DNA methylation is a class of epigenetic modification manner, which is responsible for the inactivation of various tumor suppressors. Recently, long non-coding RNAs (lncRNAs) were revealed to be implicated in a variety of malignancies, including non-small cell lung cancer (NSCLC). However, the contributions of lncRNAs to DNA-methylation-induced oncogenic effects in NSCLC remain largely unknown. In this study, we identified a DNA-methylation-repressed lncRNA DIO3 opposite strand upstream RNA (DIO3OS) in NSCLC. DIO3OS is downregulated in NSCLC, and its low expression is related to poor prognosis. Ectopic expression of DIO3OS repressed NSCLC cell growth and motility and promoted NSCLC cell apoptosis in vitro. DIO3OS also repressed NSCLC tumorigenesis and metastasis in vivo. DIO3OS knockdown exhibited opposite biological effects. DIO3OS competitively bound heterogeneous nuclear ribonucleoprotein K (hnRNPK), repressed the binding of hnRNPK to MYC DNA and MYC mRNA, reduced the promoting roles of hnRNPK on MYC transcription and translation, and led to the repression of MYC transcription and translation, and therefore remarkably decreased the expression of MYC and CDC25A, a downstream target of MYC. Additionally, depletion of hnRNPK blocked the tumor-suppressive roles of DIO3OS in NSCLC. In conclusion, these findings identified DIO3OS as an important protective factor against NSCLC via modulating hnRNPK-MYC-CDC25A axis.

INTRODUCTION

Lung cancer is the second most common malignancy and primary cause of cancer-related death according to global cancer statistics 2020.1 2,206,771 new lung cancer cases and 1,796,144 lung-cancer-related deaths were estimated to occur in 2020 worldwide, accounting for 11.4% of the total new cancer cases and 18.0% of the total new cancer deaths.2 Pathologically, lung cancer is classified into small cell lung cancer (SCLC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and large cell lung cancer. LUAD, LUSC, and large cell lung cancer were also classified as non-small cell lung cancer (NSCLC), which constitutes more than 85% of all lung cancer cases.3 Although regional early NSCLC could be cured by surgical resection, the prognosis of most unresectable NSCLC patients is still unfavorable, with 5-year survival rate of less than 10%.4 Therefore, there is a critical need to reveal the underlying molecular mechanisms in NSCLC and develop more effective therapeutic strategy against NSCLC.

Increasing evidences in gene expression profiles of NSCLC have identified many aberrantly expressed genes, such as the upregulation of oncogenes and downregulation of tumor suppressors.5 Epigenetic factors exhibit pivotal roles in the modulation of genes expression.6 Aberrant epigenetic modifications may promote NSCLC progression via regulating the expression of oncogenes or tumor suppressors.7 DNA methylation is one important type of epigenetic modifications, which silences target genes expression.8 Several protein-encoding genes have been identified as targets regulated by DNA methylation, such as ALDH2, LMX1A, and SLC22A18.9–11 The hypermethylation or hypomethylation causes downregulation or overexpression of target genes, which further regulate NSCLC tumorigenesis and progression.12

Apart from protein-encoding genes, several non-coding RNAs were also identified to be targets of DNA methylation, such as miR-21 and miR-196b.12,13 The aberrant expressions and functions of micro-RNAs (miRNAs) have been reported in various malignancies, including NSCLC.14–15 Recent transcriptomic sequencings have identified more than 58,000 long non-coding RNAs (lncRNAs).16 lncRNAs is a class of transcripts with more than 200 nt in length and limited protein-coding ability.17 Many lncRNAs were also found to be aberrantly expressed in various diseases.18–25 Additionally, lncRNAs also exhibit pivotal roles in a variety of pathophysiological processes.26–33 Several lncRNAs were revealed to play oncogenic or tumor-suppressive roles in NSCLC, such as the oncogenic lncRNA LINC01561, SNHG15, and LINC00173.v1, and the tumor-suppressive lncRNA CASC7, BRCAT54, and NORAD.34–39 However, the...
factors contributing to the dysregulation of lncRNAs in NSCLC remain largely unresolved. Furthermore, whether DNA methylation modulates lncRNA expression in NSCLC needs further investigation.

In this study, through analyzing The Cancer Genome Atlas (TCGA) dataset, we identified a DNA-methylation-modulated lncRNA DIO3 opposite strand upstream RNA (DIO3OS). We found that DIO3OS was significantly downregulated in NSCLC, and its low expression was related to poor prognosis. Gain- and loss-of-function approaches revealed that DIO3OS significantly repressed NSCLC cell growth and mobility and promoted NSCLC cell apoptosis in vitro and also repressed NSCLC tumorigenesis and metastasis in vivo. The underlying molecular mechanisms mediating the roles of DIO3OS in NSCLC were investigated.

RESULTS
DIO3OS is downregulated in NSCLC, and its low expression is related to disease progress and poor prognosis

To search the lncRNAs differently expressed in NSCLC, we analyzed TCGA dataset using the online tool Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/). The 100 most significantly downregulated lncRNAs were shown in Table S1. Among these lncRNAs, we noted DIO3OS, which was not only significantly downregulated in LUAD (Figure 1A) but also significantly downregulated in LUSC (Figure 1B). Additionally, TCGA dataset also revealed that low expression of DIO3OS was related to shorter overall survival (Figure 1C), analyzed by Kaplan-Meier Plotter (http://kmplot.com/analysis/). To further validate the clinical relevance of DIO3OS in NSCLC, we collected 78 pairs of NSCLC tissues and matched adjacent normal lung tissues. Quantitative real-time polymerase chain reaction (PCR) results showed that DIO3OS was remarkably downregulated in NSCLC tissues compared to normal tissues (Figure 1D). Correlation analyses of DIO3OS expression and clinicopathological features revealed that low expression of DIO3OS was related to local invasion, lymphatic metastasis, and advanced Tumor, Node, Metastasis (TNM) stage (Table 1). Kaplan-Meier analysis revealed that low expression of DIO3OS was related to worse overall survival (Figure 1E). Thus, these data collectively demonstrated that DIO3OS was downregulated in NSCLC, and its low expression was related to disease progress and poor prognosis.
expression (Figures 2F and 2G). Therefore, the data suggested that
the demethylation reagent 5-Aza remarkably increased DIO3OS
in normal lung tissues (Figure 2E). Treatment of NSCLC cells with
promoter were much higher in NSCLC tissues compared with those
sues. The results revealed that DNA methylation levels of
sequencing in NSCLC tissues and matched adjacent normal lung tis-
ation levels of DIO3OS
CpG456 were remarkably inversely correlated with DIO3OS expres-
Pearson correlation analyses revealed that all 17 methylation probes in
CpG456 methylation status in TCGA NSCLC samples was calculated.
heatmap/#). Then, the correlation between DIO3OS expression and
TCGA Illumina Human DNA Methylation 450k BeadChip
promoter and CpG456 with methylation data were retrieved from
mined whether low expression of DIO3OS is correlated with methyl-
aberrant expression of genes.19 Intriguingly, the DIO3OS promoter
 located on a CpG island, CpG456 (Figure 2A). Thus, we next deter-
isolation of CpG456. Seventeen methylation probes located on DIO3OS
promoter and CpG456 with methylation data were retrieved from
TCGA Illumina Infinium Human DNA Methylation 450k BeadChip
data using the online tool UCSC Xena (https://xenabrowser.net/
heatmap/). Then, the correlation between DIO3OS expression and CpG456 methylation status in TCGA NSCLC samples was calculated. Pearson correlation analyses revealed that all 17 methylation probes in CpG456 were remarkably inversely correlated with DIO3OS expression in NSCLC (Figures 2B–2D and S1). Moreover, the DNA methylation levels of DIO3OS promoter were detected using bisulfate sequencing in NSCLC tissues and matched adjacent normal lung tissues. The results revealed that DNA methylation levels of DIO3OS promoter were much higher in NSCLC tissues compared with those in normal lung tissues (Figure 2E). Treatment of NSCLC cells with the demethylation reagent 5-Aza remarkably increased DIO3OS expression (Figures 2F and 2G). Therefore, the data suggested that
methyla

| Parameter                  | No. of patients | DIO3OS | p value |
|----------------------------|-----------------|--------|---------|
| Age                        |                 |        |         |
| ≥60                        | 48              | Low    |         |
| <60                        | 30              | High   | 0.642   |
| Gender                     |                 |        |         |
| Male                       | 49              |        | 0.482   |
| Female                     | 29              |        |         |
| Histologic                 |                 |        |         |
| Squamous carcinoma         | 43              |        |         |
| Adenocarcinoma             | 35              |        |         |
| Tumor size                 |                 |        |         |
| ≤3 cm                      | 39              |        | 0.113   |
| >3 cm                      | 39              |        |         |
| Local invasion             |                 |        |         |
| T1+T2                      | 63              |        | 0.044   |
| T3+T4                      | 15              |        |         |
| Lymphatic metastasis       |                 |        |         |
| Negative                   | 47              |        | 0.037   |
| Positive                   | 31              |        | 0.015   |
| TNM stage                  |                 |        |         |
| I                          | 33              |        |         |
| II                         | 24              |        |         |
| III                        | 21              |        |         |

p values were calculated by Pearson chi square test.

**CpG456 methylation downregulates DIO3OS expression**
Epigenetic alterations in promoter region play pivotal roles in the aberrant expression of genes.19 Intriguingly, the DIO3OS promoter is located on a CpG island, CpG456 (Figure 2A). Thus, we next determined whether low expression of DIO3OS is correlated with methylation of CpG456. Seventeen methylation probes located on DIO3OS promoter and CpG456 with methylation data were retrieved from TCGA Illumina Infinium Human DNA Methylation 450k BeadChip data using the online tool UCSC Xena (https://xenabrowser.net/heatmap/#). Then, the correlation between DIO3OS expression and CpG456 methylation status in TCGA NSCLC samples was calculated. Pearson correlation analyses revealed that all 17 methylation probes in CpG456 were remarkably inversely correlated with DIO3OS expression in NSCLC (Figures 2B–2D and S1). Moreover, the DNA methylation levels of DIO3OS promoter were detected using bisulfate sequencing in NSCLC tissues and matched adjacent normal lung tissues. The results revealed that DNA methylation levels of DIO3OS promoter were much higher in NSCLC tissues compared with those in normal lung tissues (Figure 2E). Treatment of NSCLC cells with the demethylation reagent 5-Aza remarkably increased DIO3OS expression (Figures 2F and 2G). Therefore, the data suggested that

### DIO3OS represses NSCLC cell growth and motility and promotes NSCLC cell apoptosis in vitro
To explore the potential biological effects of DIO3OS in NSCLC, H1299 and H1975 cells with DIO3OS stable overexpression were constructed through transfection of DIO3OS overexpression vector. The overexpression efficiencies were confirmed by quantitative real-time PCR (Figures 3A and 3B). Cell Counting Kit-8 (CCK-8) assays revealed that cell growth of H1299 and H1975 cells was remarkably reduced after overexpression of DIO3OS (Figures 3C and 3D). Ethynyl deoxyuridine (EdU) incorporation assays validated that DIO3OS overexpressed H1299 and H1975 cells had less EdU-positive cells compared to control cells (Figures 3E and 3F), suggesting that overexpression of DIO3OS repressed NSCLC cell growth. To detect the effects of DIO3OS on NSCLC cell apoptosis, the activities of caspase-3 in cell lysates of H1299 and H1975 cells with DIO3OS stable overexpression were detected. The results revealed that caspase-3 activities were increased after DIO3OS overexpression in both H1299 and H1975 cells (Figures 3G and 3H), suggesting that DIO3OS overexpression promoted NSCLC cell apoptosis. To evaluate the effects of DIO3OS on NSCLC cell motility, transwell migration and invasion assays were conducted. Transwell migration assays revealed that cell migration abilities of H1299 and H1975 cells were both reduced after DIO3OS overexpression (Figures 3I and 3J). Transwell invasion assays revealed that cell-invasion abilities of H1299 and H1975 cells were increased after DIO3OS silencing (Figures 3K and 3L). Taken together, these findings demonstrated that DIO3OS repressed NSCLC cell growth and motility and promoted NSCLC cell apoptosis in vitro.

### Silencing DIO3OS promotes NSCLC cell growth and motility and represses NSCLC cell apoptosis in vitro
Apart from the effects of DIO3OS overexpression on NSCLC, we further investigated the effects of DIO3OS knockdown on NSCLC. A549 cells with DIO3OS stable knockdown were constructed through infection of two independent DIO3OS short hairpin RNA (shRNA) lentiviruses. The knockdown efficiencies were confirmed by quantitative real-time PCR (Figure 4A). CCK-8 assays revealed that cell growth of A549 cells was remarkably increased after DIO3OS knockdown (Figure 4B). EdU incorporation assays validated that DIO3OS-silenced A549 cells had significantly more EdU-positive cells compared to control cells (Figure 4C), suggesting that silencing DIO3OS promoted NSCLC cell growth. Caspase-3 activities in cell lysates of DIO3OS-silenced A549 cells were remarkably reduced compared to those in cell lysates of control A549 cells (Figure 4D), suggesting that silencing DIO3OS repressed NSCLC cell apoptosis. Transwell migration assays revealed that cell migration abilities of A549 cells were both reduced after DIO3OS overexpression (Figures 3K and 3L). Taken together, these findings demonstrated that silencing DIO3OS promoted NSCLC cell growth and motility and promoted NSCLC cell apoptosis in vitro.
repressed NSCLC cell apoptosis in vitro. These gain- and loss-of-function experiments suggested that DIO3OS exhibits tumor suppressive roles in vitro.

**DIO3OS represses NSCLC tumorigenesis and metastasis in vivo**

To further validate the potential roles of DIO3OS in NSCLC tumorigenesis and metastasis in vivo, luciferase-labeled H1299 cells with DIO3OS stable overexpression or control were injected into tail vein of nude mice. 7 weeks after injection, bioluminescence analysis revealed that the mice injected with DIO3OS-overexpressed H1299 cells exhibited less lung tumorigenesis and metastases compared to those injected with control H1299 cells (Figure 5A). Histological analysis of lungs revealed that DIO3OS-overexpressed H1299 cells formed a smaller number of lung metastatic nodules and smaller nodular diameter compared to control H1299 cells (Figures 5B–5D). Additionally, luciferase-labeled A549 cells with DIO3OS stable knockdown or control were also injected into tail vein of nude mice. 7 weeks after injection, bioluminescence analysis revealed that...
Figure 3. Ectopic expression of DIO3OS repressed NSCLC cell growth and mobility and promoted NSCLC cell apoptosis

(A) DIO3OS expression levels in H1299 cells with DIO3OS stable overexpression or control were measured by quantitative real-time PCR. (B) DIO3OS expression levels in H1975 cells with DIO3OS stable overexpression or control were measured by quantitative real-time PCR. (C) Cell growth of H1299 cells with DIO3OS overexpression or control was measured by CCK-8 assays. (D) Cell growth of H1975 cells with DIO3OS overexpression or control was measured by CCK-8 assays. (E) EdU incorporation assays. Scale bars, 200 μm. (F) EdU incorporation assays. Scale bars, 200 μm. (G) The activities of caspase-3 in cell lysates of H1299 cells with DIO3OS overexpression or control were measured by EdU incorporation assays. Scale bars, 200 μm. (H) The activities of caspase-3 in cell lysates of H1295 cells with DIO3OS overexpression or control were measured by EdU incorporation assays. Scale bars, 200 μm. (I) Cell migration of H1299 cells with DIO3OS overexpression or control was measured by transwell migration assays. Scale bars, 100 μm. (J) Cell migration of H1295 cells with DIO3OS overexpression or control was measured by transwell migration assays. Scale bars, 100 μm. (K) Cell invasion of H1299 cells with DIO3OS overexpression or control was measured by transwell invasion assays. Scale bars, 100 μm. (L) Cell invasion of H1295 cells with DIO3OS overexpression or control was measured by transwell invasion assays. Scale bars, 100 μm. Data are shown as mean ± SD as determined by triple assays. *p < 0.05, **p < 0.01, and ***p < 0.001 by Student’s t test.
the mice injected with DIO3OS-silenced A549 cells exhibited more lung tumorigenesis and metastases compared to those injected with control A549 cells (Figure 5E). Histological analysis of lungs revealed that DIO3OS-silenced A549 cells formed a greater number of lung metastatic nodules and larger nodular diameter compared to control A549 cells (Figures 5F–5H). Taken together, these findings demonstrated that DIO3OS repressed NSCLC tumorigenesis and metastasis in vivo, which also supports the tumor-suppressive roles of DIO3OS in NSCLC.

DIO3OS competitively binds to heterogeneous nuclear ribonucleoprotein K (hnRNPK) and abrogates the effects of hnRNPK on MYC transcription and translation

One of the most common functional mechanisms of IncRNAs is to bind proteins. To investigate this potential mechanism for DIO3OS, we utilized the online tool A database of RNA-binding proteins and associated motifs (ATRACCT) (https://attract.cnic.es/) to predict the proteins potentially interacting with DIO3OS. This analysis predicted hnRNPK as a potential

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**Figure 4. Depletion of DIO3OS promoted NSCLC cell growth and mobility and repressed NSCLC cell apoptosis**

(A) DIO3OS expression levels in A549 cells with DIO3OS stable knockdown or control were measured by quantitative real-time PCR. (B) Cell growth of A549 cells with DIO3OS knockdown or control was measured by CCK-8 assays. (C) Cell growth of A549 cells with DIO3OS knockdown or control was measured by EdU incorporation assays. Scale bars, 200 μm. (D) The activities of caspase-3 in cell lysates of A549 cells with DIO3OS knockdown or control were measured to indicate cell apoptosis. (E) Cell migration of A549 cells with DIO3OS knockdown or control was measured by transwell migration assays. Scale bars, 100 μm. (F) Cell invasion of A549 cells with DIO3OS knockdown or control was measured by transwell invasion assays. Scale bars, 100 μm. Data are shown as mean ± SD as determined by triple assays. **p < 0.01, ***p < 0.001, and ****p < 0.0001 by one-way ANOVA, followed by Dunnett’s multiple comparisons test.
DIO3OS-interacting protein (Table S2). The predicted binding motif was located on the 398–420 nt of DIO3OS (CCUACCC CUCCCCCAUCGAAUCG). The recently reported hnRNPK binding motif CCUCCCC was also located in this region. Another online tool RNA-Protein Interaction Prediction (RPISeq) (http://pridb.gdcb.iastate.edu/RPISeq/) predicted the hnRNPK-DIO3OS interaction probability score of 0.9 (probability score >0.5 was considered positive). The third online tool RBPmap (http://rbpmap.technion.ac.il/) also predicted hnRNPK binding motif in this region (Table S3). Therefore, we focused on hnRNPK. RNA pull-down assays followed by western blot analyses using sense or antisense strand of biotin-labeled DIO3OS revealed the specific enrichment of hnRNPK in sense strand of DIO3OS group (Figure 6A). Additionally, biotinylated oligonucleotide probes complementary to DIO3OS were utilized to enrich DIO3OS and its interacted proteins. The results revealed that DIO3OS was successfully enriched by DIO3OS probes and also hnRNPK was specifically enriched by DIO3OS probes (Figure 6B). In a reciprocal experiment, RNA immunoprecipitation (RIP) assays using hnRNPK-specific antibodies showed the specific enrichment of DIO3OS in hnRNPK antibody group (Figure 6C). These data supported the interaction between DIO3OS and hnRNPK.

Figure 5. DIO3OS repressed NSCLC tumorigenesis and metastasis in vivo

(A–D) Luciferase-labeled H1299 cells with DIO3OS stable overexpression or control were injected into tail vein of nude mice. Bioluminescence analyses of lung metastasis at day 49 and the representative images of mice are shown in (A). Lung metastatic nodule numbers at day 49 are shown in (B). Maximal nodule diameters are shown in (C). Representative images of lung H&E staining are shown in (D). Scale bars, 200 μm. (E–H) Luciferase-labeled A549 cells with DIO3OS stable knockdown or control were injected into tail vein of nude mice. Bioluminescence analyses of lung metastasis at day 49 and the representative images of mice are shown in (E). Lung metastatic nodule numbers at day 49 are shown in (F). Maximal nodule diameters are shown in (G). Representative images of lung H&E staining are shown in (H). Scale bars, 200 μm. Data are shown as mean ± SD, as determined by n = 6 mice in each group. *p < 0.05 and **p < 0.01 by Mann Whitney test (A–C) or Kruskal–Wallis test, followed by Dunn’s multiple comparisons test (E–G).
hnRNPK is a pivotal nucleic acid binding protein. Several reports have revealed the oncogenic roles of hnRNPK in lung cancer. Furthermore, classical oncogene MYC was identified as an important target of hnRNPK. hnRNPK is necessary for the binding of RNA polymerase II (RNA Pol II) to MYC and therefore promotes MYC transcription.

To reveal whether DIO3OS modulates the effects of hnRNPK on MYC, we first detected the binding of hnRNPK and RNA Pol II to MYC after DIO3OS overexpression or depletion using chromatin immunoprecipitation (ChIP) assays. The results revealed that the binding of DIO3OS and RNA Pol II to MYC was remarkably repressed after DIO3OS overexpression and increased after DIO3OS depletion (Figures 6D and 6E). hnRNPK knockdown abrogated the effects of DIO3OS on the binding of hnRNPK and RNA Pol II to MYC (Figure 6F). Next, the effects of DIO3OS on MYC transcription were detected. As expected, MYC mRNA levels were downregulated after DIO3OS overexpression and upregulated after DIO3OS depletion.
after DIO3OS depletion (Figures 6G and 6H), MYC expression was inversely correlated with DIO3OS expression in LUAD from TCGA dataset (Figure 6I), supporting the negative regulation of MYC transcription by DIO3OS.

Apart from the activation of MYC transcription, hnRNPK was also revealed to bind MYC mRNA 5′ UTR and promote MYC mRNA translation.47,48 Thus, we further investigated the potential influences of DIO3OS on MYC translation. RIP assays revealed that the binding of hnRNPK to MYC mRNA was significantly reduced after DIO3OS overexpression and increased after DIO3OS depletion (Figures 7A and 7B). MYC 5′ UTR was inserted into upstream of firefly luciferase encoding gene. Dual-luciferase reporter assays revealed that luciferase activities of the constructed reporter containing MYC 5′ UTR were downregulated after DIO3OS overexpression and increased after DIO3OS depletion (Figures 7C and 7D). The influences of DIO3OS on MYC 5′ UTR luciferase activities were abrogated by hnRNPK knockdown (Figure 7E). MYC protein levels were remarkably downregulated after DIO3OS overexpression and significantly upregulated after DIO3OS depletion (Figures 7F and 7G). Taken together, these data suggested that DIO3OS not only repressed MYC transcription but also repressed MYC translation through competitively binding hnRNPK.

CDC25A is an important MYC downstream target.49 TCGA LUAD dataset also showed the positive correlation between MYC expression and CDC25A expression (Figure 7H). Thus, to further validate the effects of DIO3OS on MYC, CDC25A expressions after DIO3OS overexpression or depletion were detected. The results revealed that CDC25A mRNA levels were remarkably downregulated after DIO3OS overexpression and remarkably upregulated after DIO3OS depletion (Figures 7I and 7J). CDC25A expression was inversely correlated with DIO3OS expression in LUAD from TCGA dataset (Figure 7K), further supporting the negative regulation of CDC25A by DIO3OS. Consistently, CDC25A protein levels were remarkably downregulated after DIO3OS overexpression and upregulated after DIO3OS depletion (Figures 7L and 7M). These data demonstrated that DIO3OS competitively bound hnRNPK and abrogated the positive effects of hnRNPK on MYC transcription and translation, resulting in the repression of MYC-CDC25A expression.

hnRNPK is required for the tumor-suppressive roles of DIO3OS in NSCLC

To elucidate whether hnRNPK is the critical mediator of the tumor-suppressive roles of DIO3OS in NSCLC, we detected the biological roles of DIO3OS in NSCLC at the absence of hnRNPK. CCK-8 assays revealed that hnRNPK absence abolished the reduced H1299 cell growth caused by DIO3OS overexpression (Figure 8A, compared to Figure 3C). Consistently, EdU incorporation assays revealed that hnRNPK absence abolished the reduced EdU-positive cell number caused by DIO3OS overexpression (Figure 8B, compared to Figure 3E). These results suggested that the repressive roles of DIO3OS on NSCLC cell growth were dependent on hnRNPK. Caspase-3 activity assays revealed that hnRNPK absence abolished the increased caspase-3 activity caused by DIO3OS overexpression (Figure 8C, compared to Figure 3G), suggesting that the promoting roles of DIO3OS on NSCLC cell apoptosis were dependent on hnRNPK. Transwell migration assays revealed that hnRNPK absence abolished the reduced cell migration abilities of H1299 cells caused by DIO3OS overexpression (Figure 8D, compared to Figure 3I). Transwell invasion assays revealed that hnRNPK absence abolished the reduced cell invasion abilities of H1299 cells caused by DIO3OS overexpression (Figure 8E, compared to Figure 3K). These results suggested that the repressive roles of DIO3OS on NSCLC cell motility were also dependent on hnRNPK.

DISCUSSION

DIO3OS arises from the antisense strand of DIO3OS and is located in chromosome 14q32.31. Recent reports revealed the downregulation of DIO3OS in cervical cancer and hepatocellular carcinoma but the upregulation of DIO3OS in pancreatic cancer.50–52 In this study, we first reported the downregulation of DIO3OS in NSCLC. Furthermore, we demonstrated that low expression of DIO3OS is correlated with NSCLC progression and poor prognosis. Functional experiments revealed the tumor-suppressive roles of DIO3OS. Thus, our study identified DIO3OS as a NSCLC-related IncRNA.

Through analyzing TCGA dataset, we found that DIO3OS is downregulated in both LUAD and LUSC. In our tissues, we found the DIO3OS is also downregulated in NSCLC, and its expression is not correlated with histologic types. Additionally, low expression of DIO3OS is found to be correlated with local invasion, lymphatic metastasis, and TNM stages. Kaplan-Meier survival analyses in multiple cohorts all demonstrated that low expression of DIO3OS is related to worse overall survival. Therefore, in this study, we identified DIO3OS as a downregulated and prognosis-correlated IncRNA in NSCLC. Our study provided a biomarker for NSCLC and also evidence to support the clinical significances of IncRNAs in NSCLC.

Several factors may modulate the expression of IncRNAs, such as transcription factors, histone acetylation, and histone methylation.53–58 The oncogenic IncRNA KTN1-AS1 was upregulated by STAT1 in NSCLC.59 IncRNA TUG1 was reported to be induced in p53 in NSCLC.60 SNHG3 was activated by E2F1 in NSCLC.61 AC020978 was transactivated by hypoxia-inducible factor-1z.62 Histone H3K27 acetylation induced the upregulation of LINC00467.63 Histone H3K9 demethylation upregulated MALAT1 expression.64 In this study, we found that DNA methylation also contributes to the dysregulation of IncRNAs in NSCLC. The CpG island CpG4556 methylation at DIO3OS promoter region repressed DIO3OS expression. Thus, we identified DIO3OS as a DNA-methylation-repressed IncRNA.

Gain- and loss-of-function assays revealed that DIO3OS repressed NSCLC cell growth and motility and promoted NSCLC cell apoptosis in vitro. DIO3OS was also revealed to repress NSCLC tumorigenesis and lung metastasis in vivo. Thus, our study demonstrated that
DIO3OS has tumor-suppressive roles in NSCLC and suggested that enhancing DIO3OS may be potential therapeutic strategy against NSCLC.

The combination of bioinformatic prediction and experimental verification identified hnRNPK as a critical interaction protein of DIO3OS. The expression and roles of hnRNPK in different cancers are

Figure 7. DIO3OS repressed the promoting effects of hnRNPK on MYC translation, resulting in downregulation of MYC and CDC25A
(A) RIP assays using hnRNPK-specific antibody were conducted in H1299 cells with DIO3OS overexpression or control, followed by quantitative real-time PCR to detect the binding of MYC mRNA to hnRNPK. (B) RIP assays using hnRNPK-specific antibody were conducted in A549 cells with DIO3OS knockdown or control, followed by quantitative real-time PCR to detect the binding of MYC mRNA to hnRNPK. (C) Schematic structure of the constructed MYC 5' UTR firefly luciferase reporter is shown. After transient co-transfection of the constructed reporter and pRL-TK (encoding Renilla luciferase) into H1299 cells with DIO3OS overexpression or control, luciferase activities were measured by dual-luciferase reporter assays. Results are shown as relative ratio of firefly luciferase activity to Renilla luciferase activity. (D) After transient co-transfection of the constructed MYC 5' UTR reporter and pRL-TK into A549 cells with DIO3OS knockdown or control, luciferase activities were measured by dual-luciferase reporter assays. Results are shown as relative ratio of firefly luciferase activity to Renilla luciferase activity. (E) After transient co-transfection of the constructed MYC 5' UTR reporter and pRL-TK into H1299 cells with DIO3OS overexpression or control and concurrent hnRNPK depletion, luciferase activities were measured by dual-luciferase reporter assays. Results are shown as relative ratio of firefly luciferase activity to Renilla luciferase activity. (F) MYC protein levels in H1299 cells with DIO3OS overexpression or control and concurrent hnRNPK depletion were measured by western blot. (G) MYC protein levels in A549 cells with DIO3OS overexpression or control were measured by western blot. (H) The correlation between CDC25A expression and MYC expression from TCGA LUAD dataset is shown. (I) CDC25A expressions in H1299 cells with DIO3OS overexpression or control were measured by quantitative real-time PCR. (J) CDC25A expressions in A549 cells with DIO3OS knockdown or control were measured by quantitative real-time PCR. (K) The correlation between CDC25A expression and DIO3OS expression from TCGA LUAD dataset is shown. (L) CDC25A protein levels in H1299 cells with DIO3OS overexpression or control were measured by western blot. (M) CDC25A protein levels in A549 cells with DIO3OS knockdown or control were measured by western blot. Data are shown as mean ± SD, as determined by triple assays. **p < 0.01, ***p < 0.001, and ns by Student’s t test (A, C, E, and I) or one-way ANOVA followed by Dunnett’s multiple comparisons test (B, D, and J).
different in NSCLC, hnRNPK was reported to promote NSCLC growth and metastasis. As a nucleic acid binding protein, hnRNPK exhibits a variety of complex mechanisms, such as regulating transcription, pre-mRNA splicing, mRNA metabolism, and mRNA translation. MYC is a well-known oncogene in many cancers, including NSCLC. Additionally, MYC is also revealed to be a critical downstream target of hnRNPK. hnRNPK was necessary for RNA Pol II to bind MYC and promote MYC transcription. Furthermore, hnRNPK was also revealed to promote MYC translation via binding MYC mRNA 5' UTR. In this study, we found that DIO3OS competitively bound to hnRNPK and repressed the binding of hnRNPK to MYC DNA, leading to the repression of MYC transcription. Furthermore, through competitively binding hnRNPK, DIO3OS also repressed the binding of hnRNPK to MYC mRNA, leading to the repression of MYC mRNA translation. Therefore, DIO3OS remarkably repressed the expression of MYC. CDC25A was reported as a downstream target of MYC. Consistently, through repressing MYC, DIO3OS also repressed CDC25A expression in NSCLC. The inverse correlation between the expression of DIO3OS and MYC, CDC25A supported the repressive roles of DIO3OS on MYC and CDC25A. Moreover, absence of hnRNPK abolished the tumor-suppressive roles of DIO3OS in NSCLC, suggesting hnRNPK as a critical functional mediator of DIO3OS.

In conclusion, our findings identified DIO3OS as a DNA-methylation-repressed and lowly expressed lncRNA in NSCLC, whose low expression is related with poor prognosis. DIO3OS exhibited tumor-suppressive roles in NSCLC through competitively binding hnRNPK, reducing the binding of hnRNPK to MYC DNA and MYC mRNA, reversing the promoting roles of hnRNPK on MYC transcription and translation, leading to the repression of MYC transcription and translation, and therefore significantly repressing MYC and CDC25A expression.

MATERIALS AND METHODS

Human tissue

Seventy-eight pairs of NSCLC tissues and matched adjacent normal lung tissues were obtained from NSCLC patients who received...
surgery resection at Guangzhou First People's Hospital. All tissues were examined by histopathological diagnosis and stored at −80°C until use. This study was conducted in accordance with the standards set by the Declaration of Helsinki and approved by the Review Board of Guangzhou First People's Hospital.

Cell culture and treatment
Human NSCLC cell lines H1299, H1975, and A549 were acquired from the American Type Culture Collection (ATCC) (Manassas, VA, USA). H1299 and H1975 cells were cultured in RPMI1640 Medium (Invitrogen, Carlsbad, CA, USA) added with 10% fetal bovine serum (FBS) (Invitrogen). A549 cells were cultured in F12K Medium (Invitrogen) added with 10% FBS. All cells were maintained in a humidified incubator containing 5% CO2 at 37°C. Where indicated, A549 and H1299 cells were treated with 2.5 µM or 5 µM 5-Aza (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 3 days.

RNA extraction and quantitative real-time PCR
RNA extraction and quantitative real-time PCR were conducted as we previously described.41 Briefly, total RNA was extracted from indicated tissues or cultured cells using TRizol Regent (Invitrogen), followed by reverse transcription using the M-MLV Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was conducted using the TB Green Premix Ex Taq (Applied Biosystems, Foster City, CA, USA). The primers sequences were as follows: for DIO3OS, 5'-CCGGAGACCTGAGCCACCTT-3' (forward) and 5'-CAT TCAAGACCCCGTGCAGC-3' (reverse), NCBI Reference Sequence NR_152588.1, amplifying the 99–241 bp of DIO3OS transcript with product length of 143 bp and annealing temperature 60°C; for MYC, 5'-TCTTTCCTACCCCTTCATAC-3' (forward) and 5'-CGATTTCTT CCTAATCTTCTTG-3' (reverse), NCBI Reference Sequence NM_002467.6, amplifying the 989–1,195 bp of MYC with product length of 207 bp and annealing temperature 60°C; for CDC25A, 5'-TGGAG GTGAAAGAACAATGC-3' (forward) and 5'-GGTCAAGAGAAT CAGAATGG-3' (reverse), NCBI Reference Sequence NM_001789.3, amplifying the 658–855 bp of CDC25A with product length of 198 bp and annealing temperature 56°C; and for GAPDH, 5'-GTCGGAGTCT AACCAGTTTGGG-3' (forward) and 5'-TGGGTGGAGATAT CTGACCATG-3' (reverse), NCBI Reference Sequence NM_004600.6, amplifying the 241–681 bp of GAPDH with product length of 123 bp and annealing temperature 60°C. The quantification of RNA expression was calculated using the comparative Ct method.

Vectors construction, siRNA, and transfection
To construct DIO3OS overexpression and in vitro transcription vector, DIO3OS full-length sequences were PCR amplified using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) with the primers 5'-GCGGAAACCGGCTGGCCCGCCAGC-3' (forward) and 5'-GGTGAAGAACAACAGT-3' (reverse). Next, the PCR products were inserted into the BamHI I and EcoR I sites of the pcDNA3.1 vector (Invitrogen) to produce DIO3OS overexpression vector pcDNA3.1-DIO3OS. Additionally, the PCR products were also inserted into the BamHI I and EcoR I sites of the pSPT19 vector (Roche, Mannheim, Germany) to produce DIO3OS in vitro transcription vector pSPT19-DIO3OS. To construct MYC 5' UTR luciferase reporter, MYC 5' UTR sequences were PCR amplified using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) with the primers 5'-TGGTTATGGAACCGTGAGGATCTTGTAATTAATCGGAG-3' (forward) and 5'-GGCGTTCTTCTATGTTGGATCCTAAGCAGAGC CTCTTGAGAGAAG-3' (reverse). Next, the PCR products were inserted into the BamHI I site of the pMIR-REPORT vector (Applied Biosystems) using the NovoRecom Plus One step PCR Cloning Kit (Novozyme, Shanghai, China) to produce MYC 5' UTR luciferase reporter pMIR-REPORT-MYC-5'-UTR. ON-TARGETplus Human HNRN PK small interfering RNA (siRNA) reagents were obtained from Dharmaco (Cambridge, UK). The transfection of vectors and siRNAs was conducted using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s manual.

Stable cell lines construction
To construct NSCLC cells with DIO3OS stable overexpression, DIO3OS overexpression vector pcDNA3.1-DIO3OS was transfected into H1299 and H1975 cells. 72 h after transfection, the cells were selected with neomycin (500 µg/mL) for 4 weeks. To construct NSCLC cells with DIO3OS stable knockdown, two pairs of cdNA oligonucleotides against DIO3OS were synthesized and inserted into the shRNA lentivirus-expressing vector pLV3/H1/GFP&Puro (GenePharma, Shanghai, China), which was further used to produce shRNA lentiviruses targeting DIO3OS by GenePharma. Scrambled shRNA lentiviruses were used as negative control. The shRNA sequences were as follows: for shDIO3OS-1, 5'-GATGCCGCTCTGTAATTTTGGGACCAAGAGATGGTGGTC AAATCCTCGAGGCGTCTTCCATGGTGGATCCAAGGAGAGC TCTTTTGTTTGGC-3' (forward) and 5' -AATTCAAAAAACCCCTG GATTGGGACCAACCTTCTGAAGTTGGTGGTCAAATCTTGAGGCG-3' (reverse); for shDIO3OS-2, 5'-GATGCCGCTCTGTAATTTTGGGACCAAGAGATGGTGGTC AAATCCTCGAGGCGTCTTCCATGGTGGATCCAAGGAGAGC TCTTTTGTTTGGC-3' (forward) and 5'-AATTCAAAAAACCCCTG GATTGGGACCAACCTTCTGAAGTTGGTGGTCAAATCTTGAGGCG-3' (reverse); and for negative control (NC), 5'-GATGCCGCTCTGTAATTTTGGGACCAAGAGATGGTGGTC AAATCCTCGAGGCGTCTTCCATGGTGGATCCAAGGAGAGC TCTTTTGTTTGGC-3' (forward), and for negative control (NC), 5'-GATGCCGCTCTGTAATTTTGGGACCAAGAGATGGTGGTC AAATCCTCGAGGCGTCTTCCATGGTGGATCCAAGGAGAGC TCTTTTGTTTGGC-3' (reverse). Next, the shRNA lentiviruses targeting DIO3OS were infected into A549 cells. 96 h after transfection, the cells were selected with puromycin (1 µg/mL) for 4 weeks.

Cell growth and apoptosis assays
Cell growth ability was evaluated by CCK-8 and EdU incorporation assays. For CCK-8 assays, 2,000 indicated NSCLC cells were plated per well of 96-well plates. At indicated time points, 10 µL CCK-8 solution (Dojindo, Kumamoto, Japan) was added into the wells. After incubation for another 2 h, the absorbance at 450 nm was measured to indicate cell growth. EdU incorporation assay was conducted as we previously described using the EdU Kit (RiboBio, Guangzhou, China).41 Results were quantified through counting at least ten
random fields. Cell apoptosis was evaluated through detecting the activity of caspase-3 in cell lysates using Caspase-3 Activity Assay Kit (Cell Signaling Technology, Boston, MA, USA).

**Cell migration and invasion assays**

Cell motility ability was evaluated using transwell migration and invasion assays as previously described. Briefly, indicated NSCLC cells were suspended in serum-free medium were seeded into the top chambers of 24-well transwell inserts (BD Biosciences, San Jose, CA, USA) with (invasion) or without (migration) pre-coated Matrigel (BD Biosciences). Complete medium added with 10% FBS was placed into the lower chambers. After incubation for 48 h, cells remaining on the top chambers were wiped off while the cells that had traversed the membranes were fixed, stained, and counted based on at least ten random fields.

**Mouse xenograft assays**

Indicated luciferase-labeled NSCLC cells were injected into the tail veins of 5-week-old BALB/c nude mice. 49 days post-injection, the mice were intraperitoneally injected with 4.0 mg of luciferin (Caliper Life Sciences, Hopkinton, MA, USA) in 50 μL of saline. Then, 10 min later, the lung metastases were monitored by the IVIS® Lumina II system (Caliper Life Sciences). The mice were sacrificed, and the lungs were resected and detected by hematoxylin and eosin (H&E) staining. The animal experiments were approved by the Review Board of Guangzhou First People’s Hospital.

**RNA pull-down assays**

To detect the binding of proteins to DIO3OS, sense or antisense strands of biotin-labeled DIO3OS were in vitro transcribed from pSPT19-DIO3OS using the Biotin RNA Labeling Mix (Roche) and T7 or Sp6 RNA polymerase (Roche), respectively. After removal of DNA and purification using RNeasy Mini Kit (QIAGEN, Hilden, Germany), 3 μg of biotin-labeled sense or antisense strands of DIO3OS were incubated with 1 mg of whole-cell lysate of A549 cells for 1 h at 25°C. Next, the complexes were enriched by the streptavidin agarose beads (Invitrogen). The protein present in the enrichment was detected by western blot. Furthermore, the proteins bound in the enrichment were also enriched using biotinylated oligonucleotide probes complementary to DIO3OS. The probes sequences were as follows: (1) 5′-AACGTCCGGGGAGGAAGAGACG-3′; (2) 5′-AGGACACTTACGGCTGCC-3′; (3) 5′-CTACAA GTGCTACTGCAT-3′; (4) 5′-CTACAA GTGCTACTGCAT-3′; (5) 5′-TTGGGGAGGTTAGGTAT-3′; (6) 5′-TGAGCTGCTGCCCTGGCA GAC-3′; (7) 5′-TCTGAGATGTGGCAGCAG-3′; and (8) 5′-CTGTGTT TATTGTGTTACCC-3′. The enrichment of DIO3OS and DIO3OS-interacted proteins was conducted using the Magna ChIP RNA Interacome Kits (Millipore, Bedford, MA, USA) and detected by quantitative real-time PCR and western blot, respectively.

**RIP assay**

To detect the binding of RNAs to hnRNPK, RIP assays were conducted in A549 cells using the Magna ChIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) and hnRNPK-specific antibody (Abcam, Cambridge, UK) according to the manufacturer’s manual. The enrichment of RNAs was detected by quantitative real-time PCR. To detect the binding of hnRNPK and RNA Pol II to genomic DNA, ChIP assays were conducted in indicated NSCLC cells using the EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit (Millipore) and primary antibodies against hnRNPK (Abcam) or RNA Pol II (Millipore) according to the manufacturer’s manuals. The enriched DNA was detected by quantitative real-time PCR with the primers for MYC genomic DNA 5′-CAAAAATGAG GGCTGTGTT-3′ (forward) and 5′-GGCAAGGTTGGCTTTT CAG-3′ (reverse).

**Western blot**

Western blot was conducted as we previously described. Briefly, total proteins were extracted from indicated NSCLC cells using RIPA lysis buffer (Beyotime, Shanghai, China). Proteins enriched from RNA pull-down assays or extracted from cells (30 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by being transferred into nitrocellulose membrane (Millipore). After blocking using 5% non-fat milk, the membranes were incubated with primary antibodies against hnRNPK (Abcam), GAPDH (Abcam), MYC (Cell Signaling Technology), or CDC25A (Cell Signaling Technology). The membranes were further incubated with horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse secondary antibodies, followed by visualization using enhanced chemiluminescence.

**Dual-luciferase reporter assay**

MYC 5′ UTR luciferase reporter pMIR-REPORT-MYC-5′-UTR or empty luciferase reporter pMIR-REPORT was co-transfected with pRL-TK vector (Promega, Madison, WI, USA) into indicated NSCLC cells. pRL-TK expressed Renilla luciferase and was used to monitor transfection efficiency. 48 h after transfection, the firefly luciferase activities and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

**Bioinformatics analysis**

The expressions of DIO3OS in NSCLC from TCGA dataset were analyzed by the online in silico tool Gene Expression Profiling Interactive Analysis (GEPIA) (http://geopia.cancer-pku.cn/). The correlation between DIO3OS expression and overall survival in NSCLC from TCGA dataset was analyzed by the online in silico tool R2 (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). The correlation between DIO3OS expression and overall survival in NSCLC was also analyzed in another dataset using the online in silico tool Kaplan-Meier Plotter (http://kmplot.com/analysis/). Methylation data of 17 methylation probes located on CpG456 were retrieved from TCGA Illumina Infinium Human DNA Methylation 450k BeadChip data using the online tool UCSC Xena (https://xenabrowser.net/heatmap/). The bindings between lncRNAs and proteins were predicted using three online in silico tools: a database of RNA-binding proteins and associated motifs (ATtract) (https://attract.cnic.es/); RNA-Protein Interaction Prediction (RP1seq) (http://pridb.gdcb.iastate.edu/RP1Seq/); and RBPmap (http://rbpmap.technion.ac.il/).
**Statistical analysis**

All statistical analyses were conducted using the GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Comparisons between two groups were subjected to Wilcoxon matched-pairs signed rank test, Student’s t-test, or Mann Whitney test as indicated in figure legends. Comparisons between more than two groups were subjected to one-way ANOVA followed by Dunnett’s multiple comparisons test or Kruskal-Wallis test followed by Dunn’s multiple comparisons test as indicated in figure legends. Correlations between genes’ expression and methylation probes were subjected to Pearson correlation analysis. p < 0.05 was considered as statistically significant.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.09.006.

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**AUTHOR CONTRIBUTIONS**

M.Z. conceived and designed the study. Z.Z. and W.Z. advised on the experimental design. M.Z., J.W., and W.H. carried out the experiments. M.Z. and J.W. drafted the manuscript. All authors participated in the analysis and interpretation of data. All authors read and approved the final manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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