Cancer Letters 451 (2019) 68–78

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Long noncoding RNA Linc02023 regulates PTEN stability and suppresses tumorigenesis of colorectal cancer in a PTEN-dependent pathway

Qingyuan Wang[a,b,1], Yifei Feng[b,1], Wen Peng[a,b,1], Dongjian Ji[a,b], Zhiyuan Zhang[a,b], Wenwei Qian[a,b], Jie Li[a,b], Qiou Gu[a,b], Dongsheng Zhang[b], Junwei Tang[b], Chuan Zhang[b], Sen Wang[a,b], Zan Fu[b,*], Yueming Sun[b,**]

[a] The First School of Clinical Medicine, Nanjing Medical University, PR China
[b] Department of General Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, 210029, PR China

ARTICLE INFO

Keywords:
LncRNA
CRC
Ubiquitination
P53
PTMs

ABSTRACT

Phosphatase and tensin homolog (PTEN), one of the most frequently mutated tumor suppressor genes in human cancer, is pivotal in the progression of colorectal cancer. Therefore, the regulation of PTEN has emerged as a theme of intense research in tumor biology. This study aims to show that long noncoding RNA (lncRNA) Linc02023 aberrant downregulation in colorectal cancer correlates positively with the expression of PTEN and CDKN2B but negatively with the tumor size in patients and xenografted mouse models. The gain- and loss-of-function investigation reveals that Linc02023 suppresses the proliferation of colorectal cancer cells *in vitro* and *in vivo* with apoptosis promotion and cell cycle rearrangement. Mechanistically, Linc02023 specifically binds to PTEN and blocks its interaction with and ubiquitination by WWP2, stabilizing it and suppressing its downstream expression. In conclusion, this study demonstrates that lncRNA Linc02023 may serve as a novel therapeutic target by restoring the PTEN tumor suppressor activity.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, with 1.4 million new cancer cases diagnosed and 6.9 million deaths occurring worldwide annually [1]. The development of CRC is believed to be associated with cigarette smoking, excess body weight, an unhealthy diet, and physical inactivity [2–4]. However, the molecular mechanisms underlying the occurrence and development of CRC remain to be investigated, which may contribute to the diagnosis and treatment of CRC.

Long noncoding RNAs (lncRNAs) are defined as a class of non-protein-coding transcripts composed of more than 200 nucleotides, which was considered as junk RNA [5]. LncRNAs can be classified as enhancer RNAs (eRNAs), and sense and antisense transcripts based on their relationship with the nearest protein-coding transcripts [6]. LncRNAs are crucial in the transformed phenotype of cancer cells in correlation with distinct gene sets that influence cell cycle regulation, survival, immune response, or differentiation because of their tissue-specific expression and regulation of gene expression via diverse mechanisms [7,8]. The mechanisms of lncRNAs include gene regulation in *cis/trans*, organization of nuclear architecture, and regulation of interacting proteins and RNAs [9,10]. For example, lncRNA EPIC1 promotes tumorigenesis and regulates cell cycle progression in breast cancer by directly interacting with MYC and influencing its target genes [11].

PTEN, a multidomain protein, was first identified as a tumor suppressor by three groups in 1997 [12,13]. Studies on human samples and mouse models show that loss of PTEN function alone is sufficient to cause cancer in certain tissues, and reduction of PTEN more than 50% promotes cancer progression [14,15]. PTEN suppresses cell proliferation by exerting a negative influence on PI3K/AKT pathway in the cytoplasm, regulating cell cycle proteins and stability and transcriptional activity of P53 [16–18]. Although many mechanisms of transcriptional and posttranscriptional regulation of the expression of PTEN are clear, including epigenetic silencing, transcriptional regulation, regulation by miRNAs and disruption of ceRNAs networks, posttranslational modifications (PTMs), PTEN-interacting proteins, dimerization, and secretion, the relationship between lncRNAs and PTEN remains unexplored

* Corresponding author.
** Corresponding author.
E-mail addresses: fuzan1971@njmu.edu.cn (Z. Fu), jssym@vip.sina.com (Y. Sun).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.canlet.2019.02.041
Received 17 October 2018; Received in revised form 11 February 2019; Accepted 13 February 2019

[19].

This study aims to investigate the transcriptomic associations between lncRNAs and mRNAs in patients with CRC. An aberrant downregulation Linc02023 is identified as a tumor suppressor, and the underlying mechanisms are examined. The results elucidate that Linc02023 is a PTEN-interacting RNA, maintaining its stability and restoring its activity, thus suppressing the progression of CRC.

2. Materials and methods

2.1. Clinical samples and cell lines

Sixty paired tissue samples comprising CRC tissues and adjacent normal tissues were received from patients with CRC who underwent surgical treatment at The First Affiliated Hospital of Nanjing Medical University between November 2015 and October 2016. The study was approved by the Institutional Ethics Committee of the University, and written consent was obtained from the participants. DLD-1, HCT116, LOVO, SW480, HT29, NCM460, and 293T cell lines were obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% fetal calf serum.

2.2. Quantitative real-time PCR

The total RNA was extracted with TRIzol from tissue samples and cultured cells according to the manufacturer's instructions (Qiagen, Hilden, Germany). The cDNA was synthesized from total RNA using the cDNA Reverse Transcription Kit (ABI 7900; Life Technologies). The transcriptional levels were detected in amplification by RT-PCR using the Power SYBR Green PCR Master Mix. The results were normalized to GAPDH. The primer pairs used are listed in Supplementary Table 1.

2.3. Overexpression and silence of Linc02023

The full length of Linc02023 synthesized by GenePharma Co. Ltd. (Shanghai, China) was subcloned into the lentivirus vector pGLV5. The shRNA sequence targeting Linc02023 was cloned into the lentivirus vector pGLV3 to suppress the expression of Linc02023 (Supplementary Table 1). The cells were transfected with shRNAs targeting Linc02023, or negative control hairpin shRNA, designated as shLinc02023 1, shLinc02023 2, and shControl, to generate stable knockdown cells. The efficiency of overexpression and knockdown of Linc02023 was verified by qRT-PCR.

2.4. Cell proliferation and invasion assay

The CCK8 (Dojindo Laboratories, Kumamoto, Japan), clone formation assay, and Cell-Light EDU Apollo567 In Vitro Imaging Kit (RiboBio, Guangzhou, China) were used to test the proliferation ability of CRC cells according to the manufacturer's instructions. In migration and invasion assays, the cells were cultured with serum-free media in the upper chamber (Millipore, Darmstadt, Germany) without (for cell migration assays) or with Matrigel (for cell invasion assays). A medium supplemented with 10% fetal bovine serum was added in the lower chamber and incubated at 37 °C in 5% CO_2 for 48 h. Then, cells on the lower membrane surface were fixed with methanol, stained with crystal violet, and counted under an inverted microscope in five random fileds (200×).

2.5. Flow cytometry analysis of cell cycle and apoptosis

For cell cycle analysis, the transfected cells were harvested for 24 h after serum starvation and fixed in 70% ethanol at 4 °C overnight. Before FCM detection, the cells were washed and incubated with PI staining solution for cell cycle analysis. For apoptosis analysis, the transfected cells were treated with 0.5 mM H_2O_2 overnight to stimulate apoptosis. PI and Annexin V–FITC staining were used according to the manufacturer's instructions (Roche, Basel, Switzerland). Data were acquired and analyzed on a flow cytometer.

2.6. Gene Set Enrichment Analysis

GSE8671 was an affymetrix gene chip that analyzed gene expression changes in colorectal cancer and corresponding normal tissues from 32 patients, aimed to investigate the molecular mechanisms underlying the formation of colorectal cancer. The gene profiling data of patients with CRC were obtained from the Gene Expression Omnibus site (GSE8671). Expression matrix was downloaded and genes measured using multiple probes were consolidated using average values of these probes. Expression of almost 3500 LncRNA was measured in this gene chip. All lncRNA genes were ranked using the Pearson correlation coefficient with PTEN by loop statement, cor() and cor.test() in R Studio. Then, GSEA was used to identify enriched gene sets in KEGG based on the Pearson's correlation coefficient with Linc0202 and expression of 19843 mRNA was used as input.

2.7. RNA pull-down assay and RIP

The biotin-labeled Linc02023 and the antisense RNA were transcribed *in vitro* with a Biotin RNA Labeling Mix Kit and T7 RNA polymerase (Roche), treated with RNase-free DNase I, and then isolated with the RNeasy Mini Kit (Qiagen). Then, DLD-1 cell lysates were incubated with biotinylated RNAs and 60 mL of Dynabeads MyOne Streptavidin C1 magnetic beads. The beads were boiled in 1× SDS loading buffer and the resolved protein was analyzed using Western blot analysis.

RIP was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instruction. Briefly, anti-PTEN antibodies were used for RIP and the interacting RNAs were isolated detected by qRT-PRC, and normalized to input (total RNAs). The RNA enriched with immunoglobulin G RIP of cells served as the negative control.

2.8. Ni-bead pull-down assay, immunoprecipitation, and Western blot analysis

For ubiquitination detection, Flag-PTEN, HA-WWP2, His-Ub, Linc02023, and the empty vector were co-transfected in 293T cells. The cells were lysed in 6M guanidinium denaturing solution, and PTEN-poly-Ub was purified by Ni-bead pull-down.

The immunoprecipitation was performed as follows: 293T cells were transfected with indicated plasmids, and the whole-cell lysates were extracted. Beads conjugated with Flag or HA antibody were added into the antibody lysate mixture overnight at 4 °C and washed with lysis buffer. Then, it was boiled for 10 min with 1× SDS loading buffer. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred onto a PVDF membrane, and visualized using an ECL kit. For ubiquitination detection, the ubiquitination status of PTEN proteins was detected using anti-Flag antibody, while anti-Flag and anti-HA antibodies were used to analyze the interaction between PTEN and WWP2.

The primary antibodies used were as follows: caspase-3 (CST, 1:1000, #9665), PTEN (CST, 1:1000, #9188), P53 (CST, 1:1000, #2527), AKT (Abcam, 1:500, ab8805), P-AKT (Abcam, 1:500, ab38449), C-myc (Abcam, 1:1000, ab32072), P15 (Abcam, 1:500, ab53034), CDK4 (Abcam, 1:1000, ab108357), GAPDH (Abcam, 1:2500, ab9485), HA (Roche Life Science, 1:2000 #11867423001), and Flag (Sigma, 1:2000 #F1804).

2.9. Subcutaneous xenotransplantation model

Five-week-old BALB/c nude male mice purchased from the animal center of Nanjing University were used for the xenograft model. DLD-

1 cells stably expressing Linc02023 and control cells were implanted into the right and left groins of mice separately. Tumors were measured every 5 days after the implantation, and the mice were killed 5 weeks after inoculation. All the experiments followed the protocols approved by the Institutional Animal Care and Use Committee of the Nanjing Medical University.

2.10. IHC assay

Paraffin-embedded xenografts were used for IHC detection as described in a previous study [20]. Briefly, tumor samples were embedded in paraffin and sectioned into 2- to 4-mm slices, and then, antibodies against PTEN, P-AKT, C-myc and P53 were used for IHC staining according to the manufacturer's instruction. The photographs of sections were detected using a digital microscope camera.

2.11. Statistical analysis

All experimental assays were conducted at least three times with samples in triplicates. Data were represented as means ± standard deviation. The two-tailed Student *t*-test was used to determine the statistical significance of difference between groups, and Pearson correlation analysis was used to assess the relationship among gene sets. Statistical analysis was performed using the Statistical Program for Social Sciences 22.0 software (SPSS, CA, USA) and presented with GraphPad Prism 5.0 (GraphPad Software, CA, USA). A P value < 0.05 was considered as significant and indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Downregulation of Linc02023 with a high correlation with PTEN in CRC tissues

A co-expressed lncRNA–mRNA network was reannotated and built using the expression profiles of lncRNAs and mRNAs (GSE8671) as input to identify the differentially expressed lncRNAs, which potentially interacted with the cancer-related mRNAs. Among these findings, a novel lncRNA named Linc02023 was differently expressed in CRC tumor tissues and corresponding adjacent normal tissues, with a significantly positive correlation with the expression of PTEN in both CRC and normal colorectal tissues (Supplementary Figs. 1A–1C). Then, the aberrant expression and association of closely correlated Linc02023 and PTEN were confirmed using quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR). A further Gene Set Enrichment Analysis (GSEA) was used to analyze the significant pathways associated with Linc02023 based on the Kyoto Encyclopedia of Genes and Genomes (KEGG). Among these, the most enriched cancer pathways were chosen for investigation, including cell cycling and P53 pathway (Fig. 1D).

Further, qRT-PCR was conducted in 60 pairs of tissue samples to understand the function of Linc02023 in the pathogenesis of CRC. The results indicated that both Linc02023 and PTEN were aberrantly downregulated in the CRC tumor tissues compared with the normal adjacent tissues (Fig. 1A). Next, the Pearson correlation analysis was used to verify the correlation between Linc02023 and PTEN mRNA, and the positive result ($r = 0.5460$, $P < 0.001$) (Fig. 1B). The patients were divided into Linc02023^{high} and Linc02023^{low} groups according to the expression of Linc02023 to investigate the significance of aberrant expression of Linc02023 in CRC. The statistical result showed that the expression of Linc02023 highly correlated with the tumor size rather than the TNM stage, serum CEA level, and metastasis (Table 1). The subcellular localization of Linc02023 with amplified expression of separated cytoplasm RNA and nuclear RNA was analyzed, and the result indicated that Linc02023 was mainly located in the cytoplasm of DLD-1, HCT116, and SW480 cell lines (Fig. 1E).

3.2. Linc02023 suppressed CRC cell proliferation and invasion in vitro

The gain- and loss-of-function assays were performed based on the different distribution of the expression of Linc02023 in CRC cell lines to investigate the biological function of Linc02023 in the progression of CRC (Fig. 1C). According to the endogenous expression of Linc02023, overexpression lentivirus was transfected into the DLD-1 and HCT116 cell lines while knockdown lentivirus into the SW480 cell line. The efficiency of overexpression and knockdown was confirmed using qRT-PCR, and the stable transfected cells were used for the following assays (Fig. 2A). First, in the clone formation assays, the resulting data showed that the overexpression of Linc02023 significantly inhibited the clone formation ability, whereas the knockdown of Linc02023 formed more clones (Fig. 2B). Next, the cell counting kit-8 (CCK8) assays were conducted, and the growth curve for 5 days was detected to investigate the function of Linc02023 in the proliferation of CRC cell lines. It was found that the proliferation of DLD-1 and HCT116 was suppressed by the upregulation of Linc02023, whereas the downregulation of Linc02023 induced increased proliferation of SW480 (Fig. 2C). Furthermore, EDU incorporation assays confirmed the function of Linc02023 in suppressing the cell growth and proliferation (Fig. 2D). As shown in the results of GSEA analysis and the role of PTEN in regulating cell cycling and cell apoptosis, Linc02023 was assumed to influence the proliferation of CRC cells through inhibiting G1-to-S phase transformation and promoting apoptosis. Next, whether gain or loss of Linc02023 could make a difference in cell cycle distribution and apoptosis was examined using the flow cytometry analysis. The increased percentage of G0 and G1 phases and the decreased S phase population were observed in Linc02023-overexpressed cells compared with the negative controls. In contrast, the knockdown of Linc02023 promoted the progression of G1-to-S phase transformation (Fig. 3A). Furthermore, the result of apoptosis indicated that the fraction of apoptotic cells significantly increased in Linc02023-overexpressed cells, whereas the knockdown of Linc02023 reduced cell apoptosis (Fig. 3B).

Transwell assays were conducted to further understand the functions of Linc02023 in cell migration and invasion. The results indicated that the loss of Linc02023 enhanced the migration and invasion ability of SW480, whereas the gain of Linc02023 reduced the cell migration and invasion of DLD-1 and HCT116 (Fig. 3C).

3.3. Linc02023 suppressed tumor growth in vitro

The biological effect of Linc02023 was further investigated *in vivo* using a xenotransplantation model with adoptive cells subcutaneously injected into nude mouse using DLD-1 cells stably transfected with Linc02023 expression vector and negative control vector. Tumor growth was remarkably suppressed by the overexpression of Linc02023 with lesser final tumor volume and weight compared with the controls (Fig. 4A–D). Moreover, the expression levels of PTEN, P-AKT, C-myc, and P53 were detected using immunohistochemical (IHC) staining. It was found that the expression levels of PTEN and P53 increased in the Linc02023-upregulated cells while those of P-AKT and C-myc decreased (Fig. 4E).

3.4. Linc02023 prevented PTEN ubiquitination by WWP2

A pull-down assay was conducted with biotinylated sense Linc02023 and biotinylated antisense Linc02023 transcript to investigate the potential Linc02023-interacting proteins so as to study the interaction between Linc02023 and PTEN proteins. This analysis revealed that PTEN protein could specifically be bound to biotinylated sense Linc02023 but not to biotinylated antisense Linc02023 (Fig. 5A, upper panel). The result was further validated by immunoblotting (Fig. 5A, lower panel). The RNA immunoprecipitation (RIP) assay was used to confirm the interaction between endogenous Linc02023 and PTEN protein, and the result showed that Linc02023 could be enriched

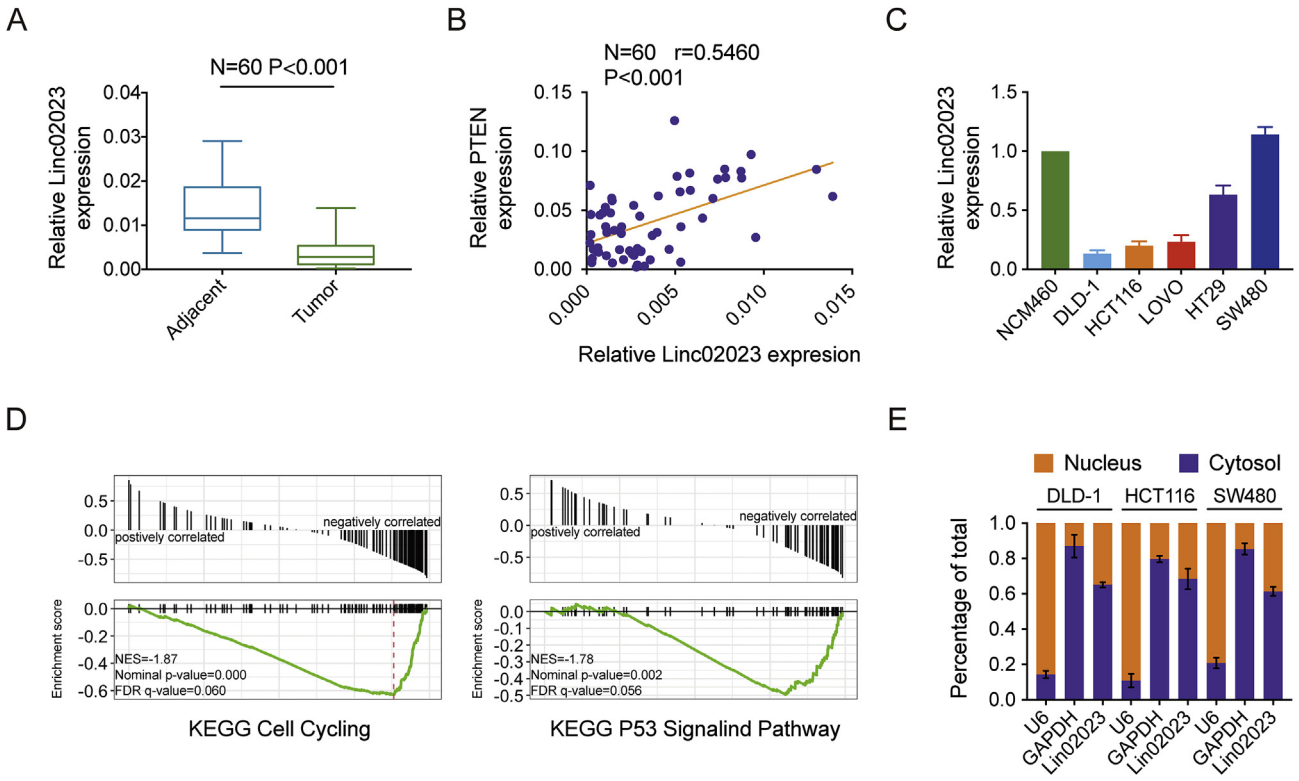

Fig. 1. Characteristics of Linc02023 in colorectal cancer. (A) Relatively decreased level of Linc02023 was confirmed in the CRC tumor tissues and corresponding adjacent tissues by qRT-PCR normalized to GAPDH ($N = 60$, $P < 0.001$). (B) Pearson correlation analysis was performed to determine the correlation between the expression of Linc02023 and PTEN ($N = 60$, $r = 0.5460$, $P < 0.001$). (C) The relative expression of Linc02023 in DLD-1, HCT116, LOVO, HT29, and SW480 cells normalized to the expression of Linc02023 in NCM460. (D) Representative gene sets from the MSigDB in the GSEA of Linc020203 correlated genes. (E) Subcellular localization indicated that the transcript for Linc02023 was located mainly in the cytoplasm of DLD-1, HCT116, and SW480 determined by qRT-PCR with U6 or GAPDH as a nuclear or cytoplasmic marker. Data are presented as means ± standard deviation.

Table 1
Relevance analysis of Linc02023 expression in CRC patients.

Variable	All patients	Linc02023		P value
		Low	High	
All Cases	60	30	30	
Age (years)				0.581
< 60	18	8	10	
≥60	42	22	20	
Gender				0.190
Male	37	21	16	
Female	23	9	14	
Tumor diameter (cm)				**0.003**
< 5	24	7	17	
≥5	36	23	13	
TNM Stage				0.795
I + II	36	17	19	
III + IV	24	13	11	
Depth of invasion				0.071
T1 + T2	25	9	16	
T3 + T4	35	21	14	
Lymphatic metastasis				0.791
Yes	21	11	10	
No	39	19	20	
Distant metastasis				0.497
Yes	11	7	4	
No	49	23	26	
CEA (ng/ml)				0.591
< 5	40	19	21	
≥5	20	11	9	
Tumor location				0.434
Colon	37	17	20	
Rectum	23	13	10	

by PTEN protein but not antisense Linc02023 or GAPDH (Fig. 5B).

On the basis of these findings, catPARID was then used to predict the potential PTEN-binding regions in Linc02023 and potential protein-binding domains for Linc02023 in PTEN (Supplementary Fig. 1D). The domain (101–179 amino acids) of PTEN was supposed to be a central protein-binding domain and also the major docking site for the E3 ubiquitin ligases WWP2 (100–187 amino acids) according to a previous study by Subbareddy Maddika in May 2011 [21]. It was apparent that Linc02023 might overlap with the interacting site between PTEN and WWP2, affecting PTEN ubiquitination and the stability of PTEN. The Flag-tagged PTEN protein, His-tagged ubiquitination protein, HA-tagged WWP2 protein, and Linc02023 were overexpressed in 293T cells to determine the effect of Linc02023 on PTEN ubiquitination, and the pull-down assay was performed to detect the degree of PTEN ubiquitination. The ubiquitination of PTEN was found to significantly decrease in a dose-dependent manner, and when Linc02023 reached a certain level, it was sufficient to inhibit WWP2-induced PTEN ubiquitination (Fig. 5C). The immunoprecipitation assay was conducted to confirm the role of Linc02023 in interfering with the interaction between PTEN and WWP2 to further understand the mechanism of how Linc02023 inhibited PTEN ubiquitination. The overexpression of Linc02023 significantly diminished the binding of PTEN with WWP2, implying that the effect of Linc02023 in the interaction of PTEN with WWP2 suppressed the ubiquitination by WWP2 and maintained the stability of PTEN (Fig. 5D).

PTEN was primarily localized in the cytoplasm and regulated Akt signaling, but also exerted its functions in the nucleus. First, the phosphorylation of Akt was examined, and it was found that the overexpression of Linc02023 enhanced Akt activation while depletion of Linc02023 resulted in an inhibition of Akt. The result of GSEA showed that P53 pathway and cell cycling were significantly enriched

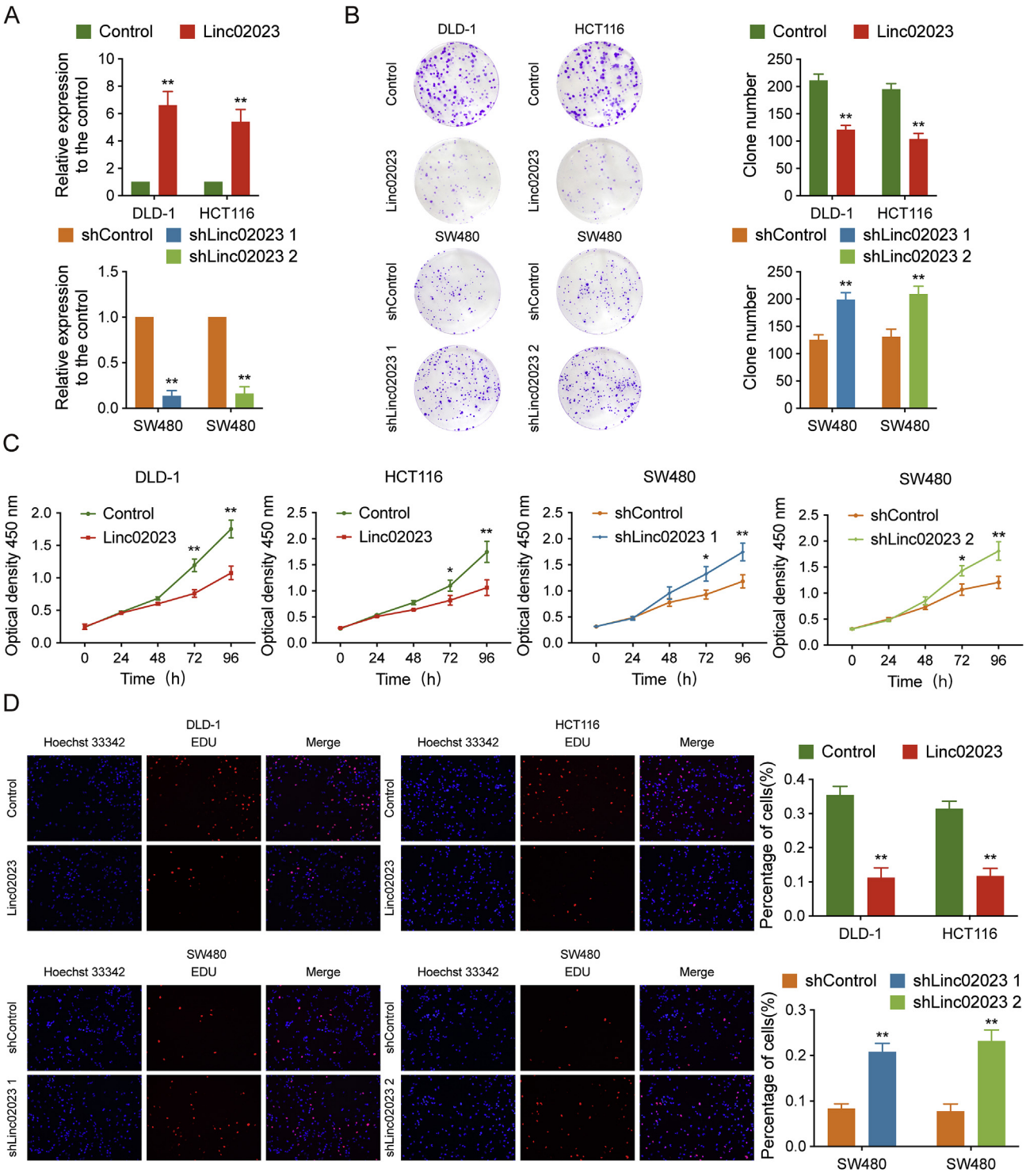

Fig. 2. Linc02023 inhibited CRC cell proliferation *in vitro*. (A) Relative expression of Linc02023 as determined by qRT-PCR in DLD-1, HCT116, and SW480 cell lines transfected with Linc02023 or Linc02023-targeting shRNA. (B) Effect of overexpression or knockdown of Linc02023 on the colony formation of CRC cells; representative graphs are shown. (C) Cells were seeded in 96-well plates after transfection with Linc02023 or control vector or Linc02023-targeting shRNA or control shRNA vector, and the cell number was detected from days 1–5 using CCK8 assays. The overexpression of Linc02023 suppressed the proliferation of DLD-1 and HCT116 cells, whereas the knockdown of Linc02023 promoted the proliferation of SW480 cells. (D) EDU immunofluorescence staining confirmed the function of Linc02023 on the proliferation of CRC cells. Original magnification 100×. Data are presented as means ± standard deviation ($^{*}P < 0.05$, $^{**}P < 0.01$).

with Linc02023-correlated genes, which correlated positively with CDKN2B but negatively with MYC and CDK4. Then, whether both the C-myc/CDKN2B/CDK4 and P53/cleaved-caspase3 pathways were altered with the expression of Linc02023 was examined. The analysis revealed that the overexpression of Linc02023 greatly increased the protein levels of CDKN2B, P53, and cleaved-caspase3 and negatively regulated C-myc and CDK4 proteins. The adverse effect could be rescued by the knockdown of PTEN, while depletion of Linc02023 was reversed. These results suggested that Linc02023 suppressed the tumorigenesis of CRC through Akt, C-myc/CDKN2B/CDK4, and P53/

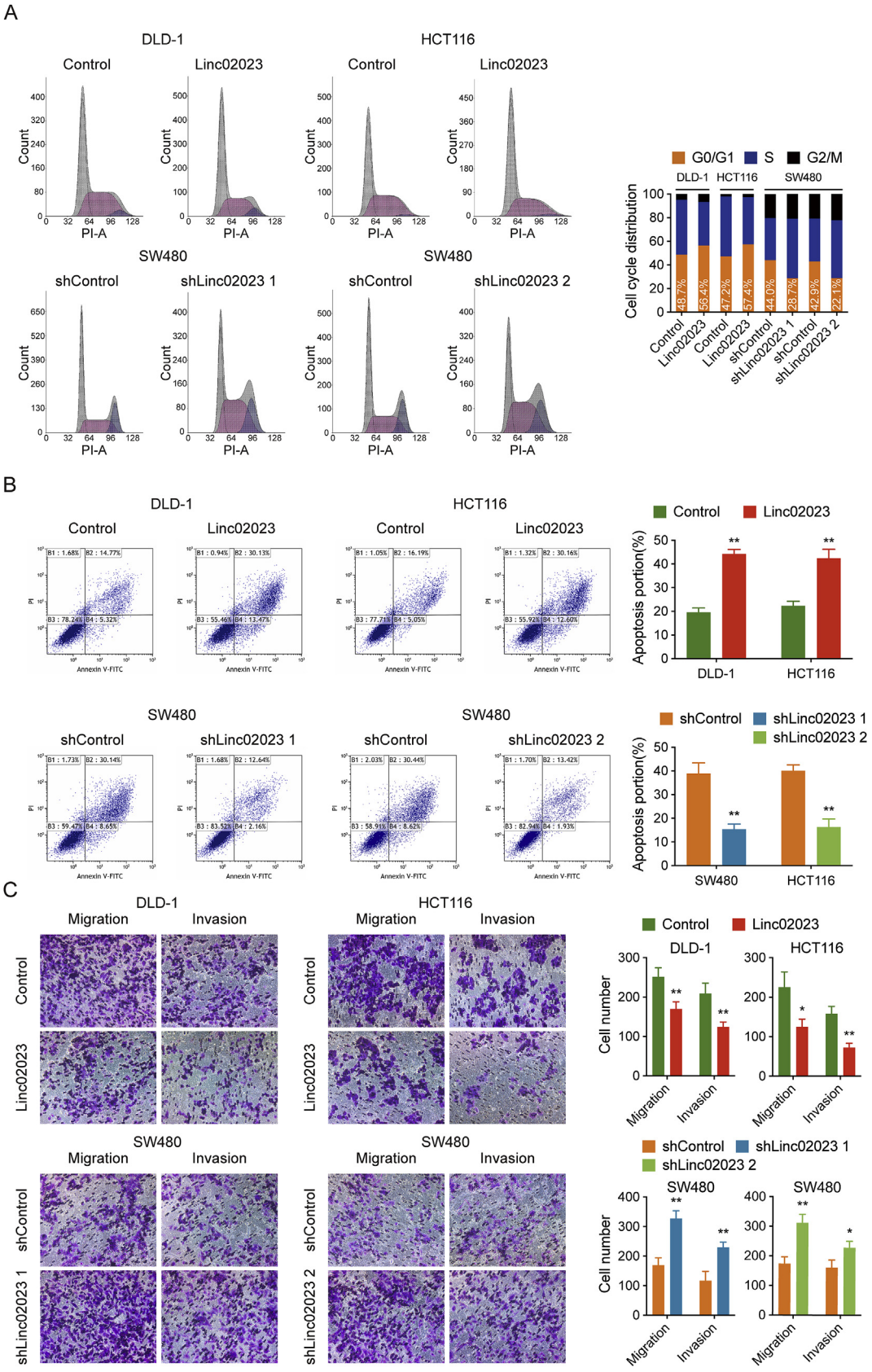

Fig. 3. Linc02023 regulated the cell cycle, cell apoptosis, and cell migration of CRC cells *in vitro*. (A) The cell cycle analysis of DLD-1, HCT116, and SW480 transfected with Linc02023 or control vector or Linc02023-targeting shRNA or control shRNA vector was performed using flow cytometry. The cell cycle distribution was listed with the percentage of G1, S, and G2 phases in the graphs. (B) Cells were treated with 0.5 mM H_2O_2 overnight and detected by flow cytometry using FITC–Annexin V/PI staining kit. The bar graph shows the percentage of early apoptotic cells and terminal apoptotic cells. (C) Representative visual fields of DLD-1, HCT116, and SW480 cells treated with Linc02023 or control vector or Linc02023-targeting shRNA or control shRNA vector. Original magnification 200×. Data are presented as means ± standard deviation ($^{*}P < 0.05$, $^{**}P < 0.01$).

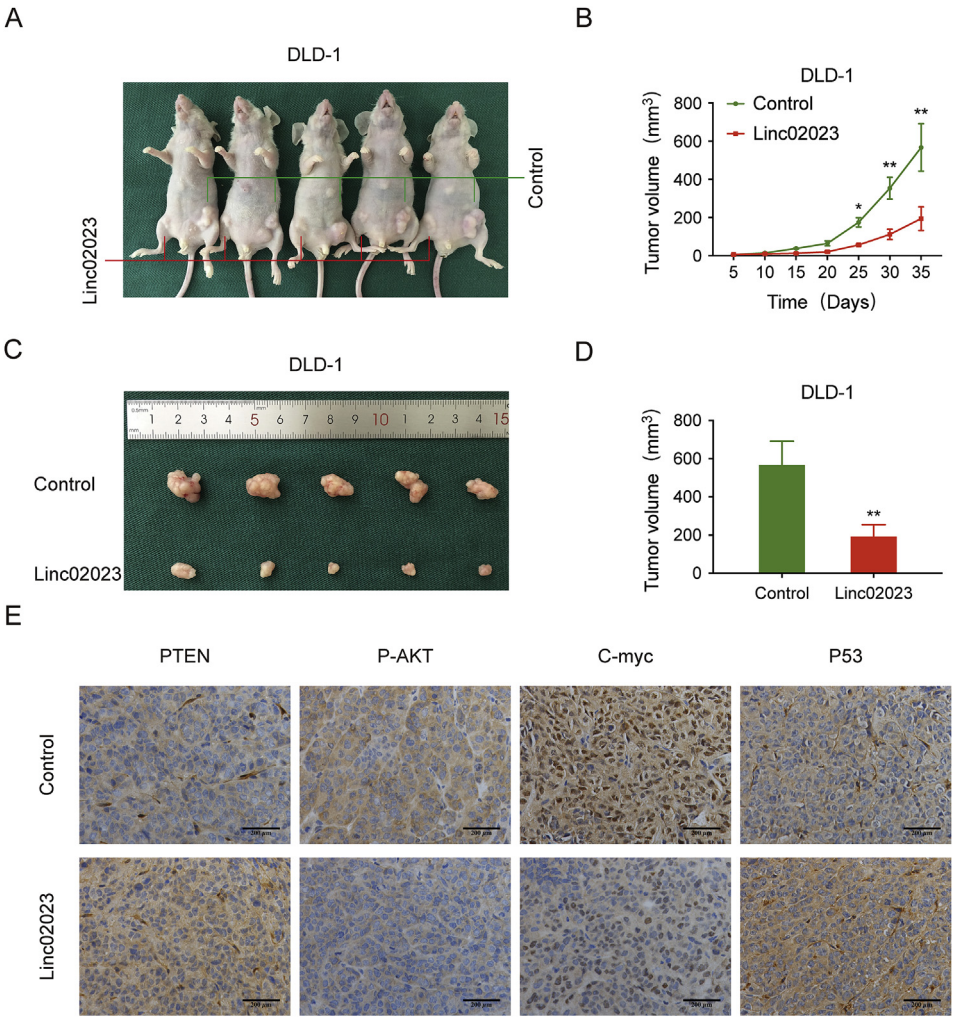

Fig. 4. Linc02023 enhanced tumor growth *in vivo*. (A–D) BABL/c nude mice (4–6 weeks of age) were subcutaneously injected with DLD-1 cells having stable overexpression of Linc02023 or control. The established tumors in the right and left groins of the mice were measured every 5 days, and the mice were killed 35 days after implantation. Linc02023 markedly inhibited the tumor growth of DLD-1 cells in nude mice. (E) Protein levels of PTEN, P-AKT, C-myc, and P53 in the tumor samples were determined by IHC. Original magnification 200×. Data are presented as means ± standard deviation ($^{*}P < 0.05$, $^{**}P < 0.01$).

cleaved-caspase3 pathways (Fig. 6A). However, the mRNA levels of PTEN were not altered in DLD-1, HCT116, and SW480 cells with stable overexpression or knockdown of Linc02023 (Fig. 6B). Mechanistically, PTEN could form a complex with histone acetyltransferase p300, up-regulating acetylation levels of P53 and enhancing P53 transcriptional activity, which, in turn, promoted PTEN transcription [22]. Moreover, we observed that there are 4 possible P53 binding sites at the Linc02023 promoter using the bioinformatic software PROMO (Supplementary Fig. 1E). We conjectured that the transcription of Linc02023 and PTEN was driven by P53 and was enhanced by the protein level of PTEN, which illustrated the correlation between mRNA levels of Linc02023 and PTEN and the negative results in alteration of PTEN mRNA levels after knockdown or overexpression of Linc02023 (see Fig. 7).

The correlation between Linc02023 and mRNA levels of Akt1, MYC, CDKN2B, CDK4, TP53, and Casp3 were further examined. Linc02023 and CDKN2B were found to be positively correlated while Linc02023 and MYC and CDK4 were negatively correlated in CRC tumor tissues (Fig. 6C).

4. Discussion

PTMs, including ubiquitination, phosphorylation, acetylation, oxidation, glycosylation, methylation, and so on, are thought to participate in the progression of cancer development. Previous studies have demonstrated their crucial role in regulating the activity, stability, and nuclear translocation of signaling proteins [23–25]. Cancer-associated altered activity of signaling proteins is due to several site-specific PTMs, of which phosphorylation is found in the central location and has the most interaction spectrum, while ubiquitination is secondary in abundance [26,27]. The regulation of PTMs is considered as an underlying molecular mechanism in tumor progression and a therapeutic target of chemotherapy involving site-selective, bioorthogonal protein-modification chemistry [28,29].

Ubiquitination, one of the most common forms of PTMs, degrades the level of labeled proteins by sequential activity of ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) [30]. Among these, the active site of E3 ubiquitin ligases or their interaction with substrates is widely selected as the target of anti-cancer drugs, such as Nutlins and RITA, influencing the interaction between Hdm2 and P53, upregulating P53, and activating the P53 signaling pathway [31–34]. However, low biological potency and off-target effects have restricted the clinical application of these inhibitors and agonists [35]. Emerging evidence has demonstrated that expression alterations of lncRNAs in cancer exhibit a strong effect on the regulation of PTMs of signaling proteins. For instance, Linc00673, a tumor suppressor lncRNA in pancreatic cancer, interacts with PTPN11 and enhances PTPN11 ubiquitination and degradation by ubiquitin

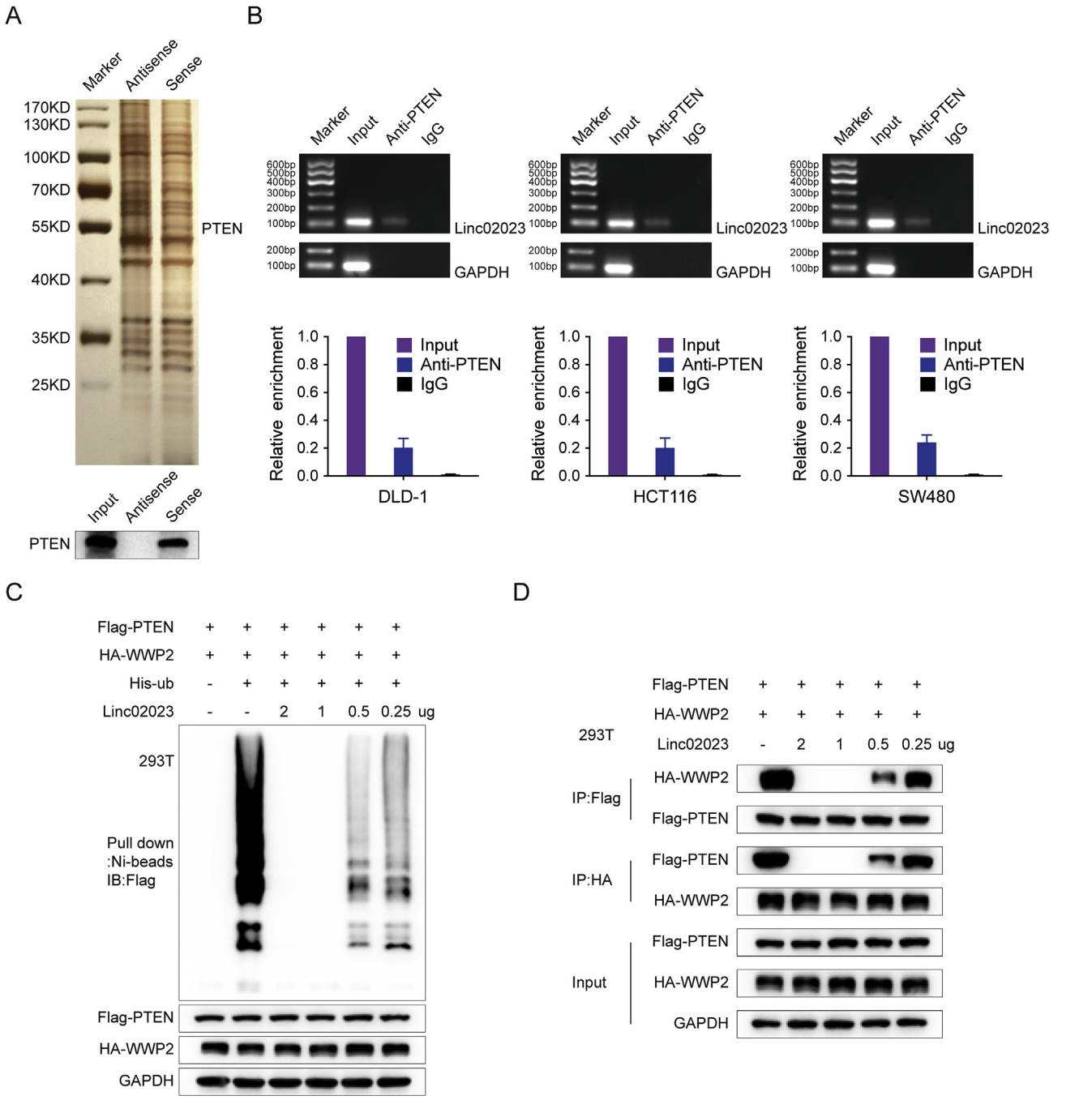

Fig. 5. Cytoplasmic Linc02023 bound specifically to PTEN. (A) Identification of Linc02023–protein complex by incubation of biotinylated sense and antisense Linc02023 with protein extracts from DLD-1 cells (upper panel). The associated proteins were further detected by immunoblot after pull-down (lower panel). (B) The RIP assay showed an association of PTEN with Linc02023 in DLD-1, HCT116, and SW480 cells. The precipitated RNAs were determined using qRT-PCR for Linc02023 and GAPDH; the RNA levels were relative to an input control from three independent experiments. Data are presented as means ± standard deviation. (C) 293T cells were transfected with indicated plasmids, lysed under denatured conditions in 6M guanidinium solution, followed by Ni-bead pull-down. The washed beads were boiled for immunoblotting to detect polyubiquitylation of exogenous PTEN. (D) The whole-cell lysates were prepared and immunoprecipitated with antibody against Flag or HA. The precipitates and input were analyzed by immunoblot.

ligase PRPF19 [36]. The present study has identified a novel lncRNA, named Linc02023, which interacts with PTEN protein and suppresses WWP2-mediated ubiquitination and degradation of PTEN.

In recent years, the role of lncRNAs in human diseases, especially in malignancy, has attracted much attention [37]. In CRC, quite a few lncRNAs have been identified to be aberrantly expressed in CRC tissues. They participate in the biological progression of tumor growth and metastasis [38,39]. However, the role of lncRNA in directly regulating signaling proteins and PTMs is still under investigation. In the present study, aberrant downregulation of long noncoding RNA Linc02023 in CRC tissues correlates positively with the expression of PTEN in human CRC samples. The overexpression of Linc02023 increases the protein level of PTEN *in vitro* and *in vivo*. The gain- and loss-of-function analysis indicates that Linc02023 inversely regulates cell cycling, anti-apoptosis, and cell proliferation. Besides, Linc02023 can suppress the migration and invasion of CRC cells. Further analysis reveals that Linc02023 directly interacts with the special site on PTEN where WWP2 bound and suppresses the ubiquitination of PTEN induced by WWP2, inhibiting

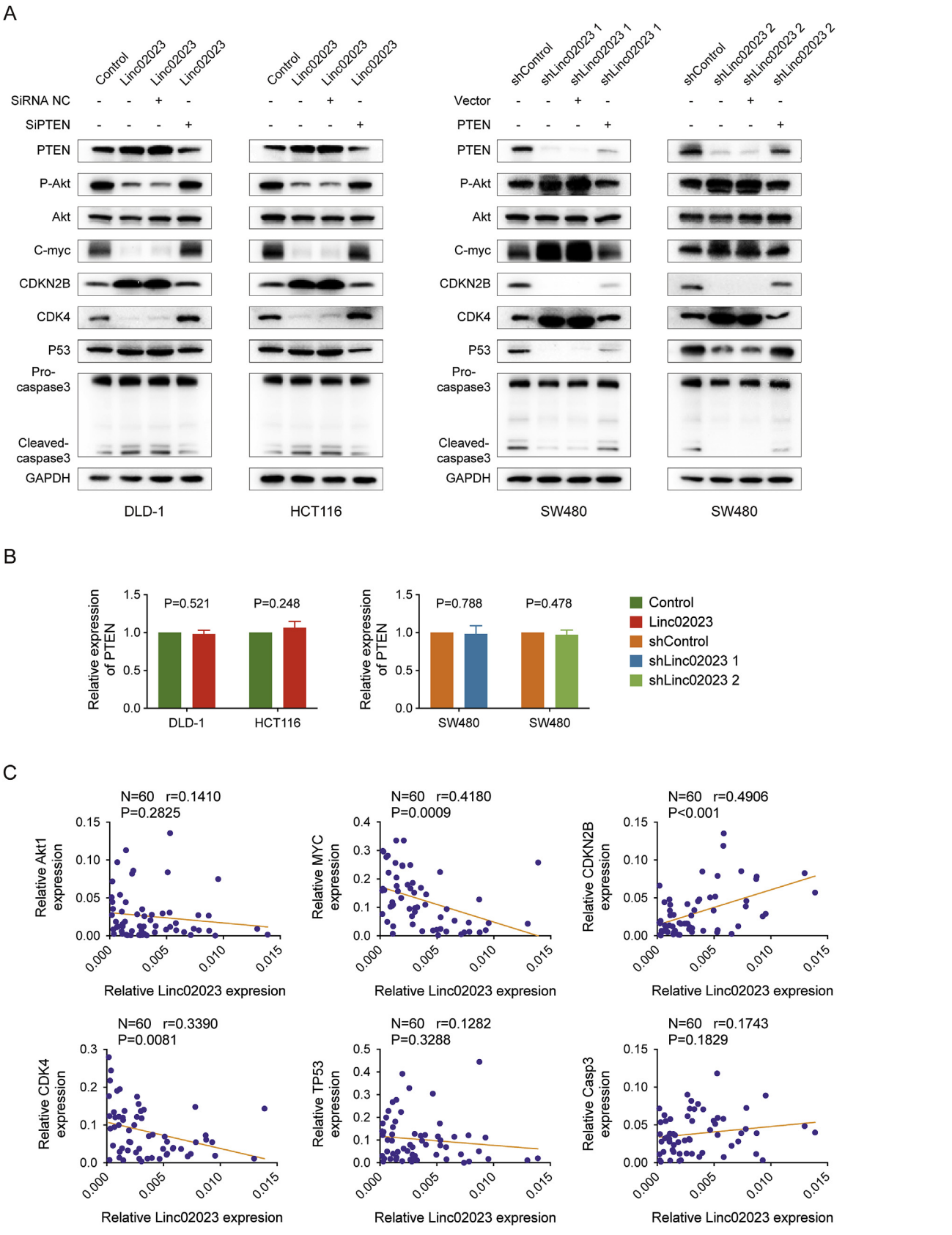

Fig. 6. Linc02023 regulated the PTEN-associated signaling module. (A) Immunoblot analysis of PTEN, total and phosphorylated Akt, C-myc, CDKN2B, CDK4, pro-caspase3, cleaved-caspase3, and GAPDH in CRC cells transfected with indicated lentiviral particles or plasmids. (B) Relative mRNA levels of PTEN in CRC cells were determined using qRT-PCR in DLD-1, HCT116, and SW480 transfected with Linc02023, Linc02023, shLinc02023 1, shLinc02023 2, Control or shControl. Data are presented as means ± standard deviation. (C) Pearson correlation analysis was performed to determine the correlation between mRNA levels of PTEN and Akt1, MYC, CDKN2B, CDK4, TP53, and Casp3 in CRC cancer tissues.

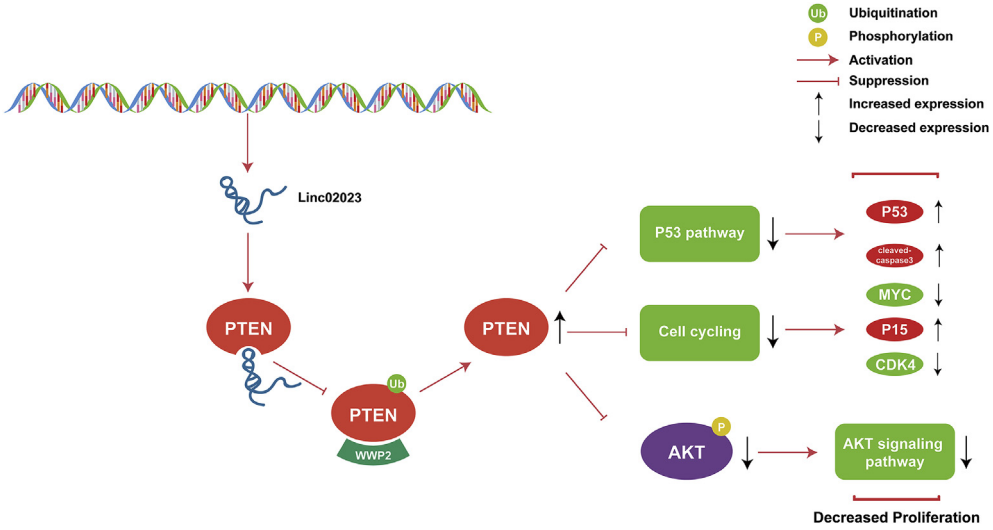

Fig. 7. Schematic model of Linc02023 in regulating CRC risk. Linc02023 specifically bound to PTEN, blocking its interaction with WWP2 and the subsequent ubiquitination and maintaining its stability, leading to a downregulation of associated module downstream and a decrease in colorectal cancer.

Akt signaling, C-myc/CDKN2B/CDK4, and P53/cleaved-caspase3 pathways.

Previous studies have demonstrated PTEN to be a bona fide tumor suppressor gene, having a critical role in the tumorigenesis of CRC. PTEN is activated by ligands, further suppressing the activation of PI3K/Akt signaling pathway, along with cell cycling and P53 signaling pathways, which are crucial in malignant transformation and metastasis. PTEN is regulated by its ubiquitination through interaction with WWP2. Blocking this interaction suppresses the ubiquitination of PTEN and upregulates its activation. The specific binding between Linc02023 and PTEN inhibits the interaction of PTEN with WWP2, thus diminishing PTEN ubiquitination, stabilizing PTEN proteins, and expanding its lifespan. The present study demonstrates that high basal levels of Linc02023 are sufficient to diminish PTEN ubiquitination–induced WWP2. These results highlight a rationale for decreased levels of PTEN protein observed in the patients of CRC and provide the evidence that PTEN protein stability is regulated by nuclear acids and interacting proteins.

In summary, using bioinformatic and GSEA analyses, an lncRNA, Linc02023, has been identified as an anti-tumor factor that specifically binds to PTEN and stabilizes it by blocking its interaction with WWP2. The subsequent ubiquitination leads to downstream inhibition and decreased proliferation of CRC cells. Moreover, the mechanistic characterization of Linc02023 and its interaction with PTEN might shed new light on developing cancer therapies that target PTEN through the restoration of Linc02023. Collectively, the findings in this study might help to investigate the mechanism of action of lncRNAs in CRC carcinogenesis and progression, contributing to cancer diagnosis and treatment.

Conflicts of interest

The authors declare no financial conflict of interest.

Acknowledgments

The authors thank Professor Yueming Sun and Professor Zan Fu for critical suggestions and Yueming Sun's lab members for their technical support. They also thank Ran Xu, Xiaoliang Xu, Jingsun Wei, Yue Wang, Fengqi, Zhou, and Ruxu Sun for helping with Pull-down assays and other experiments. This study was funded by Jiangsu Key Medical Discipline (General Surgery; grant no. ZDxKA2016005).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.041.

References

[1] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, Ca - Cancer J. Clin. 65 (2015) (2012) 87–108.
[2] A.M.D. Wolf, E.T.H. Fontham, T.R. Church, C.R. Flowers, C.E. Guerra, S.J. LaMonte, R. Etzioni, M.T. McKenna, K.C. Oeffinger, Y.T. Shih, L.C. Walter, K.S. Andrews, O.W. Brawley, D. Brooks, S.A. Fedewa, D. Manassaram-Baptiste, R.L. Siegel, R.C. Wender, R.A. Smith, Colorectal cancer screening for average-risk adults: 2018 guideline update from the American Cancer Society, Ca - Cancer J. Clin. 68 (2018) 250–281.
[3] H.G. Moore, Colorectal cancer: what should patients and families Be told to lower the risk of colorectal cancer? Surg. Oncol. Clin. 19 (2010) 693–710.
[4] F. Islami, A. Goding Sauer, K.D. Miller, R.L. Siegel, S.A. Fedewa, E.J. Jacobs, M.L. McCullough, A.V. Patel, J. Ma, I. Soerjomataram, W.D. Flanders, O.W. Brawley, S.M. Gapstur, A. Jemal, Proportion and number of cancer cases and deaths attributable to potentially modifiable risk factors in the United States, CA - Cancer J. Clin. 68 (2018) 31–54.
[5] J.M. Engreitz, N. Ollikainen, M. Guttman, Long non-coding RNAs: spatial amplifiers that control nuclear structure and gene expression, Nat. Rev. Mol. Cell Biol. 17 (2016) 756–770.
[6] T. Derrien, R. Johnson, G. Bussotti, A. Tanzer, S. Djebali, H. Tilgner, G. Guernec, D. Martin, A. Merkel, D.G. Knowles, J. Lagarde, L. Veeravalli, X. Ruan, Y. Ruan, T. Lassmann, P. Carninci, J.B. Brown, L. Lipovich, J.M. Gonzalez, M. Thomas, C.A. Davis, R. Shiekhattar, T.R. Gingeras, T.J. Hubbard, C. Notredame, J. Harrow, R. Guigo, The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression, Genome Res. 22 (2012) 1775–1789.
[7] M. Huarte, The emerging role of lncRNAs in cancer, Nat. Med. 21 (2015) 1253–1261.
[8] M. Guttman, I. Amit, M. Garber, C. French, M.F. Lin, D. Feldser, M. Huarte, O. Zuk, B.W. Carey, J.P. Cassady, M.N. Cabili, R. Jaenisch, T.S. Mikkelsen, T. Jacks, N. Hacohen, B.E. Bernstein, M. Kellis, A. Regev, J.L. Rinn, E.S. Lander, Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals, Nature 458 (2009) 223–227.
[9] F. Kopp, J.T. Mendell, Functional classification and experimental dissection of long noncoding RNAs, Cell 172 (2018) 393–407.
[10] I. Ulitsky, David P. Bartel, lincRNAs: genomics, evolution, and mechanisms, Cell 154 (2013) 26–46.
[11] Z. Wang, B. Yang, M. Zhang, W. Guo, Z. Wu, Y. Wang, L. Jia, S. Li, N. Cancer Genome Atlas Research, W. Xie, D. Yang, lncRNA epigenetic landscape analysis identifies EPIC1 as an oncogenic lncRNA that interacts with MYC and promotes cell-cycle progression in cancer, Cancer Cell 33 (2018) 706–720 e709.
[12] J. Li, PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer, Science 275 (1997) 1943–1947.
[13] P. Steck, M. Pershouse, S. Jasser, W. Yung, H. Lin, A. Ligon, L. Langford, M. Baumgard, T. Hattier, T. Davis, C. Frye, R. Hu, B. Swedlund, D. Teng, S.J.N.G. Tavtigian, Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers, 15 (1997) 356–362.
[14] M.C. Hollander, G.M. Blumenthal, P.A. Dennis, PTEN loss in the continuum of

common cancers, rare syndromes and mouse models, Nat. Rev. Canc. 11 (2011) 289–301.
[15] A. Alimonti, A. Carracedo, J.G. Clohessy, L.C. Trotman, C. Nardella, A. Egia, L. Salmena, K. Sampieri, W.J. Haveman, E. Brogi, A.L. Richardson, J. Zhang, P.P. Pandolfi, Subtle variations in Pten dose determine cancer susceptibility, Nat. Genet. 42 (2010) 454–458.
[16] M.S. Song, L. Salmena, P.P. Pandolfi, The functions and regulation of the PTEN tumour suppressor, Nat. Rev. Mol. Cell Biol. 13 (2012) 283–296.
[17] L. Salmena, A. Carracedo, P.P. Pandolfi, Tenets of PTEN tumor suppression, Cell 133 (2008) 403–414.
[18] M. Keniry, R. Parsons, The role of PTEN signaling perturbations in cancer and in targeted therapy, Oncogene 27 (2008) 5477–5485.
[19] Y.R. Lee, M. Chen, P.P. Pandolfi, The functions and regulation of the PTEN tumour suppressor: new modes and prospects, Nat. Rev. Mol. Cell Biol. 19 (2018) 547–562.
[20] S. Wang, Z. Zhang, W. Qian, D. Ji, Q. Wang, B. Ji, Y. Zhang, C. Zhang, Y. Sun, C. Zhu, Y. Sun, Angiogenesis and vasculogenic mimicry are inhibited by 8-Br-cAMP through activation of the cAMP/PKA pathway in colorectal cancer, OncoTargets Ther. 11 (2018) 3765–3774.
[21] S. Maddika, S. Kavela, N. Rani, V.R. Palicharla, J.L. Pokorny, J.N. Sarkaria, J. Chen, WWP2 is an E3 ubiquitin ligase for PTEN, Nat. Cell Biol. 13 (2011) 728–733.
[22] A.G. Li, L.G. Piluso, X. Cai, G. Wei, W.R. Sellers, X.J.M.c. Liu, Mechanistic Insights into Maintenance of High p53 Acetylation by PTEN vol. 23, (2006), pp. 575–587.
[23] A.M. Bode, Z. Dong, Post-translational modification of p53 in tumorigenesis, Nat. Rev. Canc. 4 (2004) 793–805.
[24] P.A. Konstantinopoulos, M.V. Karamouzis, A.G. Papavassiliou, Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets, Nat. Rev. Drug Discov. 6 (2007) 541–555.
[25] D. Hoeller, C.M. Hecker, I. Dikic, Ubiquitin and ubiquitin-like proteins in cancer pathogenesis, Nat. Rev. Canc. 6 (2006) 776–788.
[26] G. Duan, D. Walther, The roles of post-translational modifications in the context of protein interaction networks, PLoS Comput. Biol. 11 (2015) e1004049.
[27] D. Senft, J. Qi, Z.A. Ronai, Ubiquitin ligases in oncogenic transformation and cancer therapy, Nat. Rev. Canc. 18 (2018) 69–88.
[28] N. Krall, F.P. da Cruz, O. Boutureira, G.J. Bernardes, Site-selective protein-modification chemistry for basic biology and drug development, Nat. Chem. 8 (2016) 103–113.
[29] S. Gross, R. Rahal, N. Stransky, C. Lengauer, K.P. Hoeflich, Targeting cancer with kinase inhibitors, J. Clin. Investig. 125 (2015) 1780–1789.
[30] A. Hershko, A. Ciechanover, A.J.N.m. Varshavsky, The Ubiquitin System vol. 6, (2000), p. 1073.
[31] Y. Zhao, A. Aguilar, D. Bernard, S. Wang, Small-Molecule inhibitors of the MDM2–p53 protein–protein interaction (MDM2 inhibitors) in clinical trials for cancer treatment, J. Med. Chem. 58 (2014) 1038–1052.
[32] S. Wang, Y. Zhao, A. Aguilar, D. Bernard, C.Y. Yang, Targeting the MDM2-p53 protein-protein interaction for new cancer therapy: progress and challenges, Cold Spring Harb. Perspect .Med. 7 (2017).
[33] N. Issaeva, P. Bozko, M. Enge, M. Protopopova, L.G. Verhoef, M. Masucci, A. Pramanik, G. Selivanova, Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors, Nat. Med. 10 (2004) 1321–1328.
[34] M. Enge, W. Bao, E. Hedstrom, S.P. Jackson, A. Moumen, G. Selivanova, MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53, Cancer Cell 15 (2009) 171–183.
[35] D. Hoeller, I. Dikic, Targeting the ubiquitin system in cancer therapy, Nature 458 (2009) 438–444.
[36] J. Zheng, X. Huang, W. Tan, D. Yu, Z. Du, J. Chang, L. Wei, Y. Han, C. Wang, X. Che, Y. Zhou, X. Miao, G. Jiang, X. Yu, X. Yang, G. Cao, C. Zuo, Z. Li, C. Wang, S.T. Cheung, Y. Jia, X. Zheng, H. Shen, C. Wu, D. Lin, Pancreatic cancer risk variant in LINC00673 creates a miR-1231 binding site and interferes with PTPN11 degradation, Nat. Genet. 48 (2016) 747–757.
[37] J. Beermann, M.-T. Piccoli, J. Viereck, T. Thum, Non-coding RNAs in development and disease: background, mechanisms, and therapeutic approaches, Physiol. Rev. 96 (2016) 1297–1325.
[38] Y. Okugawa, W.M. Grady, A. Goel, Epigenetic alterations in colorectal cancer: emerging biomarkers, Gastroenterology 149 (2015) 1204–1225 e1212.
[39] D. Han, M. Wang, N. Ma, Y. Xu, Y. Jiang, X. Gao, Long noncoding RNAs: novel players in colorectal cancer, Cancer Lett. 361 (2015) 13–21.