JARID1B promotes colorectal cancer proliferation and Wnt/β-catenin signaling via decreasing CDX2 level

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Research

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Abstract

Background: Jumonji AT-rich interactive domain 1B(JARID1B) has been shown to be upregulated in many human cancers and plays a critical role in the development of cancers cells. Nevertheless, its functional role in colorectal cancer (CRC) progression is not fully understood.

Methods: Herein, JARID1B expression levels were detected in clinical CRC samples by western blotting and qRT-PCR. DLD-1 cells with JARID1B knockdown or overexpression by stably transfected plasmids were used in vitro and in vivo study. Colony formation, 5-ethynyl-20-deoxyuridine (EdU) and Real Time Cellular Analysis(RTCA) assays were used to detect cell proliferation and growth. Transcriptome and CHIP assays were used to examine the molecular biology changes and molecular interaction in these cells. Nude mice was utilized to study the correlation of JARID1B and tumor growth in vivo.

Results: Here, we first observed that JARID1B was significantly upregulated in CRC tissue compared to adjacent normal tissues. In CRC patients, JARID1B high expression was positively relation with poor overall survival. Multivariate analyses revealed that high JARID1B expression was an independent predictive marker for the poor prognosis of CRC. In addition, we found that JARID1B promoted CRC cells proliferation by Wnt/β-catenin signaling pathway. Further studies demonstrated CDX2 as a downstream target of JARID1B, and our data demonstrated that CDX2 is crucial for JARID1B -mediated Wnt/β-catenin signaling pathway. Mechanistically, we demonstrated that JARID1B regulated CDX2 expression through demethylation of H3K4me3.

Conclusions: CDX2 inhibited by JARID1B-derived H3K4me3 methylation promoted cells proliferation of CRC via Wnt/β-catenin signaling pathway. Therefore, our studies provided a novel insight into the role of JARID1B in CRC cells proliferation and potential new molecular target for treating CRC.

Background

Colorectal cancer (CRC) is the most common cancer with high morbidity in the world, which is the third leading cause of cancer-related death in men and second leading cause in women[1, 2]. In recent decades, although diagnosis and treatment techniques improve and new cancer therapeutics including molecular targeted therapies and immunotherapies occurs, cancers' prognosis is still unsatisfactory because of abnormal cancers cell death and proliferation[3, 4]. Because the cells proliferation and metastasis of CRC are the most cause of deaths, to explore the molecular mechanism of CRC cells proliferation is a vital issue in the treatment of CRC[5]. Meanwhile, epigenetic regulation of gene expression has been shown to provide new insights into the pathogenesis of CRC[6]. In particular, methylation and demethylation of histone lysine residues act as transcriptional switches for gene expression under physiological and disease conditions[6]. In this study, we showed a new target related to histone methylation modification could regulate CRC cells proliferation.

JARID1 proteins are histone demethylases that regulate the fate of normal cells and contribute to malignant transformation[8]. The JARID1 family members include JARID1A, JARID1B, JARID1C and

JARID1D[9, 10]. JARID1B(also known as KDM5B) has been studied mostly so far. It has been reported that JARID1B was originally considered as a transcription inhibitor to regulate cells cycle, cells differentiation and cells proliferation[11]. JARID1B could specifically remove H3K4 trimethylation and inhibit the relative gene transcription[12]. Recently, there have been an increasing number of studies on JARID1B in malignant tumors. JARID1B was an oncogenic epigenetic factor overexpressed in different types of cancers, such as breast cancer, lung cancer, prostate cancer, osteosarcoma, oral cancers, melanoma, glioma, hepatocellular carcinoma, gastric cancer and pancreatic cancer[12].The depletion of JARID1B has been shown to specifically inhibited H3K4 demethylation and suppressed CRC cells growth[15]. Nevertheless, clinical significance of JARID1B expression levels in CRC clinical samples has not been studied in detail, and its specific mechanism of action in the process of CRC progression is still unclear.

The Wnt family consists of 19 secretory cysteine-rich glycoproteins that all play the principal regulatory roles in many developmental and biological processes such as cell fate specification, proliferation, migration and asymmetric cell division[16]. Furthermore, the Wnt/β-catenin signaling serves important roles in tissue maintenance and regeneration[17]. Recently emerging evidence links the biological function of Wnt/β-catenin signaling to tumorigenesis and development[18]. At present, the molecular mechanism of tumorigenesis and development related to the activation of Wnt signaling which increases of β-catenin levels represents a common pathway in Wnt signaling activation, that is to enhance the translocation of β-catenin into cell nuclei where it binds to transcription factors of the TCF/LEF family. The β-catenin-TCF/LEF complex then induces transcription of downstream target genes in cancer, such as c-MYC, and cyclin D1[19]. Present studies have demonstrated that abnormal activation of the Wnt/β-catenin signaling has often been reported in colorectal cancer (CRC). For example, activation of Wnt signaling pathway is required for tumor growth in advanced CRC, activation of Wnt signaling to β-catenin contributes to the development of CRC [20, 21]. However, the regulatory mechanisms of abnormal activation of the Wnt/β-catenin signaling in CRC are not yet clear.

In the present study, we found that JARID1B was elevated in CRC tissues compared with normal colonic tissues using quantitative reverse transcription (qRT)-PCR, western blot and immunohistochemistry. JARID1B expression was correlated with patients' survival time. Furthermore, JARID1B promoted CRC cell proliferation in-vivo and in-vitro. In addition, Our further results JARID1B regulates Wnt/β-catenin signaling to influence CRC cell proliferation. Additionally, we demonstrated that JARID1B significantly inhibited CDX2 expression in CRC, and CDX2 was crucial for JARID1B - mediated Wnt/β-catenin signaling in CRC. Finally,our study verified that JARID1B decreases CDX2 expression by demethylating H3K4me3. Therefore, our findings indicate that JARID1B is believed to be a promising target in the treatment of CRC.

Materials And Methods

Clinical samples

All the specimens of 54 patients from the Second Affiliated Hospital of Nanchang University were diagnosed as CRC by pathological test from June 2018 to December 2018. Total proteins and mRNAs from these patients' clinical tissues were immediately obtained when tissues was still fresh. Moreover, formalin-fixed paraffin-embedded 130 CRC patients' tissues from December 2012 to December 2017 were randomly selected to observe the expression levels by immunohistochemistry and their corresponding follow-up data about survival was obtained from the hospital database.Informed consent was obtained from all patients, those patients' clinical data was recorded during hospitalization and the research program was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University.

EDU assay

According to what the manufacturer's protocol described, 20μM BrdU was put into CRC cells for 4h at 37°C Then washed with PBS for three times, the cells were mixed with apollo reaction for 1h. The cells were stained with 100 ul of Hoechst 33342 (5 ug/ml) for 30 min to visualize the nuclei and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Real-time proliferation assay

The xCELLigence real-time cell analysis (RTCA) system (ACEA Bioscience) was used to analyze cell proliferation. Cells were seeded on a 96-well plate (E-plate, Germany). After treated with different approaches for 12h, the growth rates were recorded every 10 min by the instrument. Under the same xCELLigence RTCA program, continue to monitor the changes of cell index for 36 hours. We used mean cell index values in real-time to present cell proliferation changes. For each experiment, we tested three biological replicates.

In vivo tumorigenicity study

After construction of a DLD-1 cell line that stably interfered with JARID1B expression, 5×10^6 cells in 200 ul of PBS were injected subcutaneously into the flanks of nude mice (male athymic BALB/c nude mice,4-6 weeks). We used the random number table as a random method to determine the experimental animals, making sure more than 40 mices. Five mice were randomly selected every 5 days to get tumor tissues, and then tumor volumes were measured according to the protocol: V=1/2 (largest diameter)×(smallest diameter)^2. In vivo imaging showed tumor growth on day 30, and fluorescent pictures were taken. After 40 days, tumors tissues from 5 mices were harvested and individually weighed after the mice were anesthetized. The data was presented as tumor weight (mean ± SD).

Cell culture

Commercialized CRC cell lines SW620, HCT116, LOVO, SW480 and DLD-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). SW620 and SW480 were cultured in L-15 medium (Gibco). LOVO was cultured in F12K medium (Gibco). DLD1-1 and HCT116 were cultured in DMEM medium (Gibco). All cell lines were cultured at 37 ℃, 5% CO_2 condition. Moreover, 10 uM XVA-939 from

American AbMole was used to inhibite β-catenin degradation. Over-expression plasmids and shRNA interference fragments are transfected into cells through lipofectamine 2000 and lipofectamine LTX (Invitrogen, Carlsbad, CA, USA).

Plasmids and reagents

SiRNA of JARID1B and CDX2 was synthesized by InvivoGen. The target sites of shRNA are detailed in Supplementary Table S1. The stable knockdown and overexpressed JARID1B CRC cells according to the Manufacturer's protocol. the shJARID1B and shCDX2 of CRC cells was selected based on resistance to hygromycin. The pcDNA3.1(+)-JARID1B-expressing CRC cells were selected using G418. The following reagents were used: Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA); dual-luciferaseassay kit (PromegaE1910); SimpleChipTM Enzymatic Chromatin IP KIT (CST, USA). EdU kit (RiboBio, China).

qRT-PCR, western blot analysis and co-immunoprecipitation (Co-IP)

qRT-PCR and western blot analysis were made as previously reported. Whether it was tissue or cells, total RNA was extracted by Trizol reagent (Invitrogen, USA) and were quantified by SYBR Green assays with RT primers and SYBR Green from Takara Biotechnology (TAKARA, Dalian, China). Human GAPDH was amplified in parallel as an internal control. For western blot, total proteins of clinical samples were obtained through RIPA lysis buffer and prepared cells were harvested by RIPA lysis buffer containing protease inhibitor cocktail(Sigma-Aldrich). All the proteins were fractionated by 10% SDS-PAGE. The antibodies anti-JARID1B(1:2000, abcam), anti-GSK-3β(1:1000, abcam), anti-Axin2(1:1000, abcam), anti-CDX2(1:1500, abcam), anti-c-MYC (1:1000, proteintech), anti-phosphorylation-β-catenin (1:2000,abcam), anti-β-catenin (1:2000, abcam), anti-H3K4me3(1:1500, abcam), anti-ubiquitin (Ub) (1:100, Nova Biomedical) and anti-tubulin(1:1000, proteintech) were used. For co-immunoprecipitation, to deal with cells for 24 hours with 10 umol/L MG132, cell lysates were incubated overnight at 4 °C with anti-β-catenin, then conjugated to protein A/G agarose beads while rocking. Immunoprecipitates were washed with washing buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton-X), re-suspended in 2 × loading buffer, and resolved by SDS-PAGE followed by immunoblotting analysis.

Luciferase reporter assay

Fragments of the CDX2 were amplified PCR using primers (Supplementary Table S2) and cloned into the luciferase reporter vector pGL3.0-Basic (Promega, Madison, WI, USA) to generate CDX2 promoter reporter constructs. Plasmids containing firefly luciferase reporters and JARID1B plasmids were cotransfected into cells. For the TOP/FOP-Flash reporter assay, the TOP/FOP-Flash reporter and JARID1B plasmids were co-transfected into cells. After transfection for 48 h, the cells were harvested for analysis with the Dual- Luciferase Reporter Assay System (Promega, Madison, WI, USA). Luciferase activity was measured using the PerkinElmer EnSpire Multilabel Reader 2300 (PerkinElmer Inc., Waltham, MA, USA). The luciferase intensity was normalized to the Renilla luciferase activity to normalize for transfection efficiency.

Chromatin immunoprecipitation Assay (ChIP)

ChIP assays were performed using the ChIP assay kit (Cell Signaling Technology). Briefly, the cells were fixed with 1% formaldehyde for 10 minutes at room temperature, then washed with PBS for three times. After a series of processing according to the protocol, Digested DNA fragments was sonicated in the range of 150-300 bp. The immunoprecipitations were mixed with H3K4me3 antibody and agarose. The input and DNA were then subjected to qPCR.

Statistical analysis

Paired t test for the two groups and one-way ANOVA for more than two groups were used to analyze JARID1B expression data and its relationship with various clinicopathological factors.When data did not follow normal distribution, Mann-Whitney U test between two groups and Kruskal-Wallis H test for three or more groups should be used. Kaplan-Meier analysis and the logrank test were used for survival analysis. Correlation between two continuous values was analyzed by pearson's correlation. Furthermore, univariate and multivariate analyses were performed using the logistic regression model. All of the data were analyzed using GraphPad Prism and SPSS 22.0. *p*-values of <0.05 indicated statistically significant changes. Each experimental design included more than three biological repeats.

Results

JARID1B was markly upregulated in CRC tissues and was closely related to CRC progression.

To investigate the role of JARID1B in the development of colorectal cancer, we investigate JARID1B expression levels in colorectal cancer, 54 paired fresh CRC tissue samples and matched adjacent non-tumor tissues were used. The qRT-PCR revealed that the mRNA expression level of JARID1B was notably elevated in CRC tissue samples compared with the corresponding adjacent tissues (Fig. 1a, b). Furthermore, western blotting showed that JARID1B protein expression in 54 paired CRC tissues was consistent with the qRT-PCR results (Fig. 1c, d). Consistently, immunohistochemistry (IHC) results showed that JARID1B protein were highly expressed in 75.38% (98 of 130) tissues, while weakly positive staining was observed in the adjacent non-tumor tissues(Fig. 1e). Then, we explored whether JARID1B expression would affect clinic pathologic parameters. As shown in Table 1, the JARID1B high expression group demonstrated a larger tumour size, higher CEA levels and greater T classification compared with the JARID1B low expression group. Furthermore, the Kaplan-Meier survival curves demonstrated that patients with high JARID1B expression levels in 130 immunohistochemical results had a significant poor overall survival (Fig. 1f), while univariate and multivariate analysis of overall survival in CRC patients also indicated that JARID1B high expression was a risk factor for 5-year survival (Table 2). Altogether, the findings demonstrated that JARID1B expression was up-regulated in CRC and implicated in the progression of CRC.

Table 1

Relationship between JARID1B protein expression and clinicopathological features in CRC patients.

Parameters	Total 54[a]	Relative JARID1B protein expression[b]	p value
Gender			0.53
Male	36	2.11 ± 0.85	
Female	18	2.34 ± 0.89	
Age (years)			0.71
≤ 60	20	2.14 ± 0.93	
> 60	34	2.22 ± 0.83	
Tumor size(cm)			0.00**
< 5	23	1.61 ± 0.80	
≥ 5	31	2.62 ± 0.62	
Preoperative CEA level			0.00**
< 5 ng/mL	40	1.86 ± 0.72	
≥ 5 ng/mL	15	3.13 ± 0.41	
Histologic grade			0.98
well differentiated	42	2.17 ± 0.89	
poorly differentiated	12	2.26 ± 0.80	
T classification			0.00**
T1 + T2	17	1.19 ± 0.54	
T3 + T4	37	2.65 ± 0.53	
TNM stage			0.38
I	4	1.57 ± 0.55	
II	14	2.28 ± 1.02	
III	28	2.18 ± 0.88	
IV	8	2.37 ± 0.51	
Lymphatic invasion			0.52
Negative	21	2.12 ± 0.91	
Positive	33	2.24 ± 0.84	

Parameters	Total 54[a]	Relative JARID1B protein expression[b]	p value
Distant metastasis			0.88
Negative	45	2.16 ± 0.92	
Positive	9	2.33 ± 0.50	

[a]: 54 fresh tissue samples from June 2018 to December 2018, [b]:protein level in Tumor tissues/protein level in Adjacent tissues(mean ± SD),**p ≤ 0.01

Table 2
Univariate and multivariate analysis of overall survival in CRC patients. (Cox proportional hazards regression model)

Parameters(130 CRC patients)[a]	Univariate analysis			Multivariate analysis		
	HR	95%CI	p value	HR	95%CI	p value
Age (≦ 60/>60)	0.70	0.34–1.41	0.32			
Gender(Male/Female)	1.13	0.57–2.22	0.73			
Histologic grade (well/poor)	0.66	0.29–1.51	0.33			
Tumor size(cm)(< 5/≧5)	1.78	0.90–3.51	0.10			
CEA(ng/ml) (< 5/≧5)	1.09	0.75–1.60	0.64			
Lymphatic invasion (Positive/Negative)	1.95	1.04–3.67	0.04*	1.52	0.52–4.43	0.03*
Distant metastasis (Positive/Negative)	2.03	1.09–3.78	0.03*	1.65	0.62–4.38	0.01**
T classification (T3 + T4/T1 + T2)	2.65	1.20–5.85	0.02*	1.80	0.67–4.84	0.02*
TNM(III,IV/I,II)	2.49	1.24-5.00	0.01**	2.12	1.13–3.98	0.02*
JARID1B protein expression (High/Low)	2.29	1.41–3.73	0.00**	2.53	1.50–4.27	0.00**

[a]: Immunohistochemical results of 130 paraffin-embedded tissues from December 2012 to December 2017, *p ≤ 0.05,**p ≤ 0.01

JARID1B promoted proliferation and tumorigenesis of CRC in vivo and in vitro.

To investigate the potential biological function of JARID1B in CRC development, we initially examined JARID1B expression levels in in CRC cells (SW620, HCT116, LOVO, SW480, DLD-1) by qRT-PCR, and western blotting, compared to the non-malignant cell line (HCoEpic). The results showed that JARID1B expression notably elevated in CRC cells (Fig. 2a, b). As shown in Table 1, the high JARID1B expression group demonstrated a larger tumour size, so that we speculate that JARID1B may be functional in CRC proliferation.. Next, we investigated the relationship between JARID1B expression and CRC cells proliferation. Colony formation, 5-ethynyl-20-deoxyuridine (EdU) and Real Time Cellular Analysis(RTCA) assays revealed that the proliferation capacity of DLD-1 cells with short hairpin RNA (shRNA)-mediated JARID1B knockdown was markly lower than the control group (Fig. 2c, d, e). In contrast, JARID1B over-expression significantly enhanced the proliferation ability of LOVO cells (Supplementary Fig. 1a, b). Furthermore, an in-vivo experiment showed that the JARID1B knockdown group had smaller volumes and lower weights than the control group (Fig. 2f, g, h). That is, knockdown of JARID1B significantly inhibited tumor size and weight in vivo. These data demonstrated that JARID1B could promote CRC cells proliferation in vivo and in vitro.

JARID1B influence CRC proliferation through regulating the Wnt/β-catenin signaling pathway

In subsequence, we explored the mechanism how JARID1B regulated CRC cells proliferation. Studies have indicated that Wnt signaling pathway played an important role in controlling the proliferation of CRC cells. Therefore, we speculated that JARID1B might regulate CRC proliferation through Wnt/β-catenin signaling pathway. First, the Gene Set Enrichment Analysis(GSEA) based on TCGA COAD RNA expression dataset revealed that JARID1B level was positively correlated with Wnt signaling pathway activity (Fig. 3a). Then, we investigated the effect of JARID1B on the activation of Wnt/β-catenin pathway in CRC cells. In JARID1B-silenced DLD-1 cells, we observed that β-catenin and its downstream target genes, c-MYC and CycinD1, were down-regulated(Fig. 3b). The Western blotting results also showed that downregulation of JARID1B expression significantly decreased the expression levels of the total and nuclear β-catenin (Fig. 3c). Consistently, TOP-Flash luciferase assay revealed that JARID1B downregulation inhibited Wnt/β-catenin pathway activity, proving that JARID1B played a key role in activation of Wnt/β-catenin signaling pathway(Fig. 3d). In contrast, upregulation of JARID1B expression significantly increased the expression protein levels of c-MYC, Cycin D1, the total and nuclear β-catenin in LOVO cells (supplementary Fig. 2a, b, c). These results indicated that Wnt/β-catenin signaling pathway may be regulated by JARID1B.

Next, we want to validate that CRC proliferation was mediated by JARID1B via Wnt/β-catenin signaling pathway. Thus, we increased β-catenin expression in JARID1B-knockdown DLD-1 cells. The western blotting results showed that β-catenin overexpression significantly inhibited the decrease in β-catenin, c-MYC and CycinD1 expression in JARID1B–knockdown DLD-1cells and rescued the decreased Wnt/β-catenin pathway activity induced by downregulating JARID1B (Fig. 3e, f). As the results shown, upregulation of β-catenin rescued the proliferation ability in DLD-1cells with JARID1B downregulation in

vivo and in vitro(Fig. 3g, h, i). Conversely, we suppressed β-catenin expression in JARID1B-overexpression LOVO cells. The results showed that β-catenin knockdown inhibited the increase of β-catenin, c-MYC and CycinD1 expression and inhibited Wnt/β-catenin pathway activity induced by JARID1B overexpression (supplementary Fig. 2d, e). And the results showed that the enhanced proliferation ability in LOVO cells caused by JARID1B overexpression was remarkably inhibited by downregulation of β-catenin (supplementary Fig. 2f,g). The above mentioned results demonstrated that JARID1B regulated CRC proliferation via the Wnt/β-catenin pathway.

JARID1B significantly inhibited CDX2 expression in CRC.

We further explored how JARID1B regulated Wnt/β-catenin signaling in CRC cells. It has been reported that CDX2 knockdown promoted the proliferation of colorectal cancer cells via Wnt/β-catenin signaling. Thus, we speculated that JARID1B might regulate the expression of CDX2 to enhance the activity of Wnt/β-catenin signaling in CRC cells. Given the role of JARID1B in the epigenetic regulation of transcription, we first performed RNA-seq to identify potential JARID1B target genes involved in cells proliferation. The results showed that CDX2 was one of the most notably upregulated transcripts when JARID1B knockdown (Fig. 4a). Next, qRT-PCR revealed that downregulation of JARID1B significantly increased CDX2 mRNA (Fig. 4b). Western blotting results showed that downregulation of JARID1B expression significantly increased the expression levels of CDX2, while the expression level of β-catenin, c-MYC and cyclinD1 were decreased (Fig. 4c). Conversely, JARID1B overexpression decreased the levels of CDX2 mRNA expression(Fig. 4d). Western blotting results showed that upregulation of JARID1B expression significantly decreased the expression levels of CDX2, meanwhile the expression level of β-catenin, c-MYC and cyclinD1 were increased (Fig. 4e). Furthermore, we examined CDX2 in 54 CRC tissue samples and the corresponding by Western blotting and qRT-PCR. The results show that the expression level of CDX2 was significantly higher in adjacent tissues compared to tumors tissues (Fig. 4f, h). Finally, the statistical analysis results revealed that CDX2 expression was negatively correlated with JARID1B expression in CRC tissues(Fig. 4g, i). Consistently, A Kaplan-Meier analysis indicated that patients with both high JARID1B expression and low CDX2 expression in 130 immunohistochemical results were predicted the worst prognosis (Fig. 4i). Collectively, these data suggested that JARID1B negatively regulated CDX2 expression to increased Wnt/β-catenin signaling activity in CRC.

CDX2 was the key protein for JARID1B-mediated Wnt/β-catenin signaling in CRC cells.

To further validate whether JARID1B activated Wnt/β-catenin signaling pathway through regulating CDX2 expression. After we first decreased the expression of CDX2 in JARID1B–knockdown DLD-1 cells, CDX2, GSK-3β, Axin2 and p-β-catenin, β-catenin, c-MYC and cyclinD1 expression levels were observed. The qRT-PCR results showed that knockdown of CDX2 inhibited the increase of CDX2 mRNA expression induced by JARID1B knockdown (Fig. 5a). The western blotting data showed that CDX2 knockdown deceased GSK-3β, Axin2 and p-β-catenin expression and markedly rescued the change of β-catenin, c-MYC and cycinD1 expression levels caused by JARID1B(Fig. 5b). Co-IP showed that JARID1B knockdown could increase ubiquitinated β-catenin level, which was rescued when CDX2 expression was knockdown

(Fig. 5c). Simultaneously, TOP-Flash luciferase assay revealed that reduced Wnt/β-catenin signaling activity induced by JARID1B knockdown was partly abolished by the knockdown of CDX2(Fig. 5d). In contrast, upregulation of CDX2 inhibited the JARID1B overexpression-induced increase in LOVO cells (Fig. 5e, f, g, h).These results demonstrated that CDX2 was required for JARID1B-mediated Wnt/β-catenin signaling pathway in CRC cells.

JARID1B regulated CDX2 expression through demethylation of H3K4me3

We then explored how JARID1B regulated CDX2 expression at the transcriptional level. Studies have shown that JARID1B affected the histone H3 lysine4 (H3K4) demethylase and exhibited a strong transcriptional repression function. We explored whether JARID1B demethylated H3K4me3 at the promoter of CDX2 in CRC cells. We first decreased the expression of JARID1B, and then observed the H3K4me3 protein expression levels and CDX2 promoter activity. Western blotting data showed that JARID1B knockdown markedly increased H3K4me3, CDX2, GSK-3β, Axin2 and p-β-catenin expression, and deceased β-catenin, c-MYC and cycinD1 expression (Fig. 6a). Simultaneously, a luciferase reporter gene assay showed that JARID1B downregulation increased CDX2 promoter activity (Fig. 6c). In contrast, overexpression of JARID1B decreased H3K4me3 protein expression and CDX2 promoter activity (Fig. 6b, c). In addition, to test whether JARID1B expression was correlated with the H3K4me3 modification at the CDX2 gene promoter in CRC cells. Meanwhile, a ChIP assay revealed that JARID1B knockdown increased H3K4me3 levels at CDX2 in DLD-1 cells (Fig. 6d), while overexpression of JARID1B decreased H3K4me3 levels at CDX2 in LOVO cells (Fig. 6d). After combining all the experimental results, we revealed a new important mechanism that JARID1B regulated CDX2 expression through H3K4me3 to indirectly activate Wnt/β-catenin pathway, leading to increased CRC proliferation(Fig. 6e).

Discussion

Tumor proliferation plays a crucial role in the development of CRC. Studies confirmed that the proliferation of CRC cells is significantly correlated with the abnormal expression of histone demethylase. JARID1B plays an important role in cell fate decision, cancer progression, and stem cell self-renewal[22, 23]. Evidence is emerging that JARID1B contribute to the epigenetic plasticity that underlies malignant transformation[24]. Studies have also shown that JARID1B is an overexpression in numerous cancers[25], JARID1B overexpression is associated with a poor prognosis in breast and prostate cancers[26, 27]. Having studies shown that histone demethylase JARID1B associated with CRC cells growth [28]. In this study, we explored the role of JARID1B in CRC proliferation. We found JARID1B is was significantly elevated in CRC, and overexpression JARID1B protein expression had significantly shorter overall survival. Furthermore, high JARID1B expression was closely associated shorter overall survival in CRC patients. In addition, we provided both evidences demonstrating that JARID1B could promote CRC cells proliferation in-vivo and in-vitro.

Next, we further explored the underlying mechanism that JARID1B regulates CRC proliferation. Present studies have demonstrated that abnormal activation of the Wnt/β-catenin signaling pathway was one of

the important causes in CRC[29].At present, the molecular mechanism of tumorigenesis and development related to Wnt/β-catenin signaling pathway always focused on four parts, separately, receptors on cell membrane, inhibitor or activator in the cytoplasm, transcription factors in the nucleus and downstream targeting genes of β-catenin[30]. In hypopharyngeal squamous cell carcinoma, JARID1B inhibited cells proliferation by activating β-catenin signaling[31]. Moreover, in hepatocellular carcinoma, basil polysaccharide was found to attenuate metastasis of the rat hepatocellular carcinoma, simultaneously resulting in the down-regulation of both JARID1B and β-catenin[32]. In line with these findings, we found a novel mechanism that JARID1B regulates CRC proliferation via Wnt/β-catenin signaling. First, we found Wnt/β-catenin signaling pathway is the downstream pathways of JARID1B, and JARID1B level was positively correlated with Wnt/β-catenin activity in CRC cells. Second, as the JARID1B expression icreased, the expression of β-catenin, c-MYC and cyclin D1 were decreased, whereas JARID1B upregulation again had the opposite effect in CRC cells. Finally, β-catenin overexpression significantly inhibited the decrease in c-MYC and cycinD1 expression in JARID1B–knockdown CRC cells and rescued the decreased Wnt/β-catenin pathway activity induced by downregulating JARID1B, rescued the proliferation ability in CRC cells with JARID1B downregulation in vivo and in vitro. In contrast, downregulation of β-catenin had the opposite results in JARID1B–overexpression CRC cells. These results revealed a mechanism by which JARID1B contributed to CRC proliferation by activating the Wnt/β-catenin pathway.

Our current studies also highlights the detailed mechanism of Wnt/β-catenin pathway regulated by JARID1B in CRC cells. Caudal-type homeobox transcription factor 2 (CDX2), an essential intestine-specific regulator, is involved in the development and differentiation of intestinal epithelial cells and regulates the balance between cell proliferation and differentiation[33]. In the present study, many studies revealed that CDX2 expression level was associated with CRC cells proliferation and poor prognosis for patients with CRC[34]. Recent evidence indicated that CDX2 inhibited the progression of CRC by suppressing the Wnt/β-catenin signaling pathway and transactivation of GSK-3β and Axin2 expression[35]. Furthermore, loss of CDX gene expression was a crucial member of the Wnt/β-catenin signaling pathway in liver metastasis of CRC[36]. Here, we revealed a novel mechanism that JARID1B regulated Wnt/β-catenin signaling pathway via inhibiting CDX2 expression. First, we found that CDX2 might be the most relevant gene in transcriptome sequencing technology of JARID1B-downregulation CRC cells. We confirmed the negative correlation between JARID1B and CDX2 expression levels by cell experiments and detection of clinical tissues. Finally, we explored the mechanism by which JARID1B regulates CDX2 expression in CRC cells. Previous study showed that JARID1B enables H3K4me3 demethylation, and the depletion of JARID1B has been shown to specifically inhibited H3K4 demethylation and suppressed CRC cells growth[28, 37]. Therefore, we proposed the underlying mechanism was that JARID1B regulated CDX2 expression through H3K4me3. This conclusion was based on the following observations: on the one hand, JARID1B knockdown markedly increased H3K4me3 protein expression and CDX2 promoter activity; on the other hand JARID1B over-expression decreased H3K4me3 levels and CDX2 promoter activity. However, future in-depth study to clarify whether other epigenetic players exist that participate in the process and have similar underlying patterns requires more investigation.

Conclusions

In summary, our study demonstrated that JARID1B acted as an oncogene to promote the CRC progression. JARID1B inhibited CDX2 transcription level by demethylation of H3K4me3, which was effected at the CDX2 promoter region. Following CDX2 low expression, GSK-3β and Axin2 expression levels were decreased, and further phosphorylated β-catenin level was inhibited. So that less β-catenin was bound by ubiquitination to degrade, and more β-catenin was transported into the nucleus, which caused downstream target genes(c-MYC and cyclinD1) to be activated (Fig. 6e). Finally, CRC cells proliferation was accelerated. Our results uncovers the diverse role of JARID1B in cell biology and function of JARID1B in cancer development, which extends foundation for the development of new anti-cancer therapeutic strategies.

Abbreviations

JARID1B: Jumonji AT-rich interactive domain 1B; CRC: colorectal cancer; EdU: 5-ethynyl-20-deoxyuridine; RTCA: Real Time Cellular Analysis; ChIP: Chromatin immunoprecipitation Assay; GSEA: Gene Set Enrichment Analysis; H3K4: histone H3 lysine4; CDX2: Caudal-type homeobox transcription factor 2; qRT-PCR: quantitative reverse transcriptase PCR; Ub: ubiquitin; Co-IP: Co-immunoprecipitation; IHC: immunohistochemistry.

Declarations

Ethical Approval and Consent to participate

The inclusion of human participants, as well as the use of human data and human tissue in this study, was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. The use of animals in this study was approved by the Animal Research Committee of the Second Affiliated Hospital of Nanchang University.

Consent for publication

All authors consent to publication.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Fanrong Liu conceived and designed the study. Da Huang, Fan Xiao , Fuzhou Hua ,Zhenzhong Luo, Zhaoxia Huang , sha Chen, and Xiuzhi Cheng performed the experiments and collected data. Xinyue Zhang and Weilan Fang performed data analysis. All authors discussed and interpreted the data. Fanrong Liu and Xiaoyun Hu wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Fig. 1

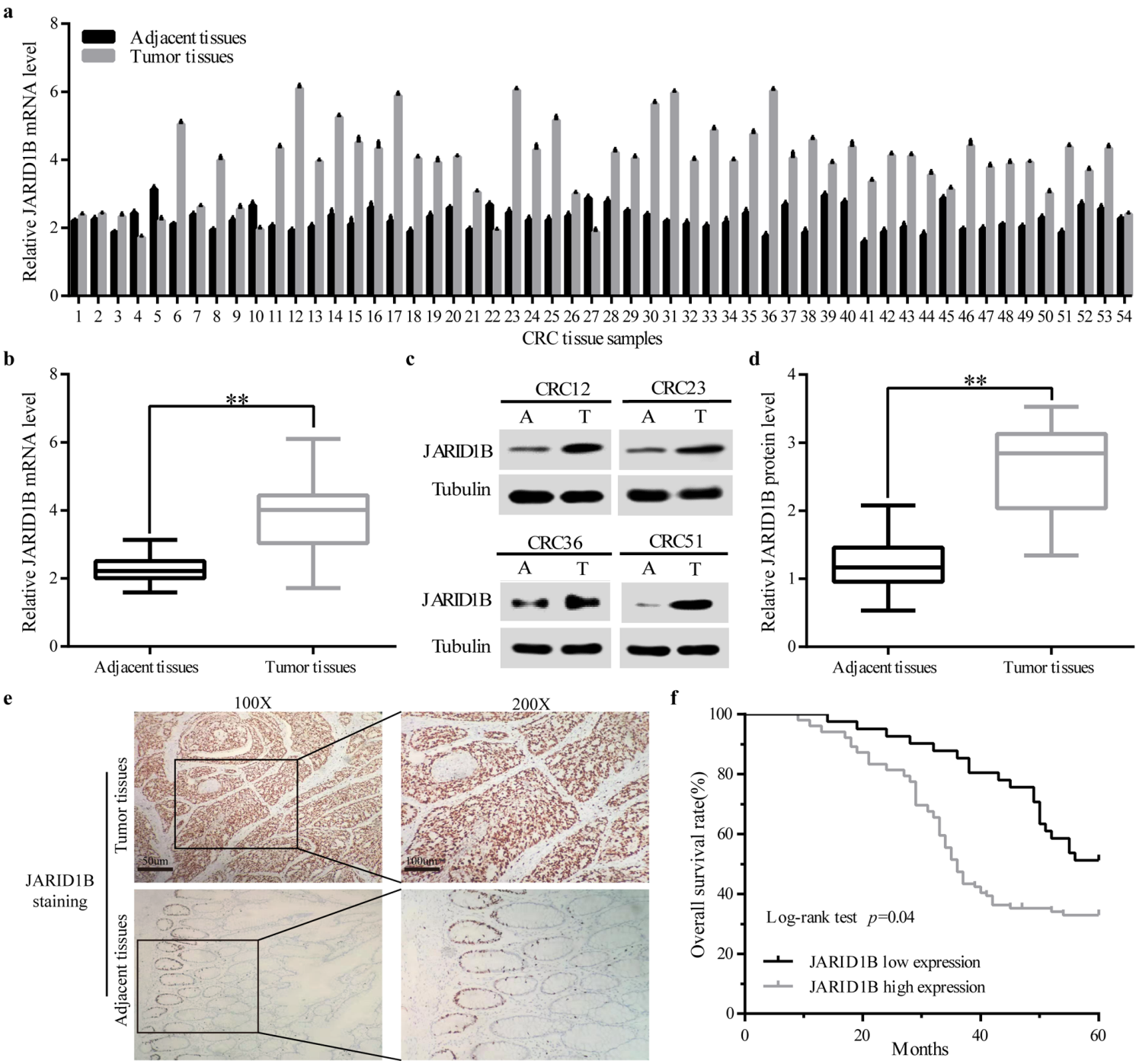

Figure 1

JARID1B overexpression was associated with poor prognosis in patients with CRC. a Relative JARID1B mRNA expression levels in fresh 54 paired colorectalcancer and para-carcinoma tissues. b JARID1B expression was measured by qRT-PCR in tumor tissues and adjacent tissues. c Representative western blotting analysis of JARID1B protein expression (T, tumor tissues; A, adjacent tissues). d Quantification of JARID1B protein expression based on western blot analyses in fresh 54 paired CRC and adjacent non-tumor tissues. e Representative images and quantification of JARID1B staining in the paired CRC tissues.

Scale bar, 50 μm. g Kaplan-Meier survival curves for 130 CRC patients showed JARID1B high expression had poor prognosis. *p<0.05, **p<0.01.

Fig. 2

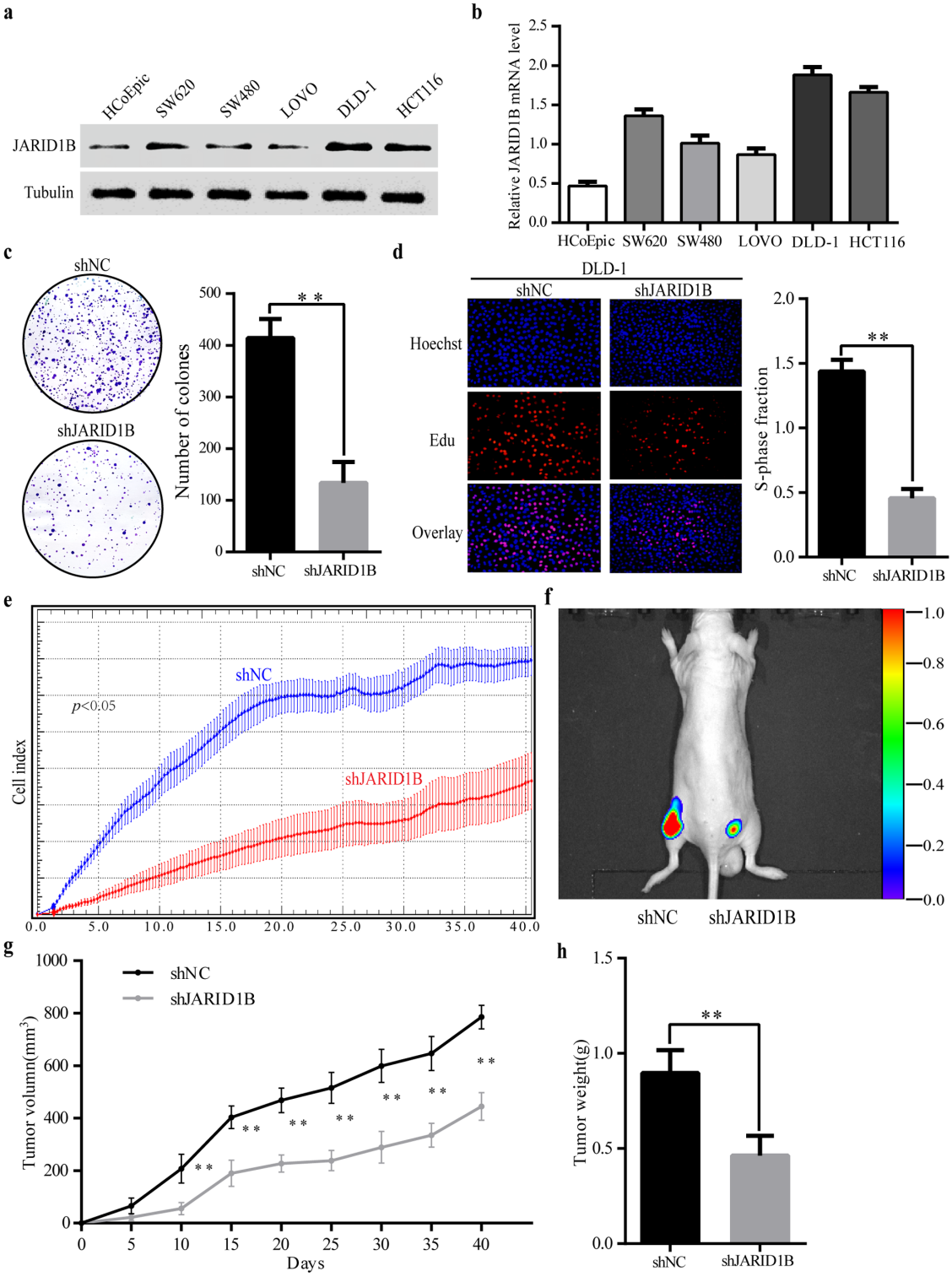

Figure 2

Downregulation of JARID1B expression led to decrease CRC proliferation in vitro and in vivo. a, b Western blot and qRT-PCR analysis of JARID1B protein expression in human nonmalignant cell line and CRC cell lines. c, d, e Cells proliferation capacities were detected by colony formation assay, EdU and RTCA assays

in CRC DLD-1-cells transfected with the shJARID1B plasmid f In-vivo tumor formation was examined by subcutaneously injecting CRC DLD-1-shNC (left) or CRC DLD-1-shJARID1B (right) cells into the flanks of nude mice. Representative images obtained by an IVIS in-vivo imaging station after inoculation were shown. Corresponding tumor growth curves were obtained(g). h Tumor weight was counted at 40 days, JARID1B knockdown inhibited tumor proliferation.*p<0.05, **p<0.01.

Fig. 3

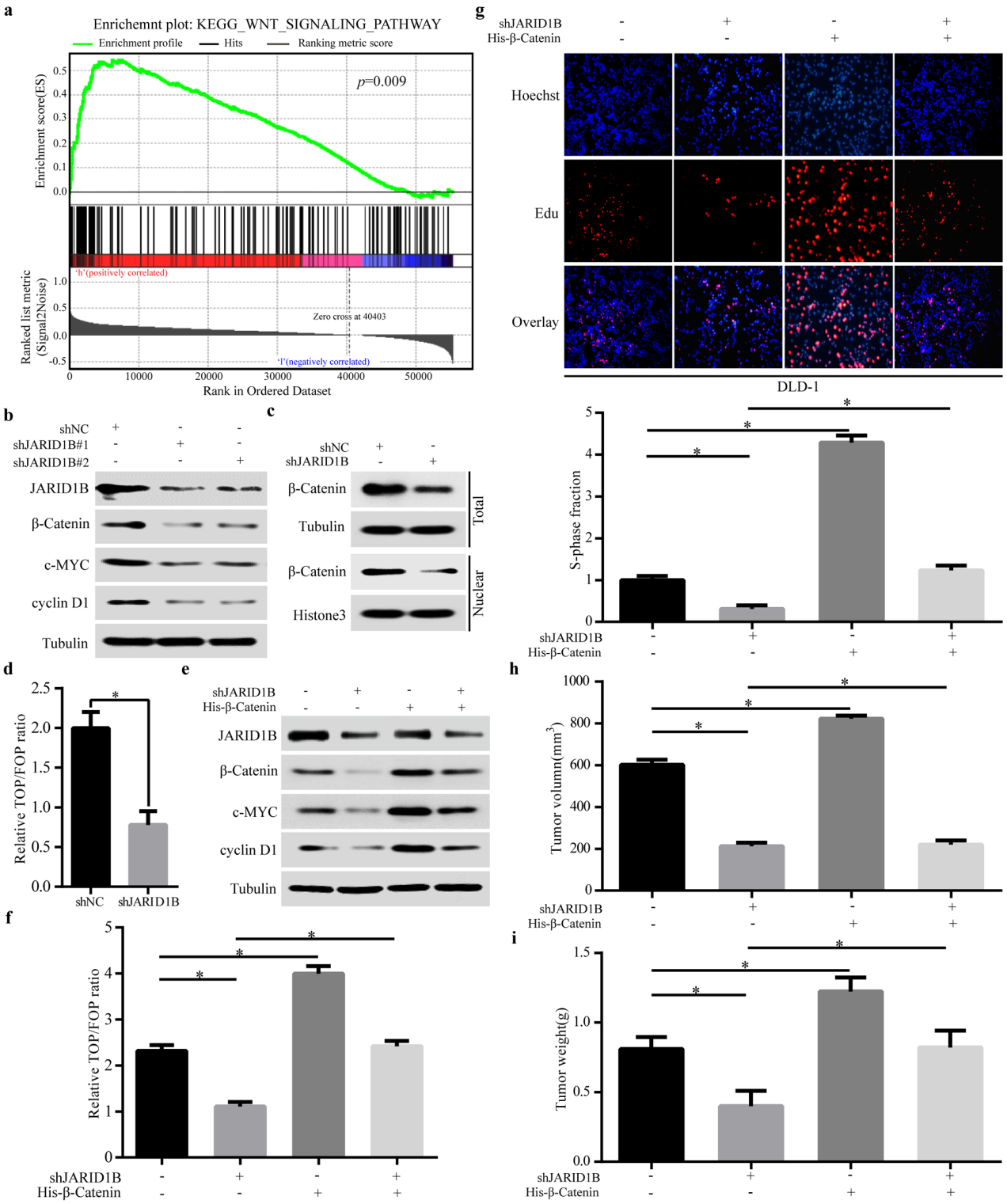

Figure 3

Stable knockdown of JARID1B repressed CRC proliferation via Wnt/β-catenin signaling pathway. a Gene set enrichment analysis of JARID1B based on TCGA COAD RNA Seq dataset showed that JARID1B expression was positively correlated with Wnt/β-catenin signaling pathway. b Western blot detected JARID1B, β-catenin c-MYC and cyclinD1 expression in DLD-1 cells transfected with shNC or shJARID1B. c The total and nuclear protein levels of β-catenin were assessed by western blotting in DLD-1 cells transfected with shJARID1B or shNC. d The inhibitive effect of JARID1B downregulation on Wnt/β-catenin pathway was detected by TOP-Flash luciferase reporter assay. e Western blot analysis showed the effects on c-MYC, cyclinD1 expression in DLD-1 cells when both JARID1B silencing and β-catenin restoration. f TOP-Flash luciferase reporter assay showing that β-catenin overexpression rescued the decreased Wnt/β-catenin pathway activity of DLD-1-shJARID1B cells. g, h EdU and in-vivo tumor formation assays showed that over-expression of ectopic β-catenin significantly rescued cells proliferation in DLD-1- shJARID1B cells. *$p<0.05$.

Fig. 4

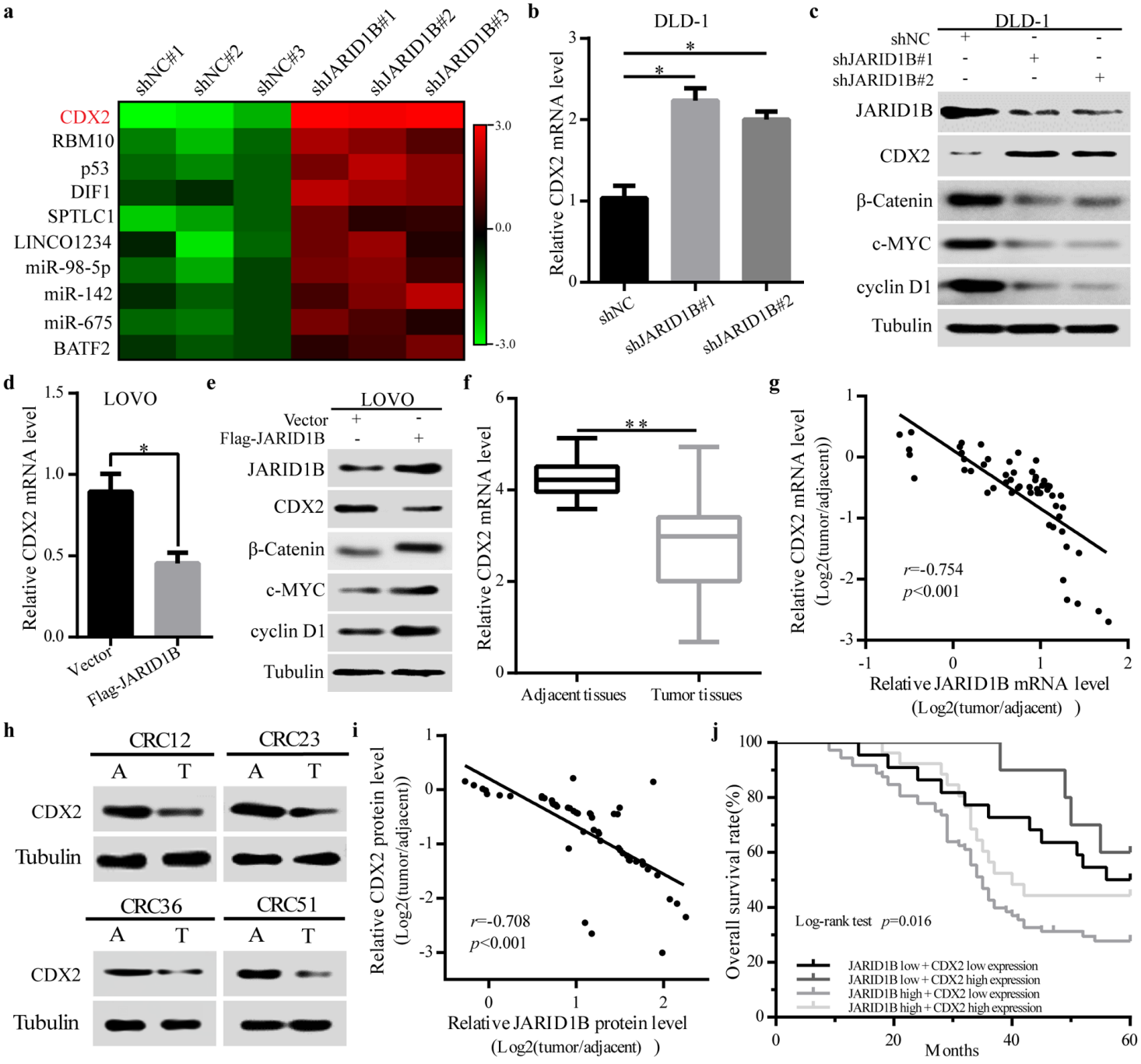

Figure 4

JARID1B inhibited CDX2 expression in CRC cells. a The microarray profiling of gene expression in DLD-1-shJARID1B cells. b, c qRT-PCR and western blot detected CDX2, β-catenin, c-MYC, cyclinD1 expression in DLD-1 cells transfected with shNC or shJARID1B. d, e qRT-PCR and western blot detected CDX2 expression in LOVO cells transfected with vector or Flag-JARID1B. f, h Western blot analysis and qRT-PCR of CDX2 protein levels in CRC tissues and in paired adjacent tissues. g, i Scatter plots showed a negative correlation between JARID1B and CDX2 at the mRNA and protein levels in fresh 54 CRC samples. j

Kaplan-Meier analysis for 130 CRC patients follow-up data showed that the prognosis was the poorest for patients with both high JARID1B expression and low CDX2 expression. *p<0.05, **p<0.01.

Fig. 5

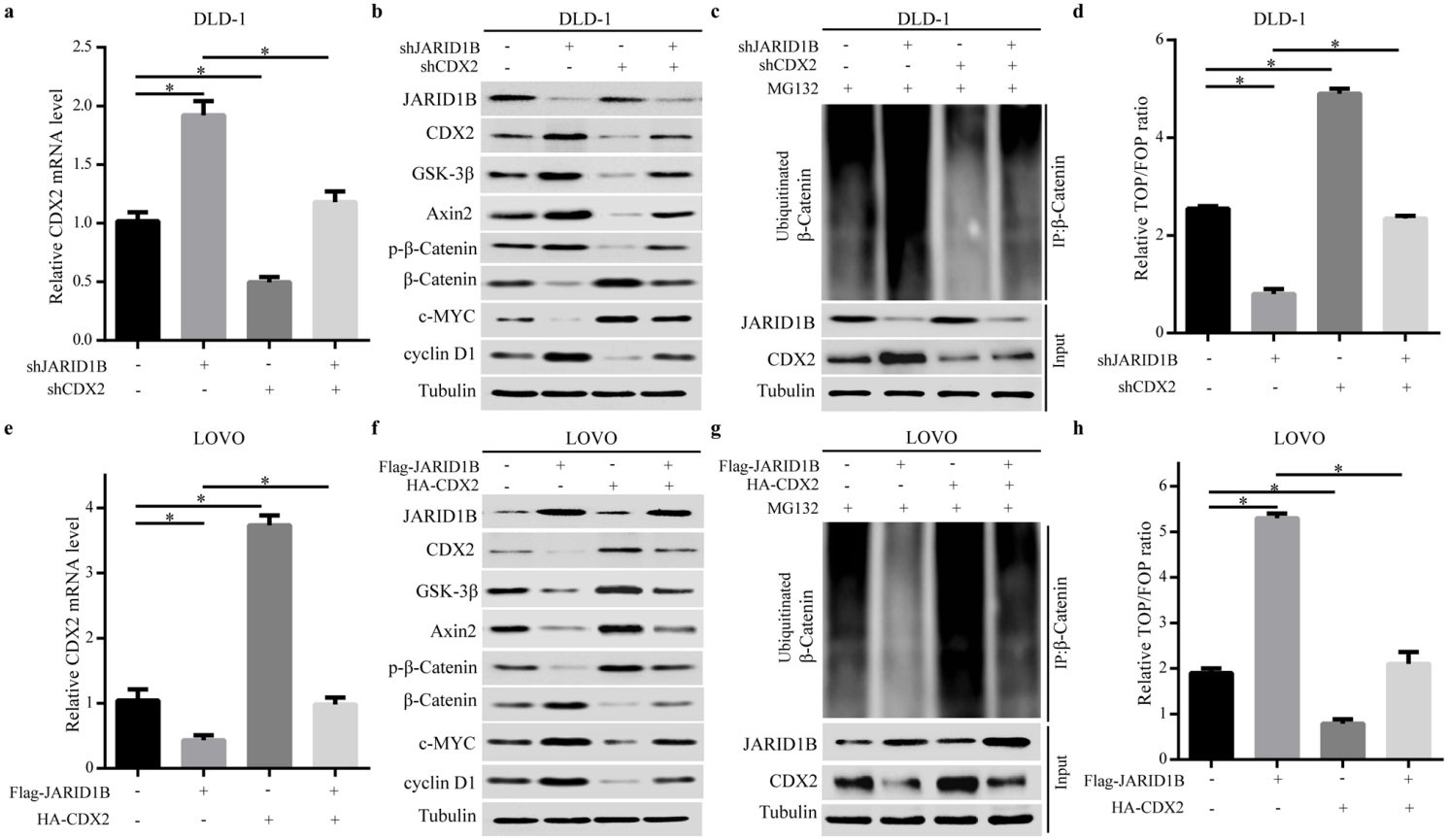

Figure 5

CDX2 was required for JARID1B-mediated Wnt/β-catenin signaling in CRC cells. a mRNA levels of CDX2 were detected by qRT-PCR. The knockdown of CDX2 expression dramatically inhibits the increase of CDX2 expression in DLD-1-shJARID1B cells. b Western blot analysis showed that the knockdown of CDX2 expression markedly rescused the increase of GSK-3β, Axin2 and p-β-catenin expression and the decrease of β-catenin, c-MYC and cyclinD1 expression induced by JARID1B knockdown. c Ubiquitinated β-catenin levels when JARID1B and CDX2 knockdown in DLD-1 cells. d Silencing CDX2 attenuated the loss of downregulating JARID1B on Wnt/β-catenin as observed by TOP-Flash luciferase assay. e The upregulation of CDX2 mRNA expression markedly inhibited the decrease in CDX2 expression observed in JARID1B-overexpression LOVO cells. f Western blot analysis showed that the levels of JARID1B overexpression and CDX2 upregulation and their effects on GSK-3β, Axin2, β-catenin, c-MYC and cyclinD1 expression in LOVO cells. g Ubiquitinated β-catenin levels when JARID1B and CDX2 over-expressed in LOVO cells. h TOP-Flash luciferase reporter assay showed the overexpression of ectopic CDX2 attenuated the increase in Wnt/β-catenin activitiy in JARID1B-overexpression LOVO cells.*p<0.05.

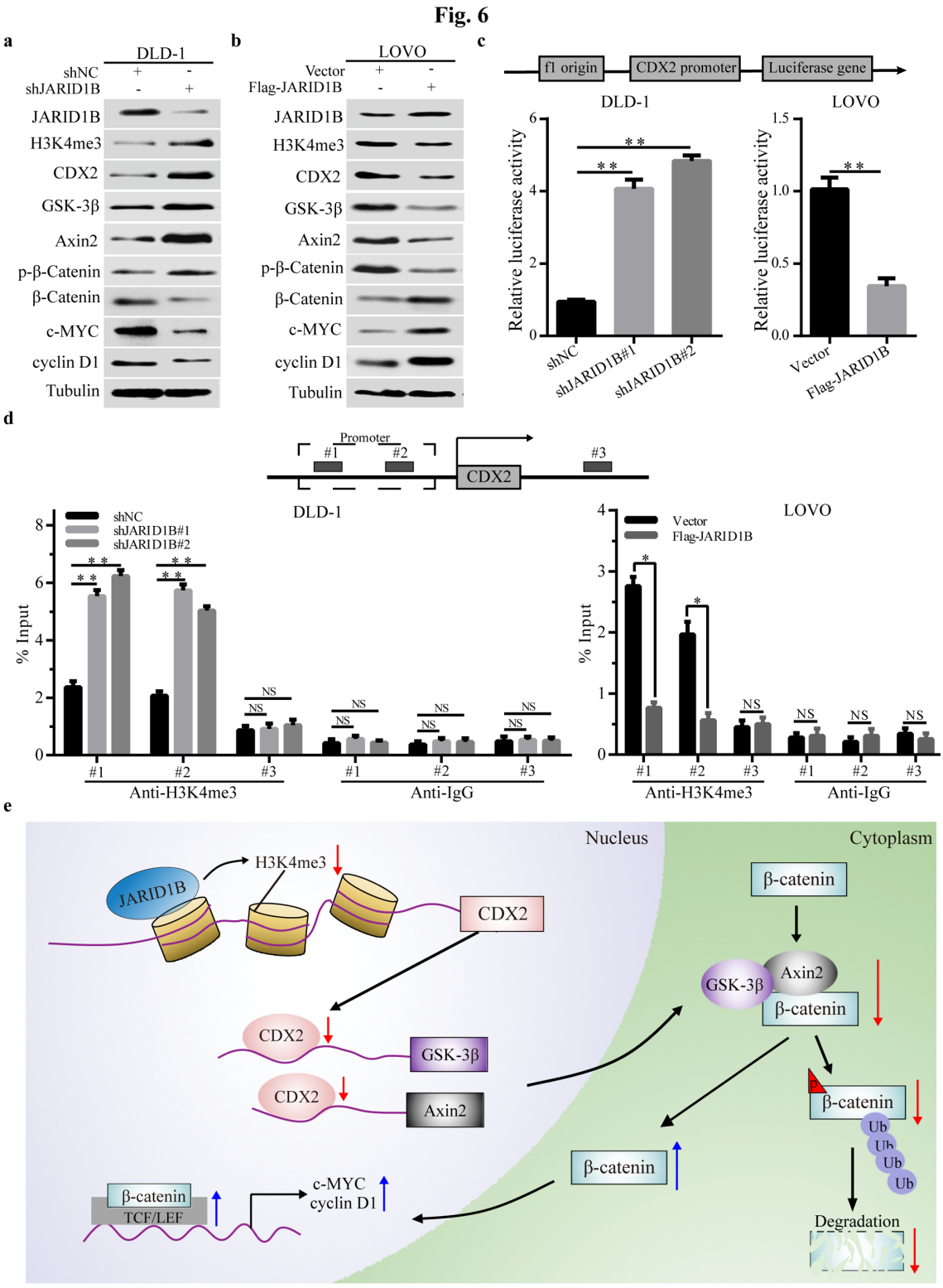

Figure 6

JARID1B demethylated H3K4me3 at the CDX2 promoter. a, b Western blot analysis was performed to detect the expression of CDX2, H3K4me3, GSK-3β, Axin2, p-β-catenin, β-catenin, c-MYC and cyclinD1 in DLD-1-shJARID1B and control DLD-1 cells, LOVO-JARID1B and control LOVO cells. c CDX2 promoter reporter luciferase assay using CRC cells transfected with the shJARID1B and shNC plasmid. Western blot was used to detect JARID1B, H3K4me3, CDX2, β-catenin and c-MYC expression in DLD-1 cells

transfected with shNC or shJARID1B and in LOVO cells transfected with Vector or Flag-JARID1B. d Schematic representation of the CDX2 promoter and the two predicted H3K4me3 binding elements in the promoter region of the CDX2 gene. Quantitative chromatin immunoprecipitation (qChIP) assays were performed in shJARID1B, control DLD-1 cells, JARID1B-overexpression and control LOVO cells. e A model of mechanism underlying JARID1B-regulated CRC Cells proliferation by decreased of CDX2 and regulation of wnt/β-catenin signaling pathway. *p<0.05, **p<0.01, NS: no significant.

Supplementary Files

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