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ORIGINAL ARTICLE

The carcinogenic complex lncRNA DUXAP8/EZH2/LSD1 accelerates the proliferation, migration and invasion of colorectal cancer

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Summary

Purpose: *To elucidate the potential role of long non-coding RNA (lncRNA) DUXAP8 in the malignant progression of colorectal cancer (CRC) and its possible molecular mechanism.*

Methods: *The expression level of lncRNA DUXAP8 in CRC tissues and matched paracancerous tissues was detected by quantitative real-time polymerase chain reaction (qRT-PCR). Meanwhile, its level in CRC patients with different tumor sizes and tumor grades was determined. The regulatory effects of DUXAP8 on the behaviors of CRC cells were evaluated by cell counting kit-8 (CCK-8), 5-Ethynyl-2'- deoxyuridine (EdU) and Transwell assay. The interaction between LSD1, EZH2 and DUXAP8 was evaluated by RNA-protein interactions and RIP assay. Linear regression analyses were conducted to examine the correlation between DUXAP8 and LSD1, EZH2.*

Results: *LncRNA DUXAP8 was upregulated in CRC tissues and cell lines. Its level remained higher in CRC with larger tumor size or higher tumor grade. Knockdown of DUXAP8 suppressed the proliferative, migratory and invasive abilities of DLD-1 and SW480 cells. Both RF classifier and SVM classifier predicted the pronounced accuracies of LSD1 and EZH2. RIP assay further demonstrated the interaction between DUXAP8 and LSD1, EZH2. Knockdown of LSD1 or EZH2 could attenuate the proliferative rate of CRC cells. Moreover, the mRNA levels of LSD1 and EZH2 were positively correlated with DUXAP8 in CRC.*

Conclusions: *LncRNA DUXAP8 accelerates the malignant progression of CRC via positively regulating EZH2 and LSD1.*

Key words: *CRC, EZH2, LSD1, LncRNA DUXAP8, progression*

Introduction

Colorectal cancer (CRC) is a common malignant tumor of the gastrointestinal tract. There are no typical symptoms of early-stage CRC. As the tumor grows, patients gradually develop symptoms such as altered bowel habit, hematochezia, diarrhea, diarrhea alternating with constipation, local abdominal pain etc. In advanced CRC stages, manifestations such as anemia and weight loss occur [1]. Annually, 700,000 people die of CRC, which has become the fourth cause of death following lung, liver and gastric cancer [2]. CRC turns out to be a modern disease, which is highly prevalent in developed countries [3]. Owing to economic development, the incidence of CRC is on the rise [4]. The mortality of CRC remains high because of high recurrence rates [5,6].

LSD1 is a histone modification factor that is upregulated in malignant tumors, leading to tumor invasion [7]. LSD1 activation is necessary for the malignant phenotypes of tumors [8,9]. In addition, silencing of LSD1 would result in proliferative ability reduction and cell cycle arrest [10]. The

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enhancer of EZH2, a histone methyltransferase, catalyzes the trimethylation of histone H3 on Lys 27 (H3K27me3) and regulates gene expressions through epigenetic mechanisms [11]. EZH2 acts as a double-sided mediation on gene expressions depending on the cellular environment [12]. EZH2 interacts with histones and non-histone proteins to regulate a variety of physiological functions, including cancer progression and malignancy [13]. In this study, we focused on the potential influences of EZH2, LSD1, and lncRNA DUXAP8 on cancer progression and malignancy. Mutant and upregulated EZH2 are observed in different types of tumors, which is related to the poor prognosis of tumors, suggesting the involvement of EZH2 in tumorigenesis and tumor progression [14]. As a result, EZH2 is considered a therapeutic target for tumor treatment [15]. Knockdown of EZH2 using siRNA or an inhibitor contributes to suppressing tumor growth.

Long non-coding RNAs (LncRNAs) are non-coding RNAs with over 200 nucleotides [16]. Numerous studies have revealed that lncRNAs are widely expressed in human tissues and cells. They exert vital functions in multiple biological activities, and have been well concerned in diverse diseases [17,18]. Numerous studies have reported the great involvement of lncRNAs in the occurrence and progression of tumors [19]. Expression patterns of lncRNAs are closely related to the malignant level of tumor cells [20]. They are capable of regulating tumor progression *via* different mechanisms, such as regulation of histone modification, protein translation and mRNA stability [21-23]. For example, overexpressed lncRNA HOXA11-AS in human gastric cancer promotes cancer cell growth and metastasis by acting as a scaffold for LSD1 and EZH2. LncRNA DUXAP8 is upregulated in gastric cancer tissues [21]. The carcinogenic complex lncRNA-HOXA-AS2 / EZH2 / LSD1 accelerates the proliferative ability of pancreatic cancer cells [24]. The specific role of DUXAP8 in CRC, however, remains unclear. This study mainly investigated the expression pattern and functional characteristics of lncRNA DUXAP8 in CRC.

Methods

Sample collection

CRC tissues and matched normal tissues were surgically resected from CRC patients. Samples were immediately transferred into liquid nitrogen for preservation. None of the enrolled CRC patients received preoperative therapy. All patients and their families signed an informed consent form. This study was approved by the Ethics Committee of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform and isopropanol. The extracted RNA was quantified and reversely transcribed into complementary deoxyribose nucleic acid (cDNA), followed by PCR using SYBR Green method. QRT-PCR was conducted at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 90 s.

Cell culture and transfection

CRC cell lines (DLD-1 and SW480) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA), and cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). Cells were maintained in a 5% CO_2 incubator at 37°C. For cell transfection, cells were seeded in a 6-well plate with 1×10^4 cells per well. 1.5 ml of serum-free medium and 500 µL of Lipofectamin™ 2000 transfection solution (Invitrogen, Carlsbad, CA, USA) were applied in each well. Complete medium was replaced 4-6 h later.

Cell counting kit-8 (CCK-8) assay

Cells were seeded in a 96-well plate with 2×10^3 cells per well. Absorbance (A) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

5-Ethynyl-2'- deoxyuridine (EdU) assay

Transfected cells were seeded in a 96-well plate with 5×10^3 cells per well. Cells were labeled with 50 µmol/L EdU at 37°C for 2 h. Subsequently, cells were subjected to 30-min fixation in 4% paraformaldehyde and 20-min incubation in PBS containing 0.5% Triton-100. After washing with phosphate buffered saline (PBS) containing 3% bovine serum albumin (BSA), 100 µL of dying solution was applied per well for 1 h incubation in the dark and cells were counter-stained with 100 µL 4',6-diamidino-2-phenylindole (DAPI) (5 µg/mL) for 30 min. The ratio of EdU-positive cells was calculated.

Colony formation assay

Cells were seeded in a 6-well plate with 50, 100 and 200 cells per well and cultured for 2-3 weeks. Subsequently, cells were subjected to 15-min fixation in 4% paraformaldehyde and 10-min staining in Giemsa solution. After removing the staining solution, colonies were air dried and observed under a microscope.

Transwell assay

3×10^4 cells were seeded in the upper side of a Transwell chamber (Millipore, Billerica, MA, USA) pre-coated with 40 µL of diluted Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). In the bottom side, 600 µL of medium containing 20% FBS was applied. After 48 h of incubation, invasive cells were fixed in methanol for 10-15 min, dyed with 0.5% crystal violet for 20 min and counted

using a microscope. Penetrating cells were counted in 5 randomly selected fields per sample. Transwell invasion assay was conducted without Matrigel pre-coating.

RNA immunoprecipitation (RIP)

Cells were collected and treated according to the instructions of the Millipore Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Cell lysate was incubated with anti-EZH2, anti-LSD1 or IgG antibody at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/mL proteinase K containing 0.1% SDS to extract RNA. The magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to mRNA level determination using qRT-PCR.

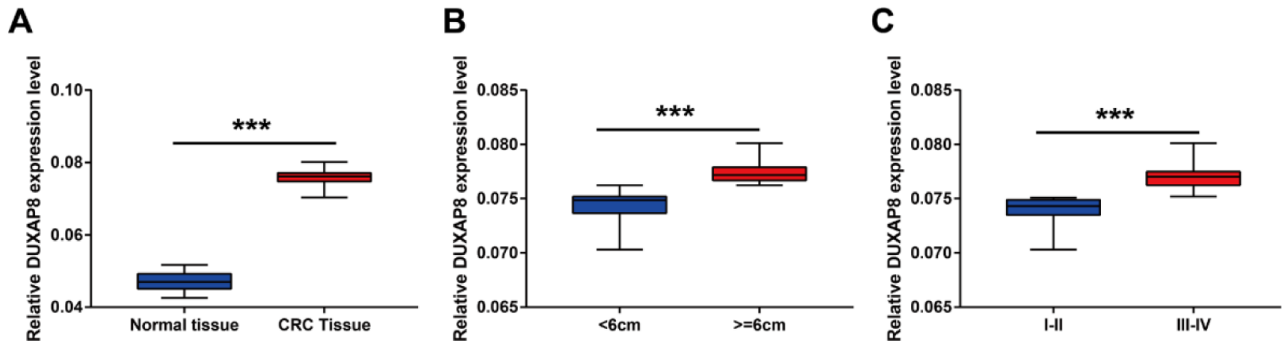

Figure 1. DUXAP8 was upregulated in CRC. **A:** Relative level of DUXAP8 in CRC tissues and matched normal tissues. **B:** Relative level of DUXAP8 in CRC tissues <6 cm and ≥6 cm. **C:** Relative level of DUXAP8 in stage I-II and III-IV CRC (***p<0.001).

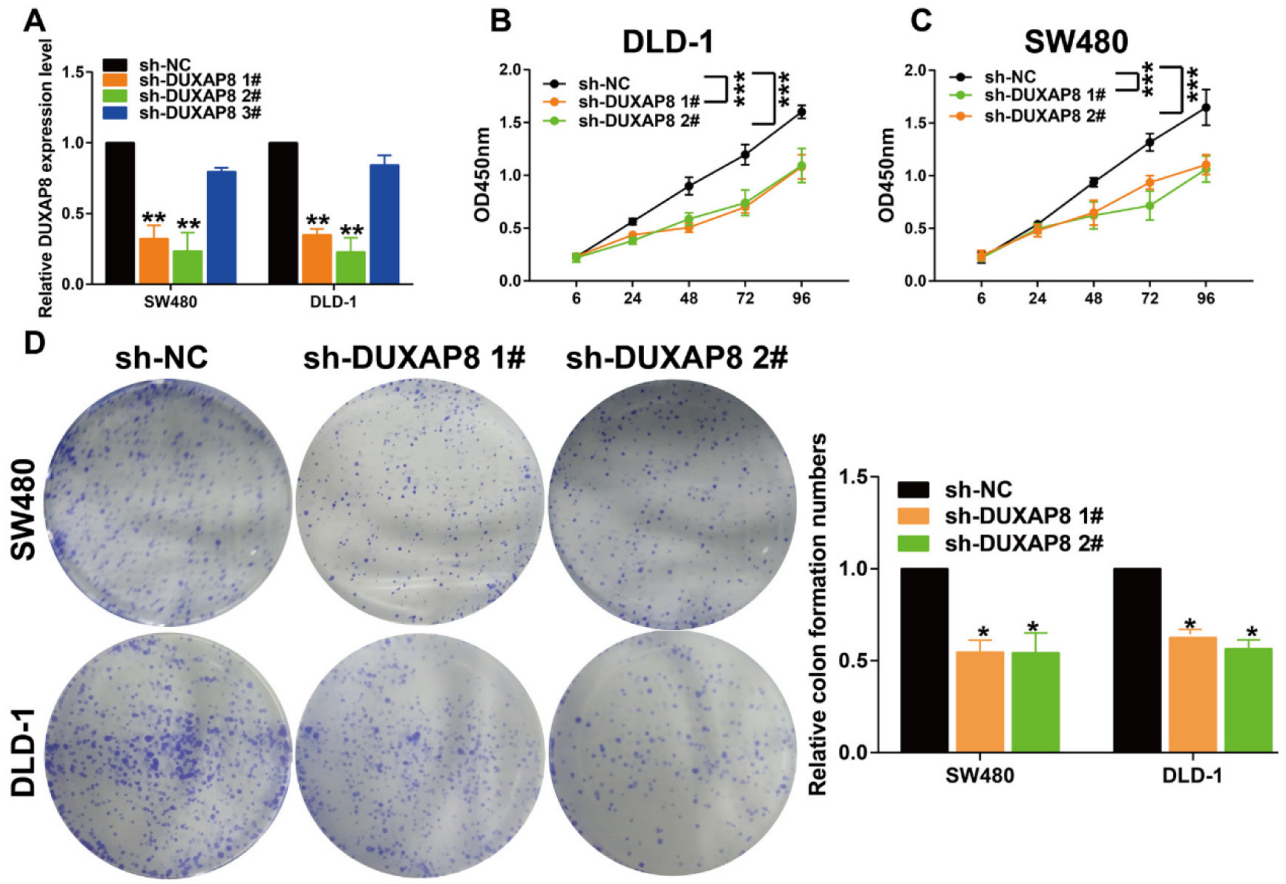

Figure 2. Knockdown of DUXAP8 suppressed the proliferative ability of CRC cells. **A:** Transfection efficacy of sh-DUXAP8 1#, sh-DUXAP8 2# and sh-DUXAP8 3# in SW480 and DLD-1 cells. **B:** CCK-8 assay revealed the viability in DLD-1 cells transfected with sh-NC, sh-DUXAP8 1# or sh-DUXAP8 2#. **C:** CCK-8 assay revealed the viability in SW480 cells transfected with sh-NC, sh-DUXAP8 1# or sh-DUXAP8 2#. **D:** Colony formation assay revealed the colony formation of SW480 and DLD-1 cells transfected with sh-DUXAP8 1# or sh-DUXAP8 2# were lower than in sh-NC group (*p<0.05, **p<0.01, ***p<0.001).

Statistics

SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. Linear regression analysis was conducted for evaluating the relationship between two genes. P<0.05 was considered statistically significant.

Results

DUXAP8 was upregulated in CRC

QRT-PCR data revealed a higher abundance of DUXAP8 in CRC tissues relative to matched normal tissues (Figure 1A). Moreover, DUXAP8 level remained higher in CRC tissues larger than 6 cm in size compared to those smaller than 6 cm (Figure 1B). CRC patients in stage I-II presented a lower level of DUXAP8 than those in stage III-IV (Figure 1C). These data suggested the carcinogenic potential of DUXAP8 in CRC.

Knockdown of DUXAP8 suppressed the proliferative ability of CRC cells

To elucidate the biological function of DUXAP8 in CRC, we constructed three sh-DUXAP8 vectors and tested their transfection in SW480 and DLD-1 cells. Among the three transfection vectors, sh-DUXAP8 1# and sh-DUXAP8 2# presented a pronounced transfection efficacy, which were selected for the following experiments (Figure 2A). CCK-8

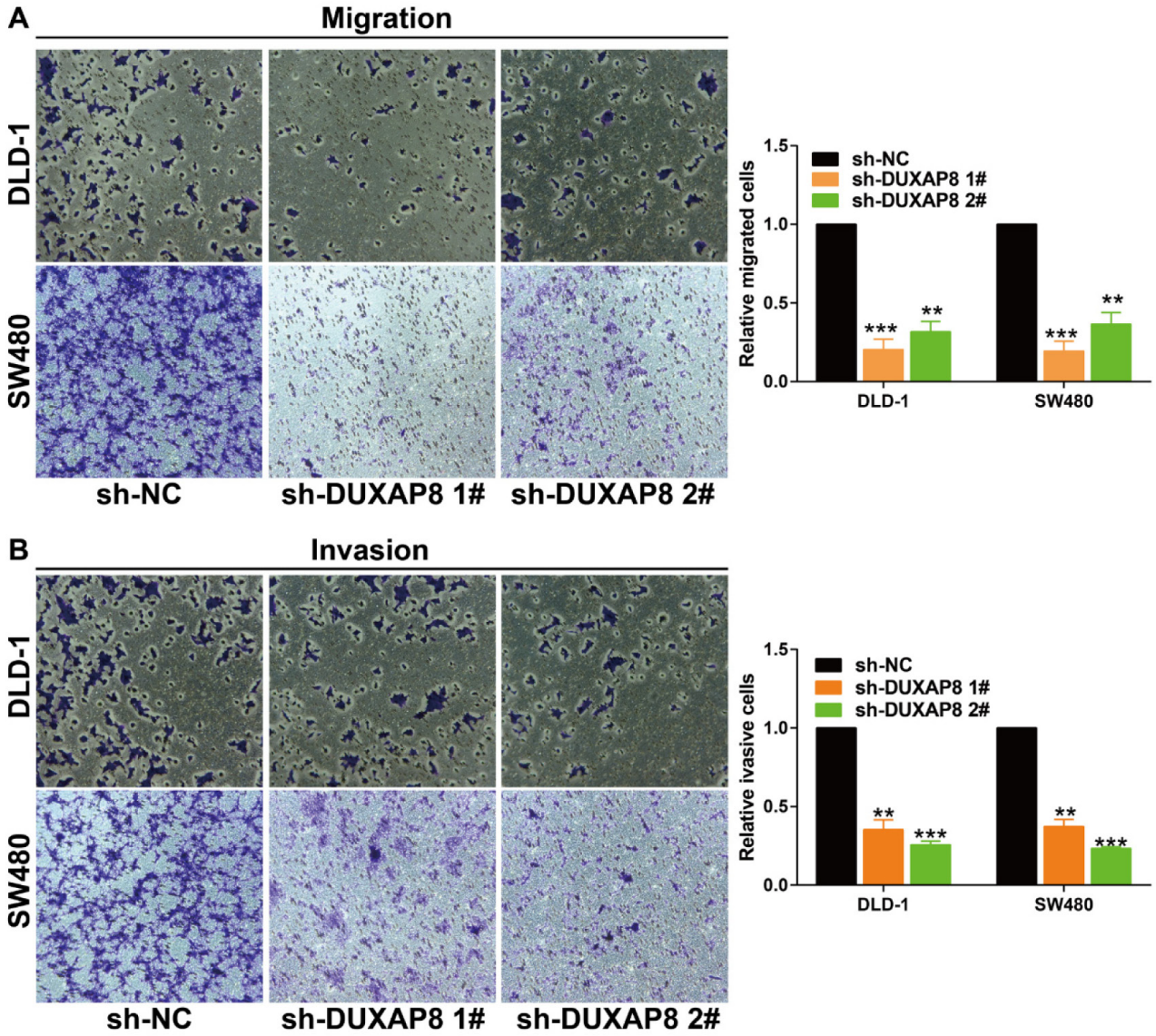

Figure 3. Knockdown of DUXAP8 suppressed the migratory and invasive abilities of CRC cells. **A:** Transwell assay revealed the migration in SW480 and DLD-1 cells transfected with sh-DUXAP8 1# or sh-DUXAP8 2# was lower than in sh-NC group. **B:** Transwell assay revealed the invasion in SW480 and DLD-1 cells transfected with sh-NC, sh-DUXAP8 1# or sh-DUXAP8 2# was lower than in sh-NC group (**p<0.01, ***p<0.001).

assay revealed the inhibited viability in DLD-1 and SW480 cells transfected with sh-DUXAP8 1# or sh-DUXAP8 2# (Figure 2B, 2C). After knockdown of DUXAP8 in CRC cells, relative colony formation numbers markedly decreased, indicating suppressed proliferative ability (Figure 2D).

Knockdown of DUXAP8 suppressed the migratory and invasive abilities of CRC cells

Transwell assay revealed that transfection of sh-DUXAP8 1# or sh-DUXAP8 2# in DLD-1 and SW480 cells attenuated migratory and invasive abilities (Figure 3A,3B). It is concluded that knockdown of DUXAP8 attenuated the proliferative, migratory and invasive abilities of CRC cells.

DUXAP8 accelerated CRC cell proliferation via binding to EZH2 and LSD1

Random Forest (RF) and Support Vector Machine (SVM) predicted the RNA-protein interactions of DUXAP8-LSD1and DUXAP8-EZH2 (Figure 4A). RIP assay revealed the pronounced occupancies of EZH2 and LSD1 in DUXAP8 of SW480 cells (Figure 4B). We constructed sh-EZH2 and sh-

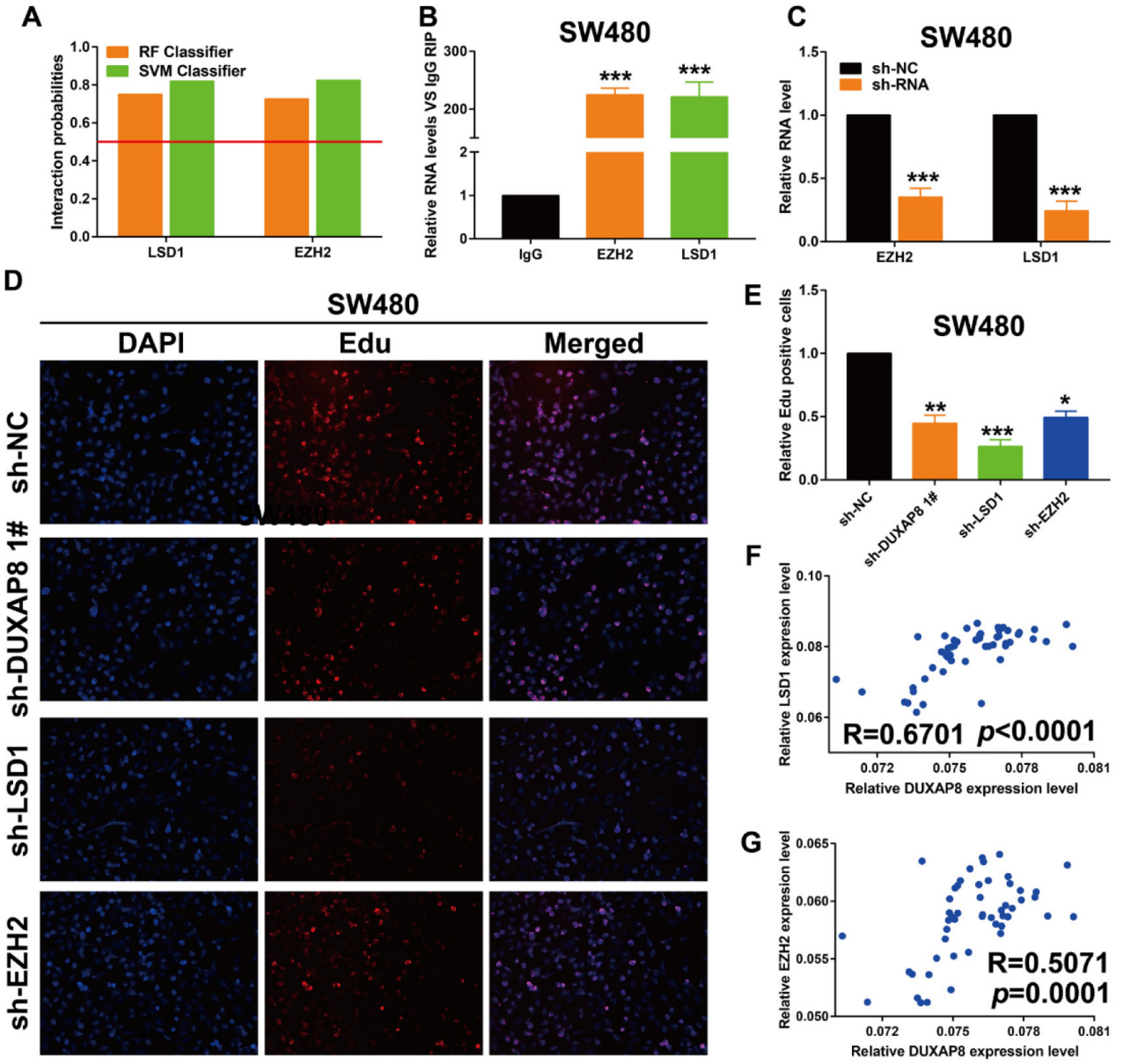

Figure 4. DUXAP8 accelerated CRC cell proliferation *via* binding to EZH2 and LSD1. **A:** RF and SVM predicted the DUXAP8 could bind to LSD1 and EZH2. **B:** RIP assay showed that DUXAP8 could bind to LSD1 and EZH2. **C:** Transfection efficacy of sh-EZH2 and sh-LSD1 in SW480 cells significantly reduced their RNA level. **D and E:** EdU assay revealed EdU-positive cells in SW480 cells transfected with sh-EZH2 or sh-LSD1 were significantly lower than in the sh-NC group. **F:** Positive correlation between LSD1 level and DUXAP8 level. **G:** Positive correlation between EZH2 level and DUXAP8 level (*p<0.05, **p<0.01, ***p<0.001).

LSD1, and their transfection efficacy was verified in SW480 cells (Figure 4C). EdU assay showed that transfection of sh-DUXAP10, sh-LSD1 or sh-EZH2 all could attenuate the proliferative rate of CRC cells (Figure 4D,4E). Correlation analyses revealed that the DUXAP8 level was correlated with LSD1 and EZH2 levels in CRC (Figure 4F,4G). Collectively, lncRNA DUXAP8 accelerated CRC cell proliferation *via* positively regulating LSD1 and EZH2.

Discussion

CRC is one of the most common malignancies of the gastrointestinal tract, ranking fourth in terms of morbidity and mortality rates [25]. Generally speaking, early-stage CRC (stage I and II) presents a relatively good prognosis, with a 5-year survival of 70% [26]. As the disease progresses, the prognosis of CRC becomes worse, especially in stage IV [27]. Tumor metastasis is the leading cause of treatment failure in CRC, resulting in 90% of CRC-related deaths [28]. The liver is the organ most frequently affected by CRC metastasis, reaching 50% of all metastatic CRC cases [29]. Current treatments for the prevention and treatment of liver metastasis from CRC are unsatisfactory. Therefore, it is of great significance to uncover the mechanisms underlying the pathogenesis of CRC.

Tumorigenesis is caused by a variety of factors, and genetic changes are considered to be major reasons [30,31]. LncRNAs stimulate tumorigenesis *via* influencing protein encoding [32]. Recent studies have demonstrated the crucial regulatory effects of lncRNAs on tumor diseases [33]. LncRNAs belong to the non-coding RNA family [34]. They are extensively expressed in humans and mediate cellular behaviors [35,36]. Importantly, thousands of lncRNAs are dysregulated in tumor tissues as revealed by RNA sequencing and tumor microarray analyses. These lncRNAs serve as vital regulators in tumor biology [37,38]. For instance, lncRNA HOXA11-AS accelerates gastric cancer growth through sponging miR-1297 as a ceRNA [39].

This study first elucidated the upregulated lncRNA DUXAP8 in CRC tissues and cell lines. Its level remained higher in CRC with larger tumor size or higher tumor stage. Furthermore, knockdown of DUXAP8 suppressed the proliferative, migratory and invasive abilities of CRC cells. These results indicated the carcinogenic potential of DUXAP8 in CRC. Through bioinformatics prediction, DUXAP8 could bind to LSD1 and EZH2. Knockdown of LSD1 or EZH2 attenuated the proliferative ability of SW480 cells. Moreover, DUXAP8 positively mediated the expressions of LSD1 and EZH2 in CRC tissues. Collectively, DUXAP8 accelerated the malignant progression of CRC *via* targeting LSD1 and EZH2.

Conclusions

LncRNA DUXAP8 is upregulated CRC. It accelerates the malignant progression of CRC *via* positively regulating EZH2 and LSD1.

Conflict of interests

The authors declare no conflict of interests.

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