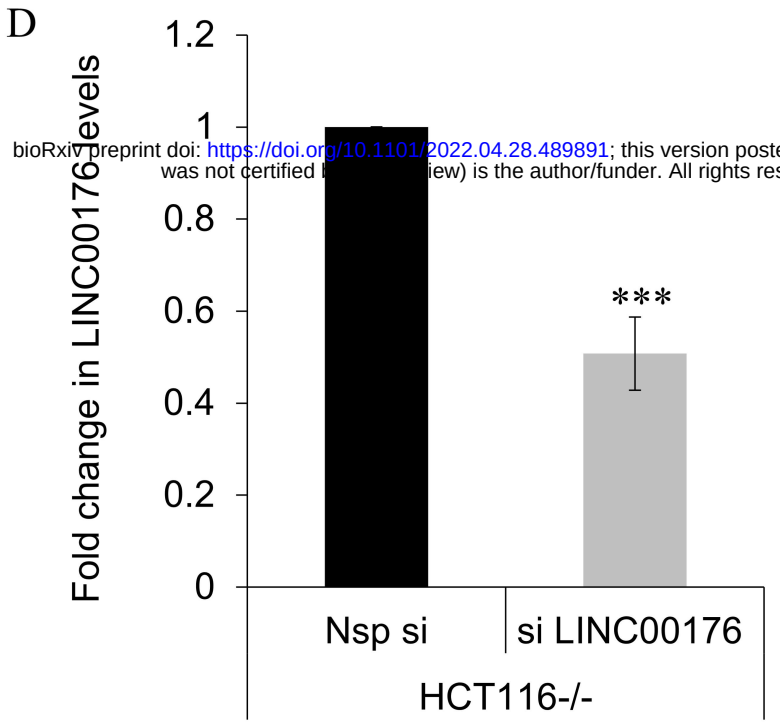
Figure: 4

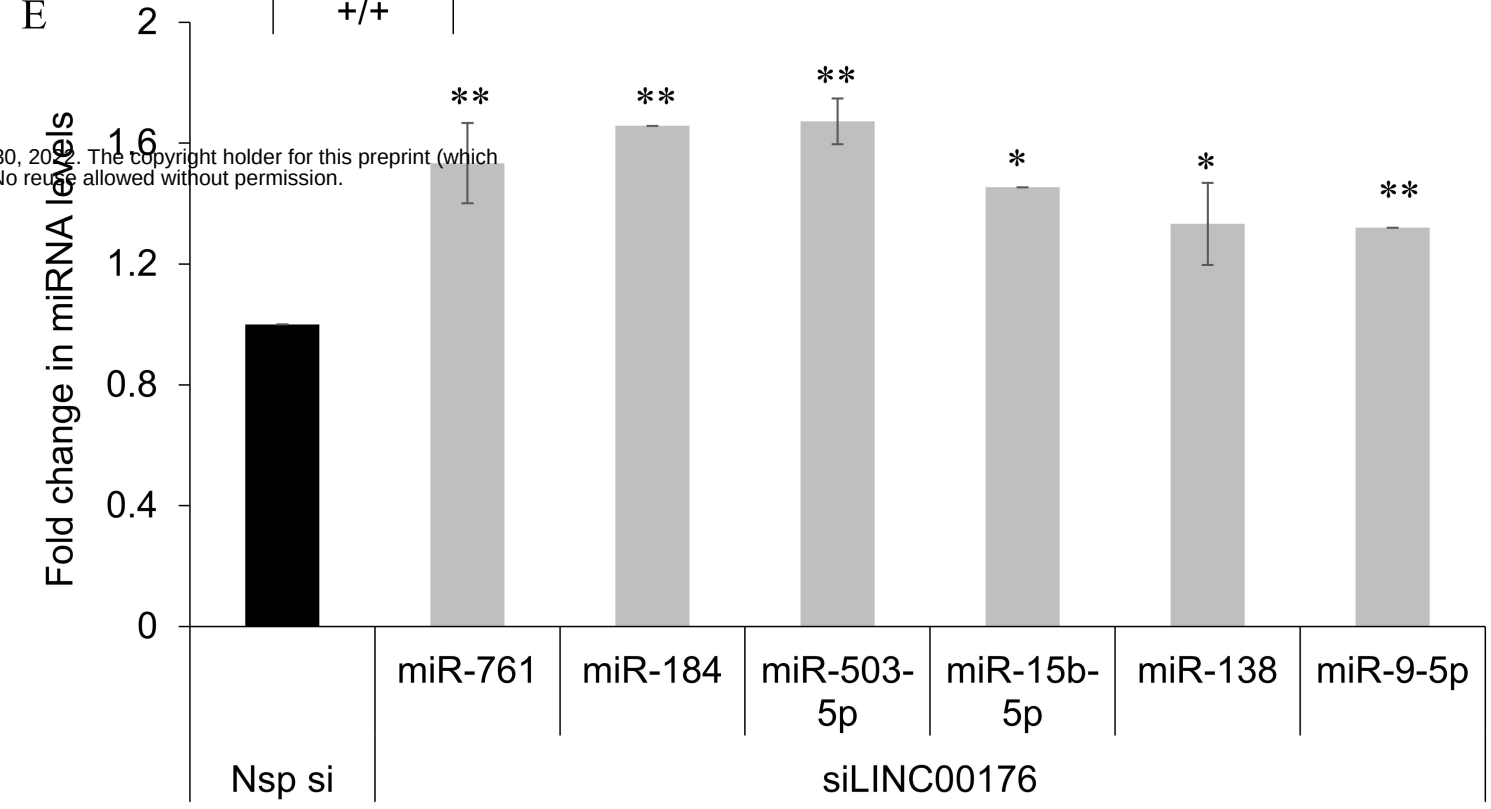


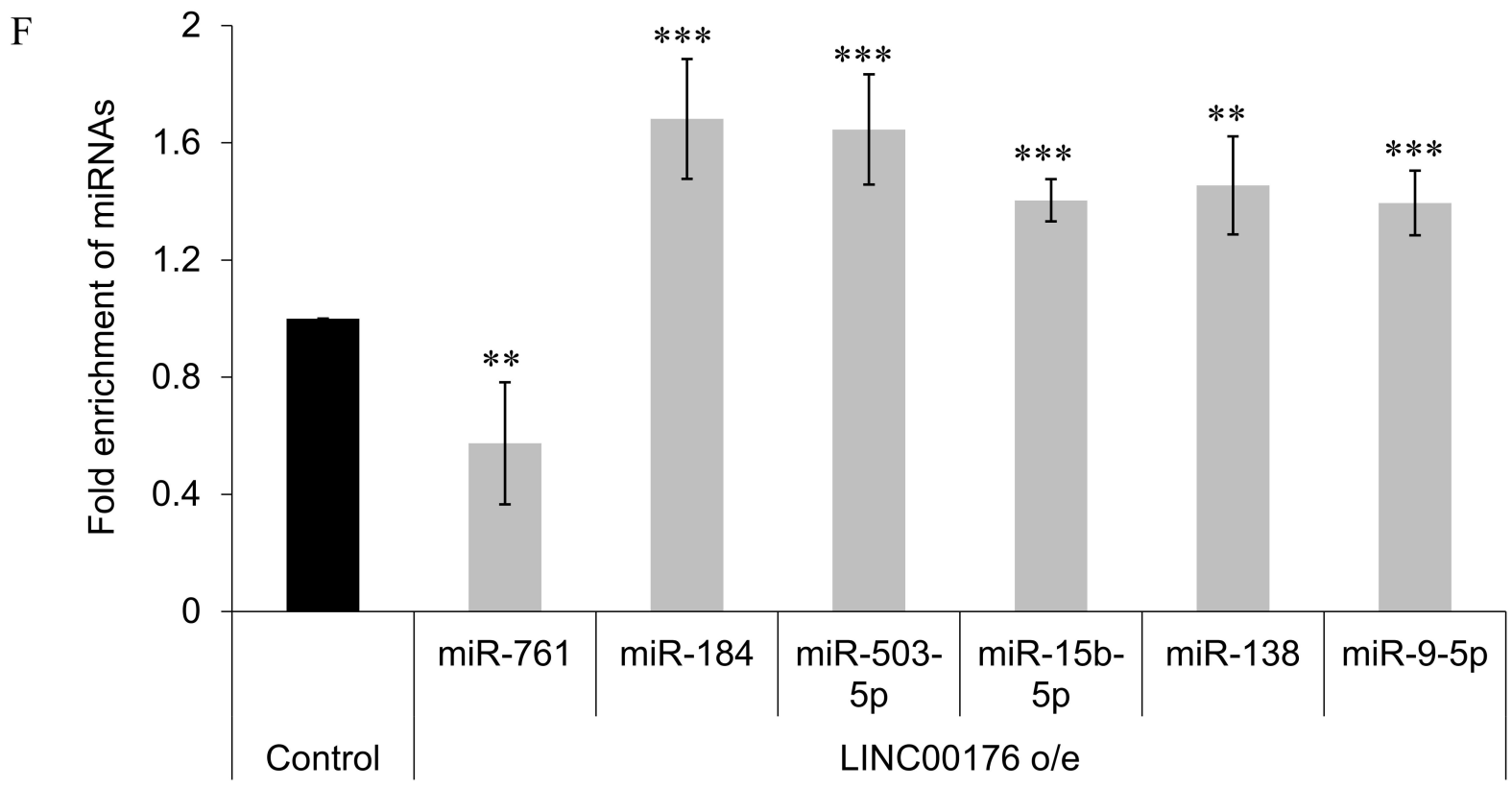
B

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









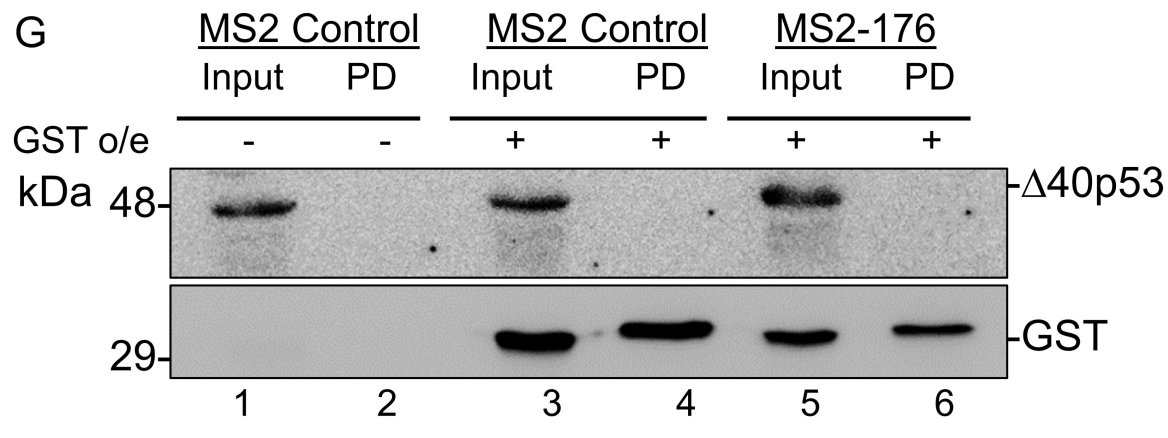
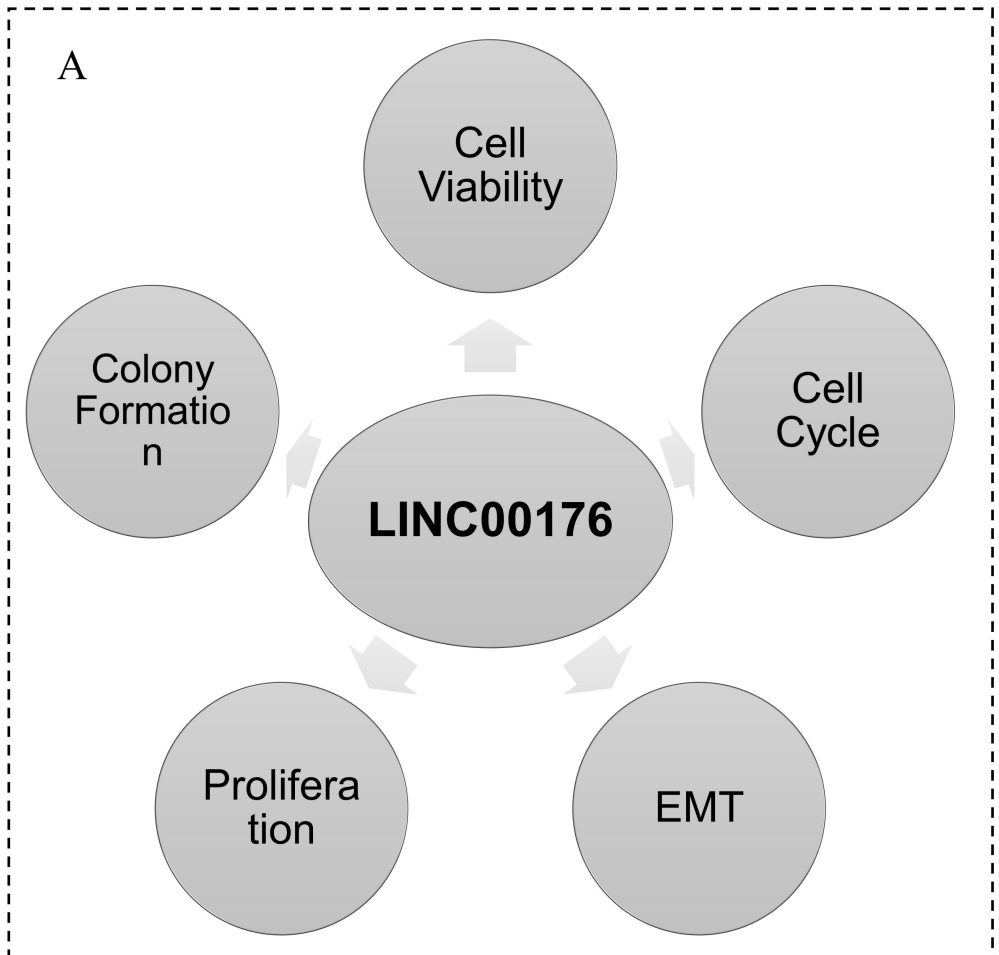
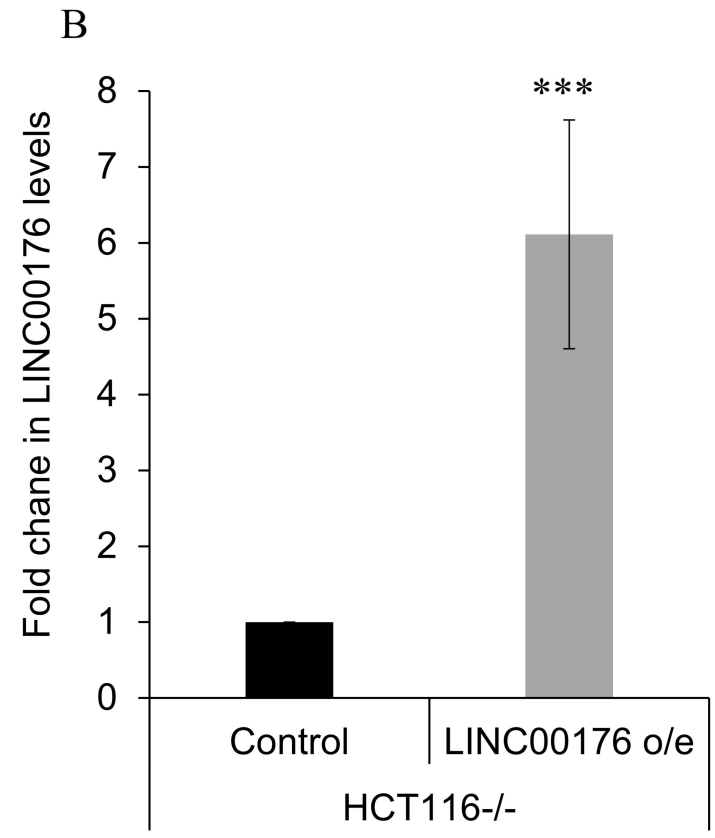
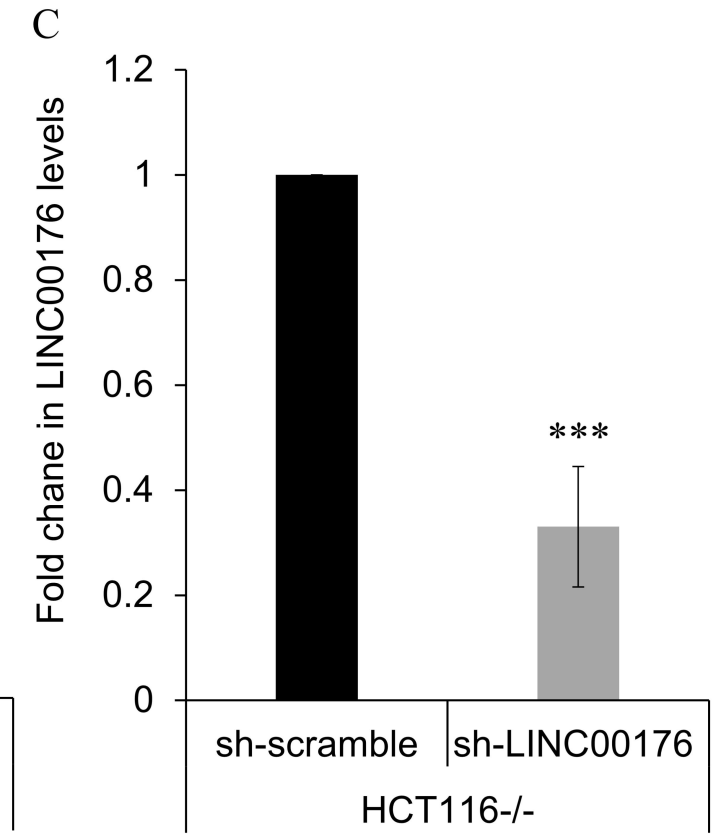


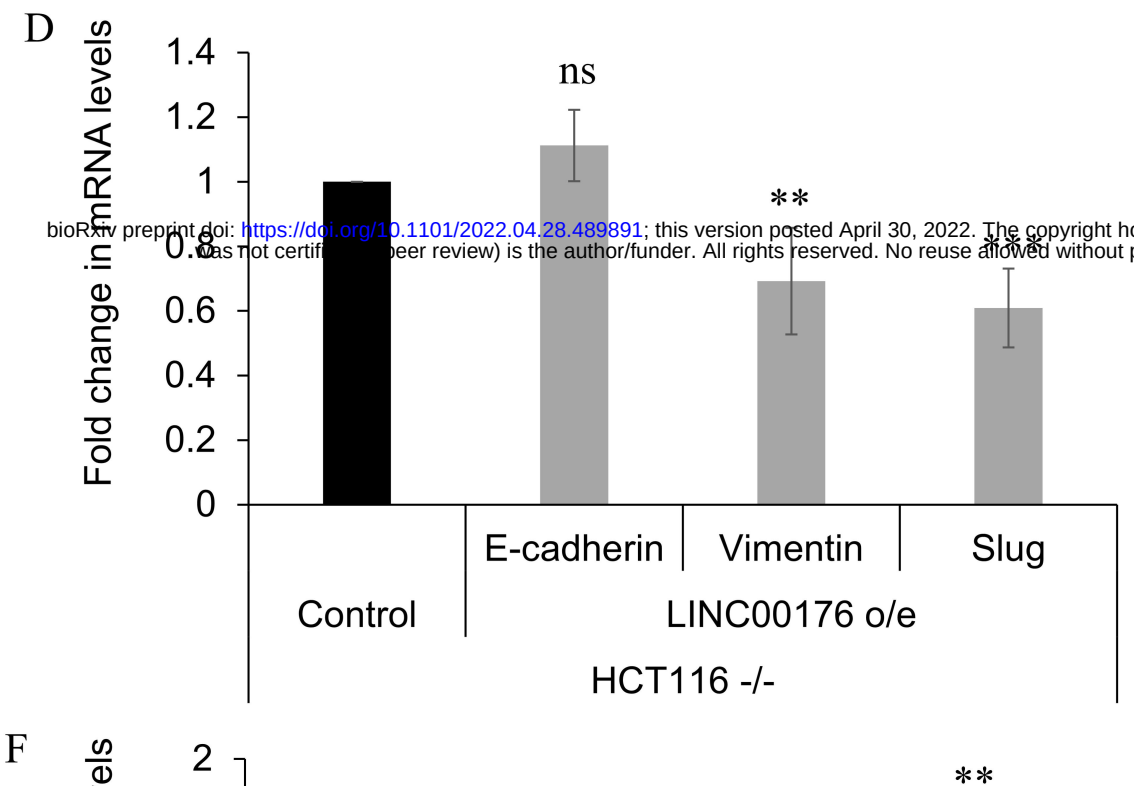
Figure 4. Deciphering the mechanism of action of LINC00176

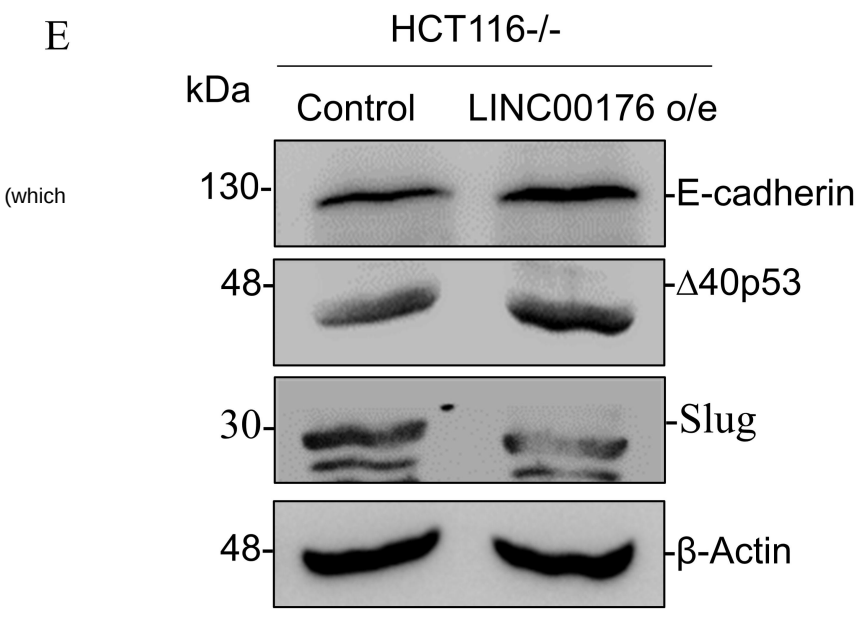
Figure: 5

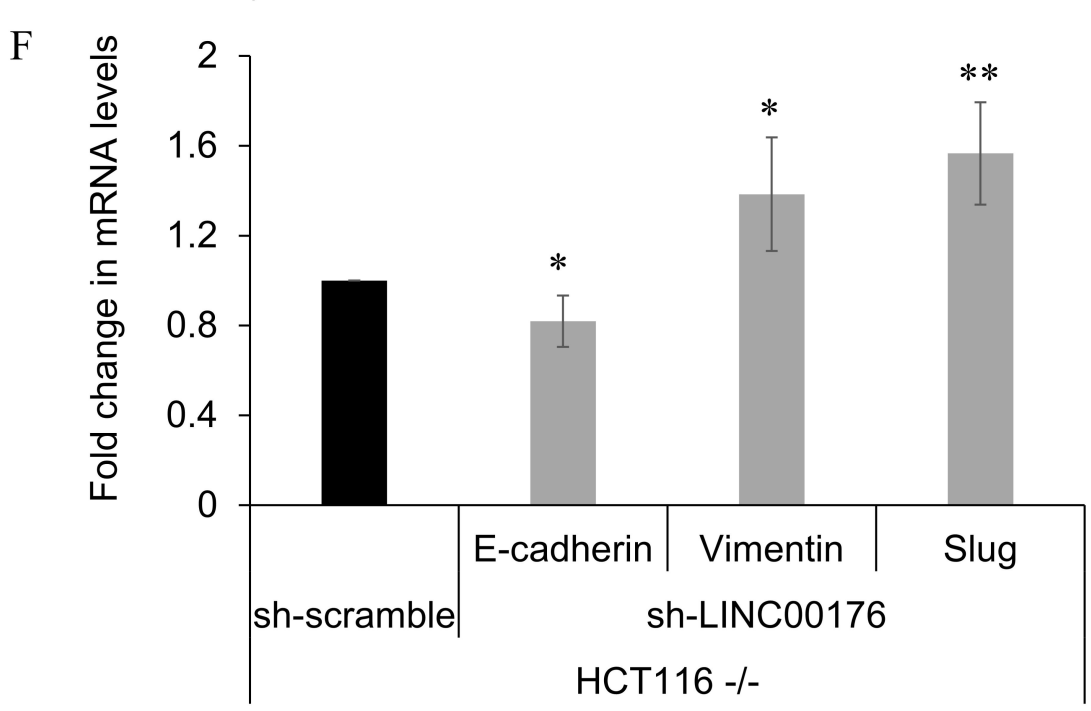


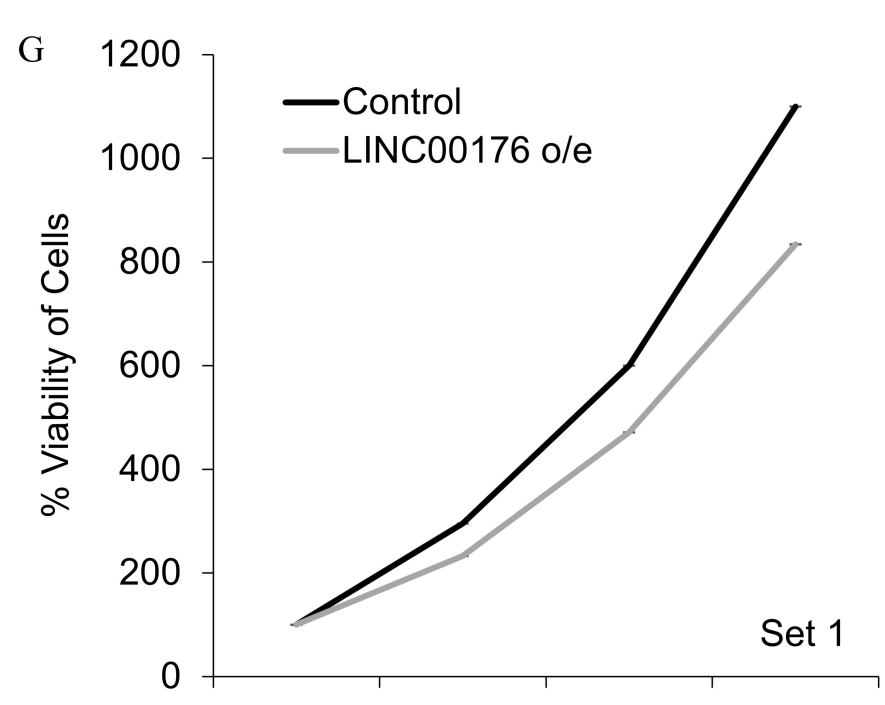


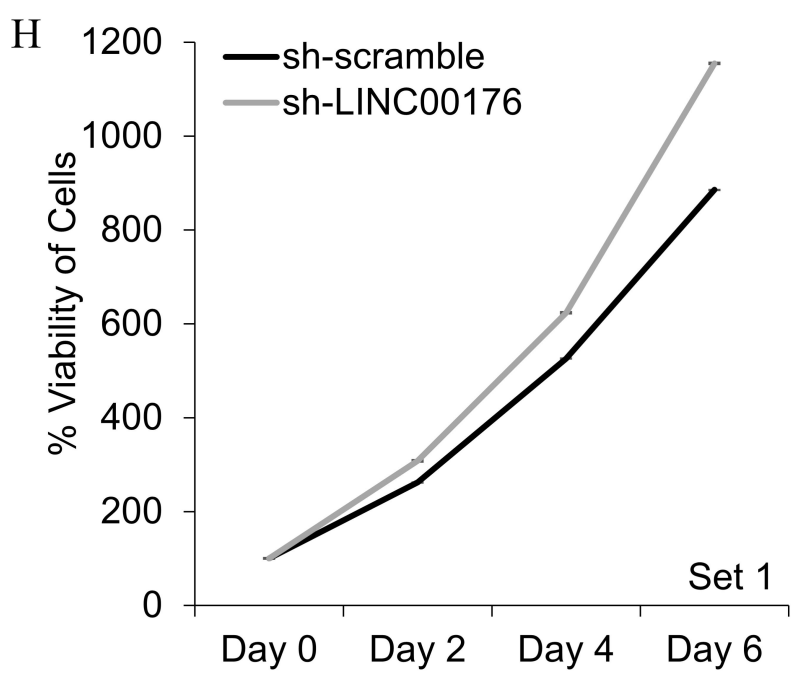












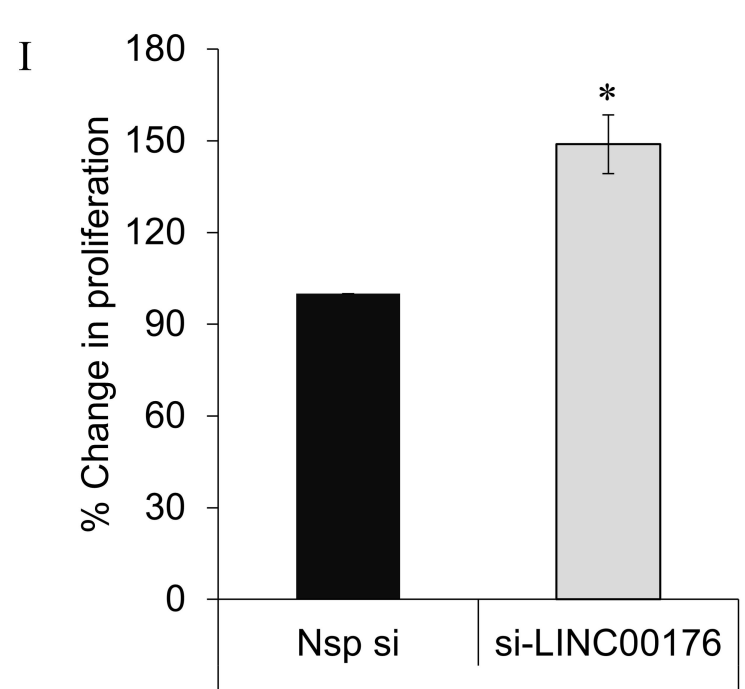
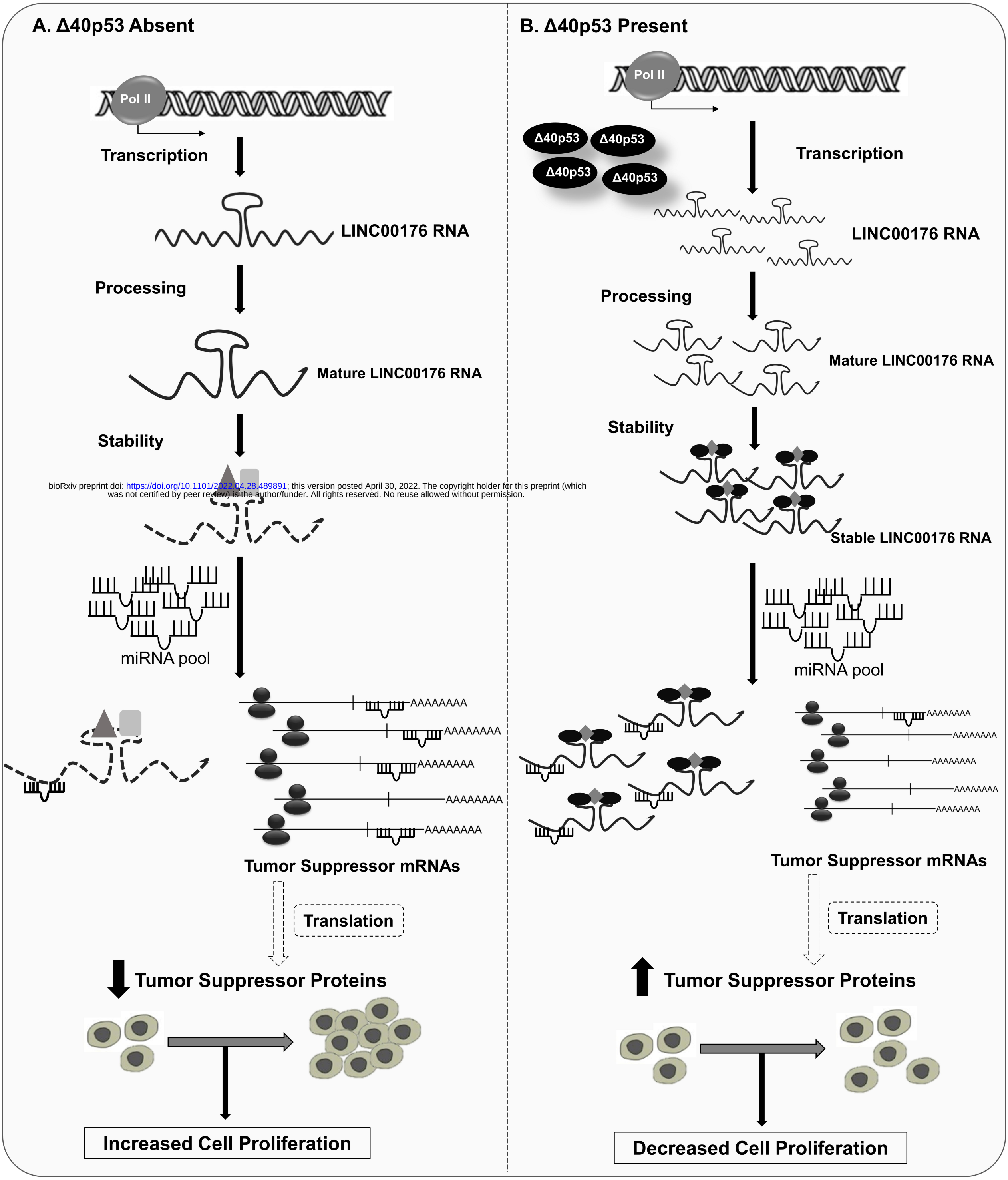


Figure 5: The impact of Δ40p53-LINC00176 axis on cellular processes

Graphical Abstract



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