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DUB3 contributes to colorectal cancer cell migration and angiogenesis via NF-κB/HIF-1α

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ABSTRACT: Colorectal cancer (CRC) is a common malignancy with poor prognosis. This study aimed to explore the role of deubiquitinase 3 (DUB3) in CRC cell migration and angiogenesis as well as its molecular mechanism. HCT116 cells were transfected with DUB3 or EZH2 siRNA, or vectors overexpressing EZH2/HIF-1α. CCK8 and colony formation assay were used to assess cell proliferation; wound healing assay and Transwell assay were performed to determine cell migration and invasion; angiogenesis in CRC was detected using tube formation assay; WB was used to measure the protein levels of DUB3, EZH2, p65, p-p65, and HIF-1α. DUB3 downregulation inhibited proliferation, migration, invasion, and angiogenesis in CRC cells. Moreover, DUB3 could positively regulate NF-κB/HIF-1α pathway through EZH2. Overexpression of HIF-1α reversed the effects of DUB3 knockdown on CRC cell proliferation, migration, invasion, and angiogenesis. DUB3 contributes to CRC metastasis and angiogenesis by regulating NF-κB/HIF-1α pathway via EZH2, which may indicate a novel insight for the pathogenesis of CRC.

KEYWORDS: colorectal cancer, deubiquitinase 3, enhancer of zeste homolog 2, nuclear factor-kappaB/hypoxia-inducible factor-1α signaling pathway, migration, angiogenesis

INTRODUCTION

Colorectal cancer (CRC) is the 3rd most common cancer and the 4th leading cause of cancer-associated death worldwide [1]. It is a complexed cancer, and its process is characterized by abnormal biological microenvironment and various genomic variations [2]. Around 55% CRC cases occur in developed countries [3], and the underlying mechanisms of CRC are multifactorial [4]. Although the awareness, addressability, and screening measures have been developed, 25% of the cases were diagnosed as CRC at advanced stages [5]. Thus, a better understanding of the potential molecular mechanism is critical for inhibiting CRC progression and developing efficient treatment.

Ubiquitin-induced protein degradation is known to play a critical role in multiple cancer-related cellular processes. Ubiquitination is a reversible process and ubiquitin moieties conjugated onto protein can be removed by deubiquitinases (DUBs) [6]. DUB3, which belongs to the DUB/ubiquitin-specific processing protease (USP) family, has been revealed to participate in regulating many cellular reactions [7]. DUB3 is known to be overexpressed in various human cancers, such as non-small cell lung cancer [6], ovarian cancer [8], and breast cancer [9]. Recently, the ectopic expression of DUB3 has been reported in CRC cells, and it was revealed that highly expressed DUB3 caused chemotherapy resistance in CRC cell lines [10]. However, the underlying molecular mechanism remains largely unknown.

Enhancer of zeste homolog 2 (EZH2) is a component of polycomb repressive complex 2, which can catalyze histone H3K27me3 to modulate gene expression [11]. Loss of EZH2 at tumor invasion front has been shown to be associated with higher aggressiveness in CRC cells [12]. Interestingly, it has been reported that DUB3 promotes growth and reduces apoptosis by overexpressing EZH2 in oral squamous cell carcinoma (OSCC) [7]. Thus, we supposed that DUB3 may regulate EZH2 to affect cancer progression. Nuclear factor-kappaB (NF-κB) is a transcription factor that induces signal transduction between nucleus and cytoplasm in many cell types [13]. Hypoxia-inducible factor-1α (HIF-1α) is a subunit of HIF-1 transcriptional factor [14]. NF-κB has been demonstrated to regulate HIF-1α expression [15, 16]. Moreover, the NF-κB/HIF-1α signaling pathway are known to be involved in the progression of BC [17] and hepatocellular carcinoma [18]. Ma et al [19] have found that zinc finger protein 91 activates HIF-1α via NF-κB/p65 to promote proliferation and tumorigenesis of colon cancer. Nevertheless, the role of NF-κB/HIF-1α pathway in CRC is scarcely studied.

This study was designed to explore the effect of DUB3 on CRC progression, and we speculated that DUB3 may regulate EZH2 to facilitate the progression of CRC through the NF-κB/HIF-1α signaling pathway (Fig. S1). Our research may provide novel biomarkers for the treatment of CRC.

MATERIALS AND METHODS

TIMER2.0

TIMER (https://cistrome.shinyapps.io/timer) is a comprehensive resource for the analysis of immune infiltrates across different cancer types and can be used to explore clinical and genomic features of cancers. In this study, we used TIMER 2.0 to analyze DUB3 (also known as USP17) expression in cancer.

Cell culture

Human umbilical vein endothelial cells (HUVECs), normal colon mucosa cells (NCM 460) and CRC cell lines, (SW480, SW1116, HT29, and HCT116) were obtained from ATCC, VA, USA. All cell lines were cultured in RPMI1640 medium (Thermo Fisher Scientific, MA, USA) with 10% fetal bovine serum (FBS), streptomycin (100 mg/ml), and penicillin (100 unit/ml) (all from Gibco, CA, USA) in a 37 °C incubator containing 5% CO_2.

RNA interference

Small interfering RNAs (siRNA, si-DUB3, and si-EZH2; 20 nM) or pcDNA overexpression vector plasmids (pcDNA-EZH2 and pcDNA-HIF-1α, 1.6 μg/ml) and matched negative control (NC) (all from GenePharma Technology Co., Ltd., Shanghai, China) were transfected into HCT116 cells using Lipofectamine 3000 reagent (Thermo Fisher Scientific). Transfected cells, or cell supernatant, were used for subsequent experiments [20].

Analysis of cell proliferation and colony formation ability

In CCK8 assay, HCT116 cells that were transfected with si-DUB3, pcDNA-HIF-1α, and their corresponding NCs for 24, 48, and 72 h were collected; and CCK8 (Dojindo, Kyushu, Japan) was used to determine cell proliferation according to the manufactures' information. For colony formation assay, cells that have been transfected with si-DUB3 and si-NC for 48 h were collected; and 1500 cells were cultured on 6-well plates for 2 weeks and stained with 0.1% crystal violet. The colonies (> 50 cells) were imaged and counted.

Wound healing and Transwell assays

Cells that had been transfected with si-DUB3, pcDNA-HIF-1α, and their corresponding NCs for 48 h were harvested for wound healing and Transwell assays. Cells (5×10^6) were planted onto 6-well culture dishes for cell migration analysis. A 200 μl sterile pipette tip (1-mm-wide) was used to scratch on the cell monolayer after 90% confluence. The wound healing rate was monitored under a microscope after 24 h. For invasion assessment, Transwell chambers pre-coated with Matrigel (Corning, NY, USA) were used to detect the cell invasion ability. The apical chambers containing serum-free medium were planted with 5×10^4 cells, while the basolateral chambers were added with DMEM (Gibco) containing 10% FBS. After 48 h, the invasive cells were stained with crystal violet, and 5 fields of view were selected from each chamber for photographing under a microscope (Olympus, Tokyo, Japan).

Tube formation assay

HUVECs (5×10^4) were seeded onto 24-well plates pre-coated with cold Matrigel (BD Biosciences, NJ, USA). The supernatant of HCT116 cells after 48-h transfection with si-DUB3, pcDNA-HIF-1α, and their corresponding NCs was incubated with HUVECs for 24 h. A microscope was used for imaging of angiogenesis and ImageJ software (NIH, MD, USA) was used to calculate the number of branch points.

Western blotting (WB)

Cells that had been transfected with si-DUB3, si-EZH2, pcDNA-EZH2, and their corresponding NCs for 48 h were collected for WB assay. Total protein was extracted using RIPA buffer solution, and the protein concentration was determined using BCA kits (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were performed with 10%SDS-PAGE and transferred onto PVDF membranes (Millipore, MA, USA), which were blocked with 5% skim milk and TBST for 1 h. Then, the membranes were incubated overnight with primary antibodies: DUB3 (1 μg/ml, NBP1-79745, Novus Biologicals, CO, USA), EZH2 (1:1000, ab283270, Abcam, MA, USA), p65 (1:1000, ab32536, Abcam), p-p65 (1:1000, ab109458, Abcam), HIF-1α (1:1000, ab179483, Abcam), and GAPDH (1:1000, ab8245, Abcam); and incubated with relative secondary antibody (1:1000, Cell Signaling, MA, USA) for visualization.

Statistical analysis

All data analyses were conducted using SPSS 22.0 software, and the measurement data were expressed as mean ± standard deviation. The unpaired *t*-test was performed for comparisons between two groups, and one-way analysis of variance (ANOVA) was used for comparisons among multiple groups, followed by Tukey's multiple comparisons test. p-value < 0.05 was indicative of statistically significant difference.

RESULTS

DUB3-silencing inhibits CRC cell proliferation

DUB3 has been reported to be overexpressed in various cancers and promote cancer cell proliferation [6, 7]. We also found through Timer database that DUB3 (also known as USP17) expressed at a high level in CRC (Fig. 1A). We detected DUB3 expression in normal colon mucosa cells (NCM 460) and human CRC cell lines (SW480, SW1116, and HCT116). It was found that DUB3 was highly expressed in CRC

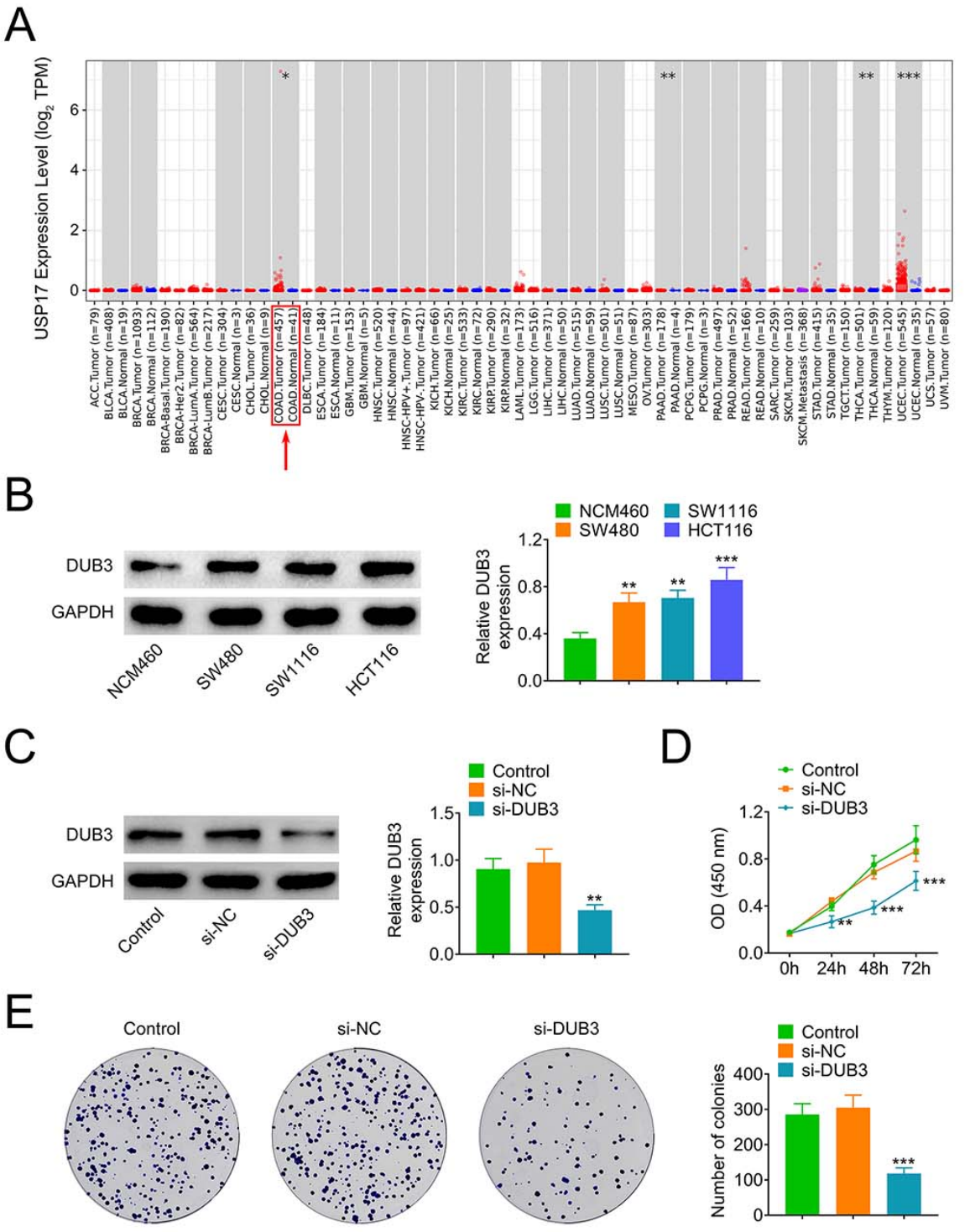

Fig. 1 DUB3 (also known as USP17) silencing inhibits CRC cell proliferation. HCT116 cells were transfected with si-NC or si-DUB3. (A) Timer database indicated that DUB3 expressed at a high level in CRC (indicated by arrows); (B) WB was used to detect DUB3 expression in HCT116 cells; (C) cell viability was measured through CCK8; (D) colony formation assay was performed to determine the number of formed-colonies. Repetition = 3, ** $p < 0.01$, *** $p < 0.001$ vs. si-NC. The measurement data were expressed as mean ± standard deviation. One-way ANOVA was used for comparisons among multiple groups, followed by Tukey's multiple comparisons test.

cell lines, among which HCT116 showed the greatest difference in DUB3 expression when compared with NCM 460 cells (Fig. 1B). Therefore, we selected HCT116 cells for subsequent experiments. In this study, HCT116 cells were transfected with si-DUB3 to inhibit DUB3 expression to explore the potential biological function of DUB3 on CRC. Cells transfected with si-DUB3 for 48 h were collected for WB assay. It was found that the transfection of si-DUB3 significantly downregulated DUB3, indicating the successful knockdown (Fig. 1C). CCK8 (Fig. 1D) and colony formation assay (Fig. 1E) were used to assess the effect of DUB3 knockdown on HCT116 cell proliferation. The results showed that DUB3 inhibition markedly reduced viability and colony formation ability of HCT116 cells (all $p < 0.05$). These data suggested that DUB3 knockdown inhibits CRC cell proliferation.

DUB3-silencing suppresses CRC cell migration and invasion

Wound healing and Transwell assays were performed to investigate the role of DUB3 knockdown in HCT116 metastatic ability. Our findings (Fig. 2A,B) indicated that the wound healing area significantly increased while the number of invasive cells decreased after downregulation of DUB3 (all $p < 0.05$). Therefore, DUB3 knockdown inhibits the metastatic ability of HCT116 cells.

DUB3-silencing CRC cell reduces angiogenesis

Tube formation assay was used to determine the effect of DUB3 inhibition on CRC angiogenesis. It was found that (Fig. 3) the repression of DUB3 decreased the angiogenesis of CRC cells.

DUB3 regulates NF-κB/HIF-1α pathway through EZH2

To further study the molecular mechanism of DUB3 regulating CRC progression, we knocked DUB3 down and detected the protein expressions of EZH2, p65, p-p65, and HIF-1α using WB. Cells transfected with si-DUB3 for 48 h were collected for WB assay. The results implied that (Fig. 4A) EZH2, p-p65, and HIF-1α expressions decreased after DUB3 inhibition. It has been uncovered that HIF-1α promoter contains the binding site of NF-κB, and NF-κB/p65 promotes CRC progression via upregulating HIF-1α [19, 21, 22]. Moreover, inhibiting EZH2 represses NF-κB activity and HIF-1α expression. On this basis, we transfected cells with si-EZH2 for 48 h, cells were collected for WB assay, and we found that the expression of p-p65 and HIF-1α significantly decreased (Fig. 4B). Then, cells transfected with si-DUB3 and/or pcDNA-EZH2 for 48 h were collected for WB assay. We overexpressed EZH2 after DUB3 knockdown and it was discovered through WB that (Fig. 4C) EZH2 reversed the inhibition effect of DUB3 knockdown on expressions of p-p65 and HIF-1α. The above data showed that DUB3 increased EZH2 and NF-κB/HIF-1α expressions, and it could regulate the NF-κB/HIF-1α pathway via EZH2.

DUB3 facilitates CRC cell migration and angiogenesis via HIF-1α

pcDNA-HIF-1α and si-DUB3 were co-transfected into HCT116 cells to explore whether DUB3 could regulate HIF-1α to affect CRC migration and angiogenesis. We found in CCK8, wound healing assay, and Transwell assay that (Fig. 5A-E) HIF-1α reversed the role of DUB3 downregulation in inhibiting HCT116 cell migration, angiogenesis, and invasion. Tube formation assay revealed that (Fig. 5F,G) the overexpression of HIF-1α increased the number of tube formation after DUB3 reduction. These findings implied that DUB3 facilitates CRC cell migration and angiogenesis via HIF-1α.

DISCUSSION

CRC is one of the most dangerous cancers. CRC's global incidence and mortality have increased markedly over the past decades. Although epigenetic and genetic changes of CRC have been broadly reported, the molecular mechanism in CRC progression remains to be further studied [23]. We aim to investigate the role of DUB3 in metastatic viability and angiogenesis in CRC cells, and our results suggested that DUB3 contributes to CRC metastasis and angiogenesis by regulating NF-κB/HIF-1α pathway via EZH2.

HCT116 cell populations are composed mainly of stem-like cancer cells and are commonly used for CRC study [24]. Higher expression of DUB3 than normal colon mucosa cells (NCM 460) was also observed in various human CRC cell lines tested (SW480, SW1116, and HCT116), with the highest in HCT116 cells. As previously reported, DUB3 is strongly expressed in a variety of cancers; for example, in BC [25] and OC [8]. Therefore, DUB3 was knocked down in HCT116 cells to evaluate its role during the progression of CRC. In our study, CCK8 and colony formation assay were used to assess cell proliferation, wound healing assay and Transwell assay were performed to determine cell migration and invasion, and angiogenesis in CRC was detected using tube formation assay. Results of these assays indicated that the knockdown of DUB3 inhibits proliferation, migration, invasion, and angiogenesis in CRC. Similarly, Hu et al [6] have found that DUB3 regulates cell cycle through deubiquitinating cyclin A that links to NSCLC cell proliferation, and DUB3 knockdown reduces cyclin A level to inhibit the proliferation of NSCLC cells. It has also been identified that DUB3 interacts with Twist and Slug and prevented them from degradation, thereby facilitating migration and invasion of BC cells [26]. Moreover, DUB3 inhibition has been reported to repress BC invasion and metastasis

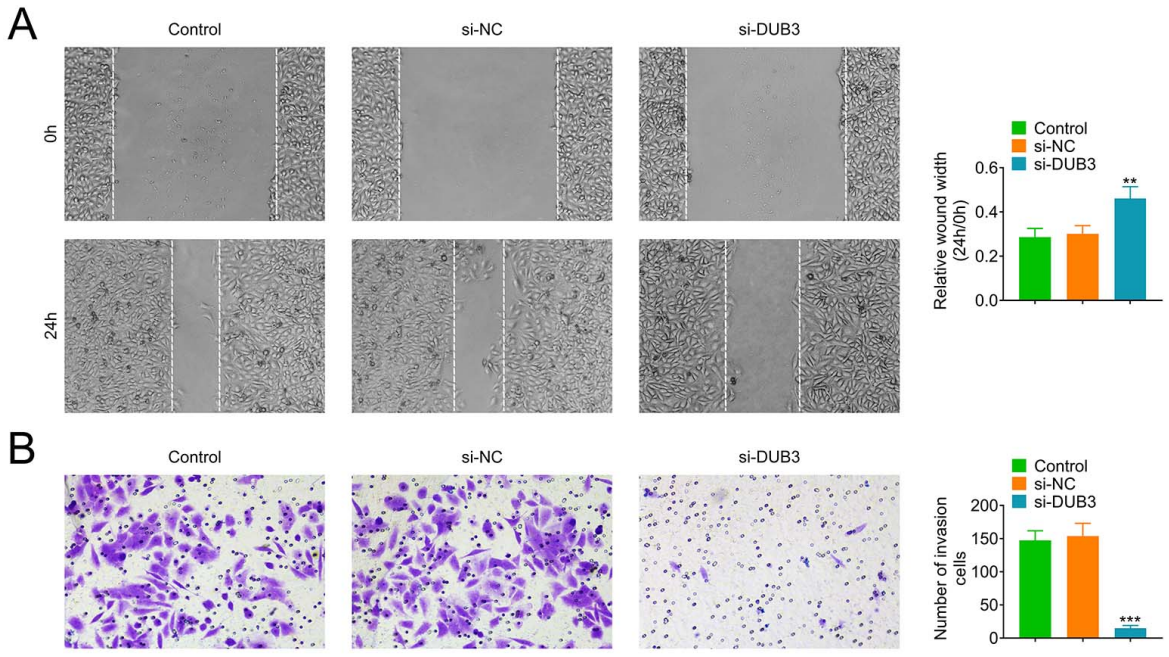

Fig. 2 DUB3 silencing suppresses CRC cell migration and invasion. HCT116 cells were transfected with si-NC or si-DUB3. (A) Cell migration ability was detected using wound healing assay; (B) cell invasion ability was assessed through Transwell assay. Repetition = 3, ** $p < 0.01$, *** $p < 0.001$ vs. si-NC. The measurement data were expressed as mean ± standard deviation. One-way ANOVA was used for comparisons among multiple groups, followed by Tukey's multiple comparisons test.

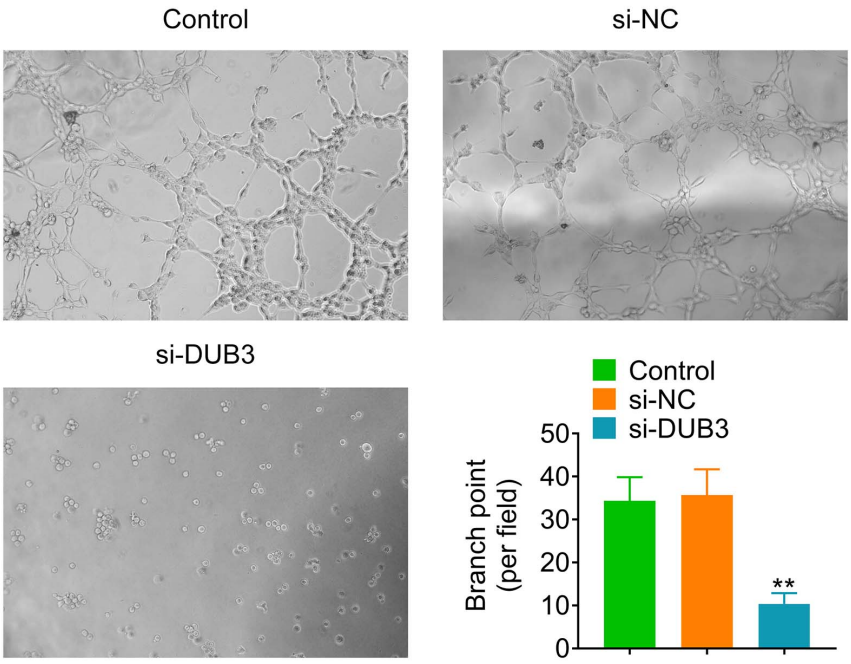

Fig. 3 DUB3 silencing reduces angiogenesis. HCT116 cells were transfected with si-NC or si-DUB3. Tube formation assay was utilized to determine angiogenesis in CRC cells. Repetition = 3, ** $p < 0.01$ vs. si-NC. The measurement data were expressed as mean ± standard deviation. One-way ANOVA was used for comparisons among multiple groups, followed by Tukey's multiple comparisons test.

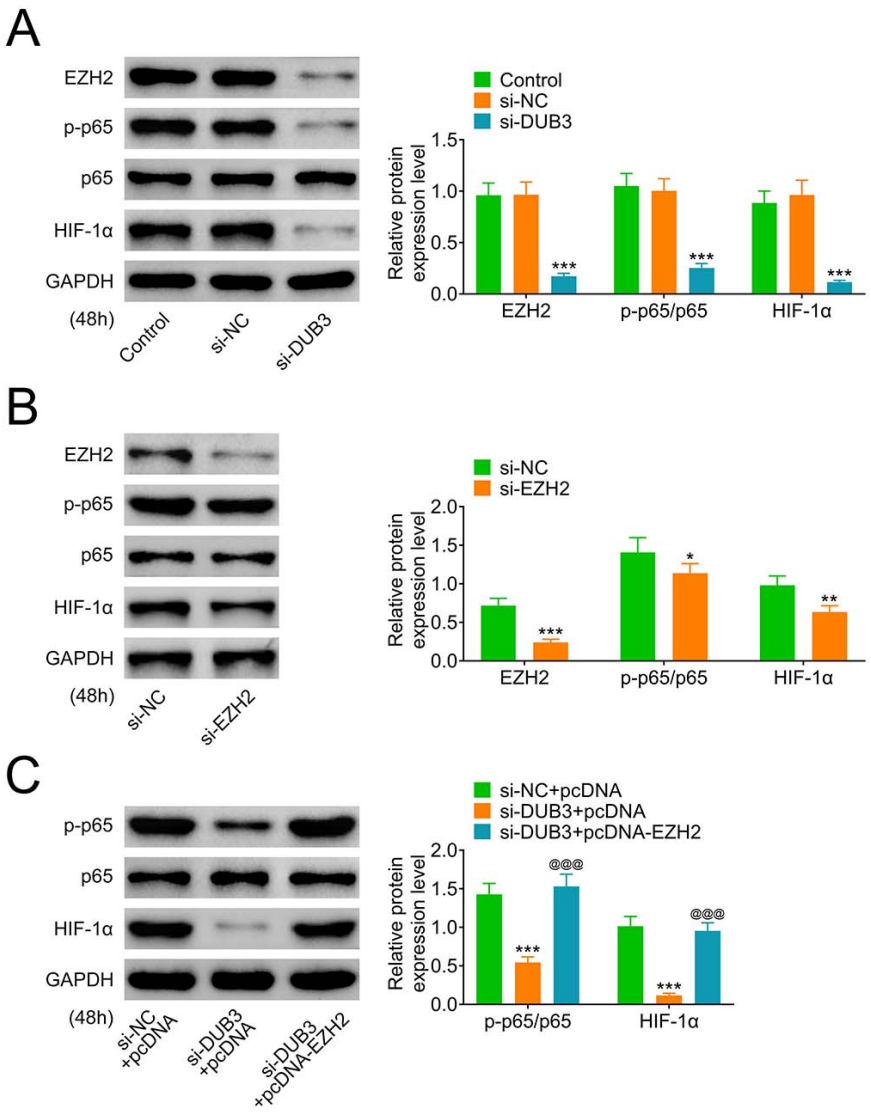

Fig. 4 DUB3 regulates NF-κB/HIF-1α pathway through EZH2. HCT116 cells were transfected with si-NC, si-DUB3, si-EZH2, pcDNA, or pcDNA-EZH2. A&B&C, WB was used to detect the protein expression of EZH2, p65, p-p65, and HIF-1α. Repetition = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. si-NC, $^{@@@}$ $p < 0.001$ vs. si-DUB3 + pcDNA-EZH2. The measurement data were expressed as mean ± standard deviation. The unpaired t-test was performed for comparisons between two groups and one-way ANOVA was used for comparisons among multiple groups, followed by Tukey's multiple comparisons test.

via promoting Snail1 degradation [25]. As for the role of DUB3 in CRC, a recent study has clarified that DUB3 deubiquitinates and stabilizes NRF2 in chemotherapy resistance of CRC [10].

HIF-1α has been shown to be implicated in angiogenesis, metastasis, and migration in CRC [19, 27, 28]. Additionally, a publication has revealed that DUB3 facilitates growth and inhibits apoptosis through overexpressing EZH2 in OSCC [7]. To further study the molecular mechanism of DUB3 regulating CRC progression, we knocked DUB3 down and detected the protein expression of EZH2, p65, p-p65, and HIF-1α using WB. The results implied that EZH2, p-p65, and HIF-1α expression decreased after DUB3 inhibition. Consistently, Zhao et al [21] have uncovered that EZH2 regulates PD-L1 expression via HIF-1α in NSCLC cells. HIF-1α has also been reported to regulate proliferation and tumorigenesis of colon cancer via NF-κB/p65 [19]. In the present study, we found in WB that EZH2 knockdown decreased the expressions of p-p65 and HIF-1α, and EZH2 reversed the inhibition effect of DUB3 knockdown on the expressions. These data

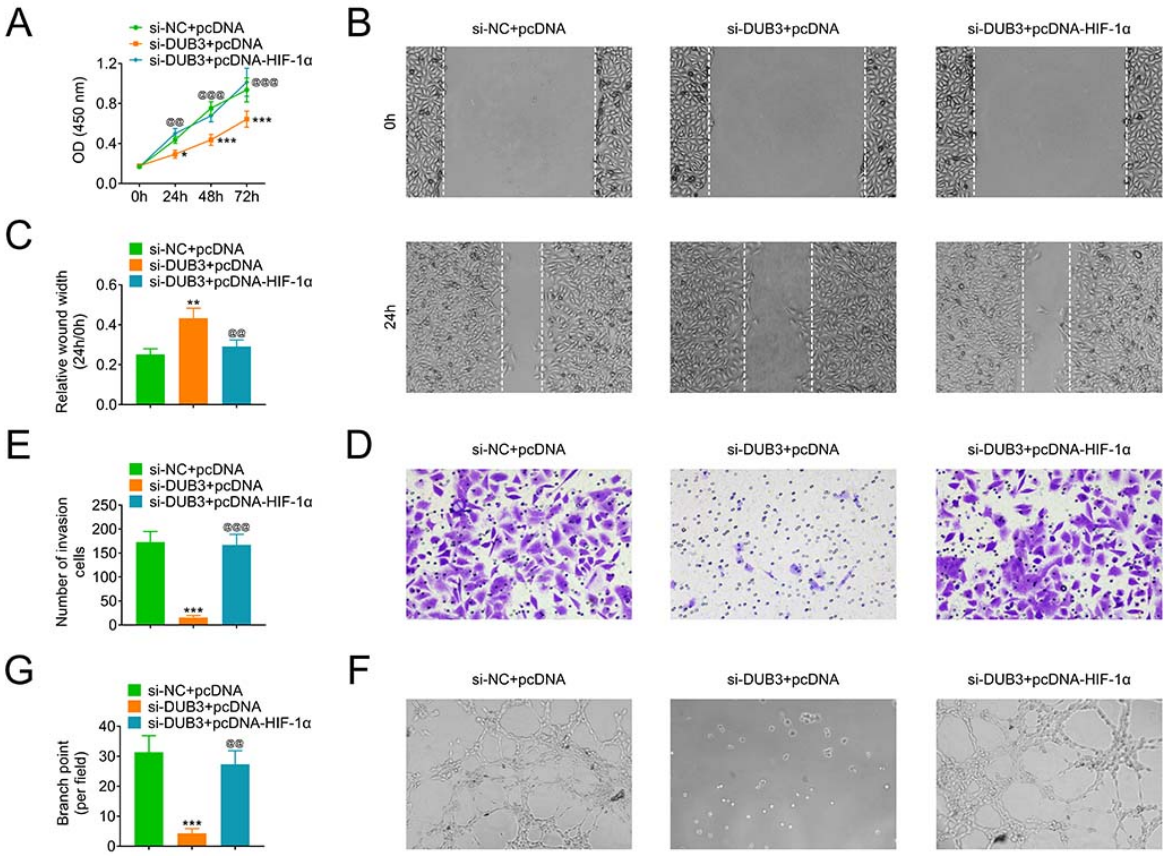

Fig. 5 DUB3 facilitates CRC cell migration and angiogenesis via HIF-1α. HCT116 cells were transfected with si-NC, si-DUB3, pcDNA, or pcDNA-EZH2. (A) Cell viability was measured through CCK8; (B, C) cell migration ability was detected using wound healing assay; (D, E) cell invasion ability was detected using Transwell assay; (F, G) tube formation assay was utilized to determine angiogenesis. Repetition = 3, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. si-NC + pcDNA, $^{@@}p < 0.01$, $^{@@@}p < 0.001$ vs. si-DUB3 + pcDNA. The measurement data were expressed as mean ± standard deviation. One-way ANOVA was used for comparisons among multiple groups, followed by Tukey's multiple comparisons test.

showed that DUB3 regulates NF-κB/HIF-1α pathway through EZH2. Then, HCT116 cells were transfected with si-NC, si-DUB3, pcDNA, or pcDNA-EZH2 to further explore the relationship between DUB3 and HIF-1α in CRC. The results of CCK8, colony formation assay, wound healing assay, Transwell assay, and tube formation assay reflected that HIF-1α reversed the role of DUB3 downregulation in inhibiting HCT116 cell migration, invasion, and angiogenesis. Similar to our findings, HIF-1α knockout could inhibit migration, invasion, and proliferation of oral cancer cells [29], and Jiang et al [30] have found that HIF-1α accumulation leads to the upregulation of vascular endothelial growth factor (VEGF), thus promoting tumor angiogenesis.

In conclusion, this research indicated that DUB3 contributes to CRC metastasis and angiogenesis by regulating NF-κB/HIF-1α pathway via EZH2, which may provide a better understanding of the molecular mechanisms on CRC. However, *in vivo* experiments are needed to further explore the detailed mechanism, and we would make effort to that in our future work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.054.

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Appendix A. Supplementary data

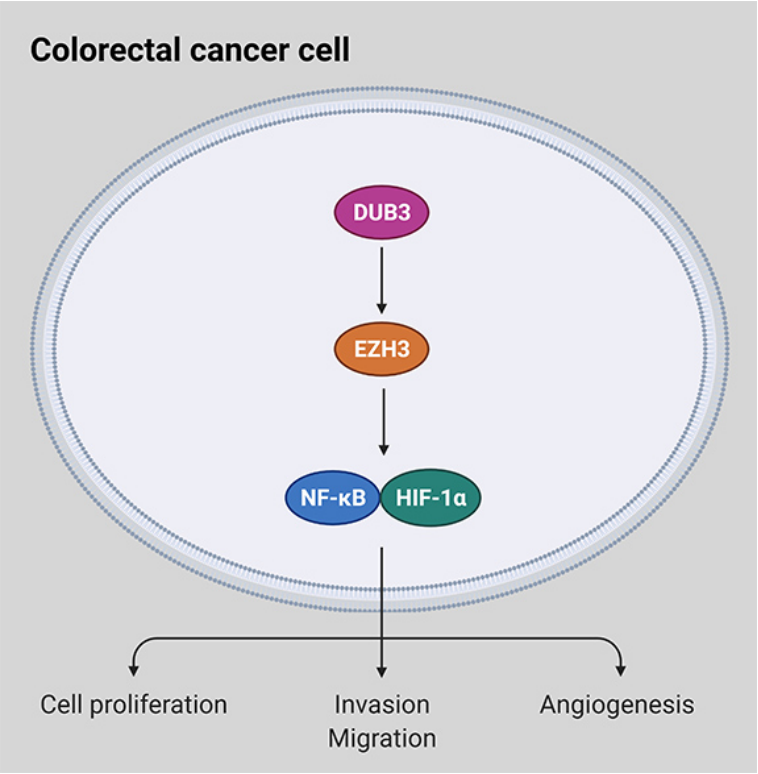

Fig. S1 Possible mechanism of DUB3 contribution to colorectal cancer cell migration and angiogenesis via NF-κB/HIF-1α.