

Role of STAT3 and vitamin D receptor in *EZH2*-mediated invasion of human colorectal cancer

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Abstract

The polycomb group protein enhancer of zeste homologue 2 (*EZH2*), which has histone methyltransferase (HMT) activity, is overexpressed in malignant tumours. However, the role of *EZH2* in colorectal cancer (CRC) invasion is little known. Here we investigated the clinical significance, biological effects, and mechanisms of *EZH2* signalling. Knockdown of *EZH2* significantly reduced cell invasion and secretion of matrix metalloproteinases 2/9 (MMP2/9) in *in vitro* studies. Knockdown of *EZH2* dramatically increased overall survival and decreased metastasis of lung in *in vivo* studies. Conversely, overexpression of *EZH2* significantly increased lung metastasis and shortened overall survival when compared with control tumours. *EZH2*-induced CRC cell invasion may depend on down-regulation of vitamin D receptor (VDR), which is considered to be a marker of CRC invasion. *EZH2* regulates the histone trimethylation of lysine 27 (H3K27me3) in the VDR promoter. Moreover, we found that STAT3 directly binds to the *EZH2* promoter and regulates VDR down-regulation in CRC cells. Significant inverse correlations were observed between the expression of *EZH2* and pSTAT3 and that of VDR in CRC tissues compared with normal tissue in patients. We show the role of *EZH2* in CRC metastasis and identify VDR as a target gene of *EZH2*. *EZH2* expression may be directly regulated by STAT3, and STAT3 may play an important role in *EZH2*-mediated VDR down-regulation in CRC. This pathway may provide potential targets in aggressive CRC.

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Introduction

Although colorectal cancer (CRC) mortality has decreased in the past decade, it remains a leading cause of morbidity and mortality [1,2]. Genetic and epigenetic alterations are important in the pathogenesis of CRC [3–5]. In addition to well-defined promoter DNA hypermethylation and histone deacetylation, deregulation of polycomb-induced gene silencing has been reported [6–8]. The enhancer of zeste homologue

2 (*EZH2*) protein is a core component of the polycomb repressive complex 2 (PRC2), which modifies transcription at the epigenetic level by affecting both histone and DNA methylation [9]. Recent studies have shown that *EZH2* silences tumour suppressor gene expression, via the modification of lysine 27 in H3 histone tails in the promoter region of the target gene [10,11]. Overexpression of *EZH2* correlates with invasiveness in several cancers [12,13] but the role of *EZH2* in the invasion of CRC remains unclear.

As a member of the steroid hormone receptor superfamily, the vitamin D receptor (VDR) regulates gene expression in a ligand-dependent manner [14]. VDR was initially isolated from the nuclei of chicken small intestinal cells [15]. The active metabolite of vitamin D, $1\alpha,25$ -dihydroxyvitamin D₃, induces the expression of E-cadherin, which plays a causal role in the invasion of carcinoma cells that express the VDR [16,17]. Aggressive CRC cells with a high metastatic potential have low levels of VDR expression [18]. In addition, *snail2* and *snail1*, the inducers of CRC cell invasion, may cooperate to repress expression of the VDR in CRC [19]. Therefore, down-regulation of the VDR may be involved in the pathogenesis of CRC and could be a predictive marker of malignant potential [20].

Overexpression of *EZH2* is associated with a poor outcome [21]. It has been reported that the mitogen-activated protein kinase (MAPK) pathway regulates *EZH2* overexpression in subtypes of aggressive breast cancer [22]. The expression and function of *EZH2* may be regulated by microRNA [23] and non-coding RNA [24]. However, the mechanism by which *EZH2* is overexpressed in CRC remains unclear.

Accumulating evidence indicates that abnormalities in the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway are involved in the oncogenesis of CRC [25,26]. As a key component of the JAK/STAT pathway, STAT3 is constitutively activated in CRC [27]. Yeh *et al.* postulated that *EZH2* may be regulated by STAT3 in prostate cancer [28]. Furthermore, *EZH2* has been shown to regulate VDR gene expression in germinal centre B cells [29]. Whether *EZH2* mediates VDR expression and the transcription factor(s) responsible for overexpression of *EZH2* in CRC cells is not known. Our goal was to investigate this hypothesis.

Materials and methods

Cell culture and treatment

Human colon cancer cell lines HT29, SW1116, HCT116, and SW620 (ATCC, Manassas, VA, USA) were maintained in 1640 medium (Gibco, Carlsbad, CA, USA), respectively, supplemented with 10% fetal bovine serum (FBS). CRL-1790 cells, a 'normal' human colon epithelial cell line (ATCC), were also used. CRL-1790 cells were grown in F-12 medium enriched with 0.02 mg/ml insulin, 0.01 mg/ml transferrin, 25 nM sodium selenite, 50 nM hydrocortisone, 1 ng/ml epidermal growth factor, 0.01 mM ethanolamine, 0.01 mM phosphorylethanolamine, 100 pM triiodothyronine, 0.5% (w/v) bovine serum albumin, 10 mM HEPES, 0.5 mM sodium pyruvate, and an extra 2 mM L-glutamine (final concentration 4.5 mM). Cell density was maintained at approximately 100 000 cells per millilitre of medium. Cells were then plated in fibronectin- and collagen type I-coated

T-25 flasks. All of the cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere.

Immunohistochemical staining

All specimens (tumour and parallel normal) were obtained from 129 patients with primary CRC, who underwent surgery at the Shanghai Renji Hospital from July 2009 to May 2012. All patients provided written informed consent before enrolment, and the study protocol was approved by the Ethics Committee of Shanghai Jiao-Tong University School of Medicine, Renji Hospital. The study was carried out according to the provisions of the Helsinki Declaration of 1975. None of the patients received pre-operative treatment. The expression levels of pSTAT3, STAT3, *EZH2*, and VDR were examined with primary antibodies (STAT3, dilution 1 : 100; pSTAT3, *EZH2*, and VDR, dilution 1 : 50) using the LSAB + kit (DakoCytomation, Copenhagen, Denmark) according to the manufacturer's instructions. Antibodies used in this study were purchased from Cell Signaling Technology Inc (Beverly, MA, USA).

The slides were examined independently by two investigators, who were blinded to the clinical and pathological data. Protein expression was quantified using a visual grading system based on the extent and intensity of staining (percentage of positive tumour cells was graded on a scale of 0–4: 0, none; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%) and the intensity of staining (graded on a scale of 0–3: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining). For further analysis, an index value was calculated as a product of the grades of the extent and intensity of staining to define the cut-off value for high expression of the proteins, and protein expression was classified into two categories: high (grades 4–12) and low (grades 0–3).

Animal experiments

We developed a CRC metastasis model in nude mice. HCT116 cells were infected with lenti-GFP-control virus to construct HCT116-GFP-control stable cell lines. HCT116 cells and HCT116-GFP-control stable cells (2.5×10^6) were suspended in 500 μ l of PBS and injected through the tail veins of 6-week-old nude mice [30]. Forty days later, the NightOWL II LB 983 *in vivo* imaging system (Berthold Technologies, Bad Wildbad, Germany) was used to image the metastases. Preliminary data showed that GFP-positive subcutaneous metastasis was successfully detected after the injection of HCT116-GFP-control stable cells, but almost no GFP-positive subcutaneous metastasis was detected after the injection of PBS or HCT116 cells. All of the lentiviruses were provided by Shanghai Sunbio Biomedical Technology (Shanghai, China).

SW1116 cells were infected with lenti-GFP-*EZH2* virus or lenti-GFP-control virus and HCT116 cells were infected with lenti-GFP-*EZH2* shRNA virus or lenti-GFP-control shRNA virus to construct the

SW1116-GFP-*EZH2*, SW1116-GFP-control, HCT116-GFP-sh*EZH2*, and HCT116-GFP-control shRNA stable cell lines, respectively. We injected PBS, SW1116 cells, SW1116-GFP-control cells, SW1116-GFP-*EZH2* cells, HCT116 cells, HCT116-GFP-control shRNA cells or HCT116-GFP-sh*EZH2* cells into the tail veins of nude mice once a week. At 40 days, photographs were taken with the NightOWL II LB 983 imaging system. During this time, we recorded the survival time of the nude mice and survival curves were generated. The livers and lungs of the nude mice were excised post-mortem for haematoxylin and eosin staining [31]. Details of the CRC xenograft animal models may be found in the Supplementary methods. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Chromatin immunoprecipitation (ChIP) and real-time ChIP

ChIP assays were performed using the ChIP assay kit (Upstate, Charlottesville, VA, USA) according to the manufacturer's instructions. The protein-DNA complexes were precipitated using 5 µg of antibodies against trimethyl-histone 3-Lys27 (3meH3K27; #07-449, BD San Jose, CA, USA), STAT3 (#9132, CST), *EZH2* (#4905, CST, San Jose, CA, USA) or IgG control (#12-370, Santa Cruz, Dallas, TX, USA). Approximately 2–5 µl of ChIP-enriched chromatin was subjected to a standard ChIP-PCR reaction, and the enrichment of specific genomic regions was assessed relative to either control IgG or control cells.

Real-time PCR was performed using an Applied Biosystems 7900 quantitative PCR system. Each PCR reaction was carried out in a 10 µl reaction volume using 3 µl of the eluted immunoprecipitated DNA. The amount of genomic DNA that co-precipitated with the specific antibody was calculated in comparison to the total input DNA used for each immunoprecipitation as follows: $CBTB = CBTB(\text{genomic input}) - CBTB(\text{specific antibody})$, where $CBTB(\text{genomic input})$ and $CBTB(\text{specific antibody})$ are the mean threshold cycles of PCR performed in triplicate on DNA samples from the genomic input samples and the specific antibody samples, respectively. The ChIP primers for the *VDR* gene promoter were *VDR-F* (5'-CCCTTGGGTGAGATT-3') and *VDR-R* (5'-CTCCGCACGAATGG-3'). The ChIP primers for the *EZH2* gene promoter were *EZH2-F* (5'-GGCGTCCTGTGAA-3') and *EZH2-R* (5'-CCGCCAACAACTG-3'; Figure 6C). A ChIP primer pair for *GAPDH* was used as a negative control for STAT3 binding: (5'-CACCGTGTGCCCAAGACCTC-3') and (5'-CAGCCCTGTAGCCTGGACCT-3').

Statistical analysis

Statistical analysis was performed with SPSS 13.0 software (SPSS Inc, Chicago, IL, USA). For clinicopathological analysis, the chi-squared test or Fisher's exact

test (two-sided) was performed. The Kaplan–Meier method was used to estimate the overall survival, and the log-rank test was used to evaluate the differences between survival curves. Correlation analyses were performed for the expression of pSTAT3, STAT3, *EZH2*, and VDR. Statistical tests and *p* values were two-sided. Results were considered significant if the *p* value was less than 0.05.

Results

Expression of *EZH2* in CRC tissues and cells

As shown in Figure 1A, *EZH2* staining was mainly detected in the nuclei of cells in CRC tissue samples, with almost no *EZH2* staining observed in normal epithelial tissues. Furthermore, the expression of *EZH2* was much higher in tissues from CRC patients with metastasis than in those without metastasis. We found an association between *EZH2* expression and several known clinicopathological features in CRC. *EZH2* expression was positively correlated with location, TNM stage, histological grade, vascular invasion, and lymph node metastasis (Supplementary Table 1). Western blot analysis (Figure 1B) showed that *EZH2* was not detectable in normal human colon epithelial cells (CRL-1790), but *EZH2* expression was significantly increased in all of the CRC cell lines tested, especially in highly invasive CRC cells.

EZH2 plays an important role in CRC cell invasion

EZH2 may mediate cell proliferation and invasion in human cancers [8,32,33]. To study this in CRC, first, we introduced lenti-*EZH2* shRNA-1/2 viruses or a lenti-*EZH2* overexpression virus into CRC cells. Lenti-*EZH2* shRNA-1/2 viruses successfully decreased *EZH2* expression, respectively (Supplementary Figure 1A). Moreover, introduction of the lenti-*EZH2* overexpression virus remarkably increased *EZH2* expression (Supplementary Figure 1B). There was no significant change of *EZH2* expression after transduction of the control shRNA or control viruses when compared with control CRC cells.

In Transwell cell invasion assays, representative data showed that knockdown of *EZH2* expression significantly reduced the invasion ability of CRC cells (Figure 1C). Moreover, overexpression of *EZH2* dramatically increased the invasion ability of CRC cells (Figure 1C), indicating that *EZH2* may have a significant effect on cell migration and invasion in CRC cells. In wound healing assays, less wound closure was observed in CRC cells with knockdown of *EZH2* compared with control. Furthermore, more wound closure was observed in CRC cells with overexpression of *EZH2* compared with control (Figure 1D). This result was further confirmed by ELISA assays, as knockdown of *EZH2* significantly reduced the secretion of MMP2 and MMP9 in CRC cells (Supplementary Figure 2).

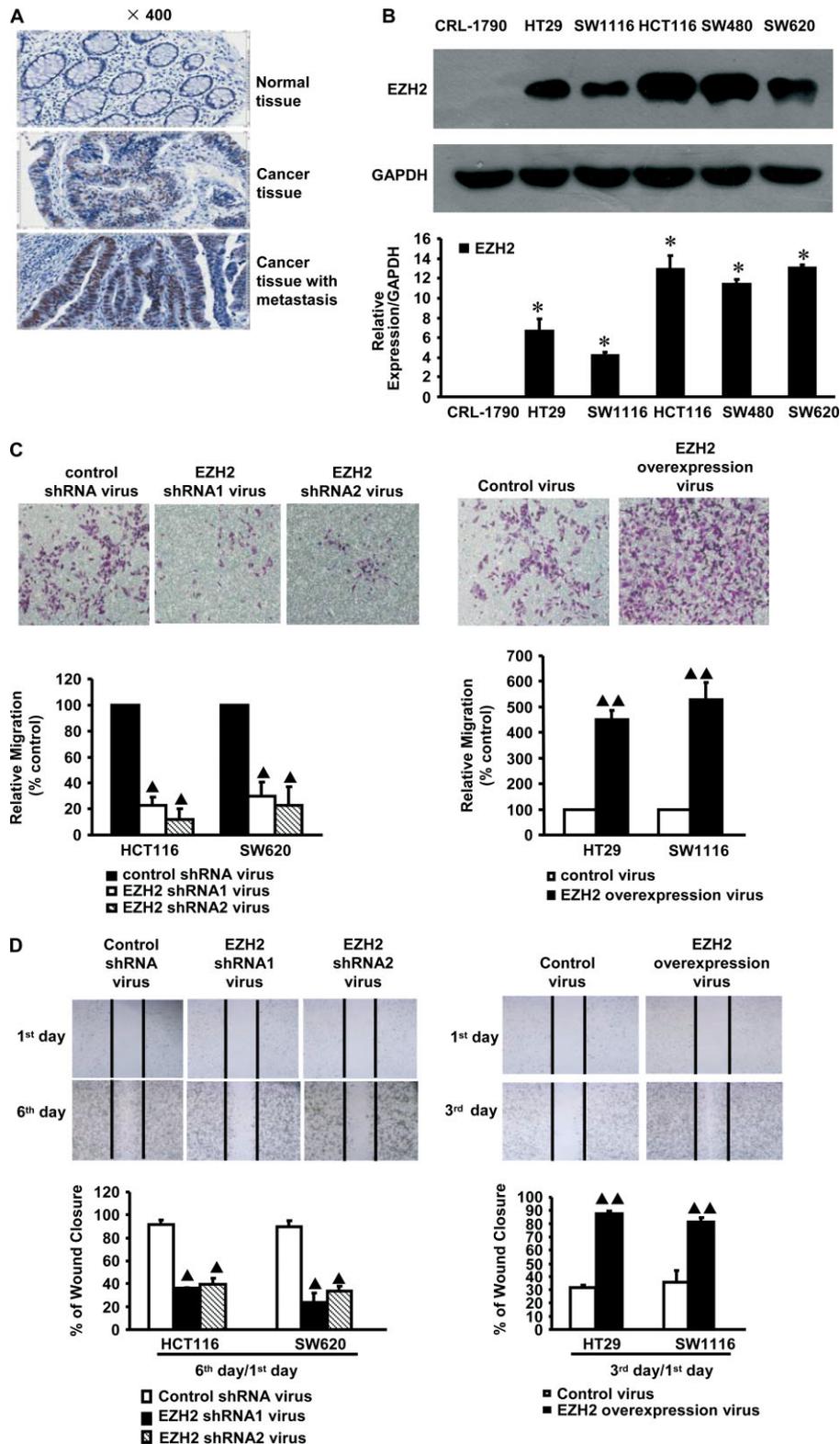


Figure 1. Expression and function of *EZH2* in CRC tissues and cells. (A) Immunohistochemical analysis of *EZH2* in normal colorectal mucosa, CRC tissues, and CRC tissues from patients with metastasis (original magnification $\times 400$). (B) Western blot analysis showing almost no expression of *EZH2* in a normal human colon epithelial cell line, CRL-1790; however, the expression of *EZH2* was significantly increased in all CRC cell lines, especially in highly invasive CRC cells. (C) Transwell Matrigel invasion assays were performed in CRC cells infected with control shRNA or *EZH2* shRNA viruses or control or *EZH2* overexpression virus. Cells were observed under a light microscope and photographed. Cells were counted from five random microscopic fields ($200\times$) per insert in triplicate. The migrated cell numbers were normalized to that of the control group. Data are shown as mean \pm SD from three separate experiments. (D) Wound healing assays were performed in CRC cells after infection with the *EZH2* overexpression virus, control virus, control shRNA virus or *EZH2* shRNA virus. $n = 3$, ANOVA, $*p < 0.05$, compared with CRL-1790 cells; $n = 3$, ANOVA, $\blacktriangle p < 0.05$, compared with the control shRNA virus; $n = 3$, $\blacktriangle\blacktriangle p < 0.05$ (Student's *t* test).

We then explored the functional impact of *EZH2* overexpression/knockdown. Different stable cells were transplanted into BALB/c-nu/nu mice via tail vein injection. Increased numbers of subcutaneous metastases were detected after injection of SW1116-GFP-*EZH2* cells, compared with mice injected with control cells (Figure 2A). Mice inoculated with SW1116-GFP-*EZH2* cells had a shorter overall survival time (mean 65.7 days) compared with those injected with control cells (mean 76.4 days; Figure 2C). Haematoxylin and eosin staining showed that fewer metastatic CRC cells were observed in the lungs of nude mice at 17 weeks after injection of SW1116-GFP-control cells, while most of the mice injected with SW1116-GFP-*EZH2* cells displayed significant lung metastases (Figure 2E and Supplementary Table 2). Fewer subcutaneous metastases were detected after down-regulation of *EZH2* (Figure 2B). Knockdown of *EZH2* remarkably increased the overall survival time and significantly decreased lung metastasis of tumour cells *in vivo* (Figures 2D, 2E, and Supplementary Table 2). These data suggest that *EZH2* expression plays a critical role in CRC cell motility and metastasis.

We compared the survival time in patients with *EZH2*-expressing tumours ($n = 17$) with that of patients with *EZH2*-negative tumours ($n = 16$; Figure 2F). The cumulative survival rate was significantly better in patients with *EZH2*-negative tumours than in those with *EZH2*-positive tumours ($p < 0.05$, HR = 0.1973, 95% CI 0.06–0.66), indicating that *EZH2* overexpression is significantly associated with patient survival in CRC.

EZH2 negatively regulates VDR expression in CRC cells and *EZH2*-induced CRC cell invasion depends on the repressing of VDR

E-cadherin expression is regulated by *EZH2* via the histone methylation of *E-cadherin* promoter [34,35]. Velichutina *et al.* predicted that VDR is a target of *EZH2* [29]. We examined whether *EZH2* regulates VDR expression in CRC cells. Real-time PCR data showed that up-regulation of *EZH2* significantly down-regulated VDR expression in SW1116 cells (Figures 3A and 3C). Conversely, down-regulation of *EZH2* significantly increased the mRNA level (Figure 3B) and protein levels (Figure 3D) of VDR in HCT116 cells. The data suggest that *EZH2* may regulate VDR expression in CRC cells. In addition, overexpression of VDR significantly decreased the invasion ability of CRC cells under basal conditions. Up-regulation of VDR blocked *EZH2*-induced cell invasion in SW1116 cells (Figure 3E) and down-regulation of VDR restored the *EZH2* shRNA-induced decrease in cell invasion ability in HCT116 cells (Figure 3F), indicating that *EZH2*-induced CRC cell invasion may depend on the suppression of VDR expression.

To explore the mechanism of *EZH2*-induced VDR down-regulation in CRC cells, we constructed a luciferase reporter plasmid containing part of the VDR

promoter region. Knockdown of *EZH2* significantly increased the transcriptional activity of the VDR promoter in HCT116 cells (Figure 4A), indicating that *EZH2* may mediate VDR expression by regulating the transcriptional activity of the VDR promoter. We then examined the H3K27 methylation level of the VDR promoter after knockdown of *EZH2*. As shown in Figure 4B, VDR genomic DNA was detectable in a ChIP assay when either an H3K27me3 antibody or *EZH2* antibody was used, and Figure 4C shows that knockdown of *EZH2* significantly decreased the H3K27 histone methylation level of the VDR promoter, suggesting that *EZH2* may regulate the expression of VDR by modulating the H3K27 histone trimethylation in the VDR promoter region.

Supplementary Tables 1 and 4 show the frequencies of *EZH2* and VDR expression in 129 paired samples of normal and CRC tissues from patients undergoing colorectal surgery. In consecutive tissue sections (Figure 4D), *EZH2* was expressed at higher levels in the CRC samples than in the control tissues. In contrast, VDR was expressed at lower levels in the tumour tissues than in the normal colon epithelial samples. Our data suggest that *EZH2* is significantly up-regulated in CRC (chi-squared test, $p < 0.0001$), while VDR is significantly down-regulated (chi-squared test, $p = 0.012$). Additionally, the expression levels of *EZH2* and VDR were inversely correlated (Spearman's correlation coefficient $r = -0.65720$; $p < 0.0001$).

STAT3 regulates *EZH2* expression in CRC cells

STAT3 mediates the invasion of cancer cells [36,37]. Since *EZH2* mediates CRC cell invasion and *EZH2*-induced CRC cell invasion depends on the suppression of VDR expression, we next determined whether STAT3 regulates the expression of *EZH2* and VDR in CRC cells. Western blot analysis showed that knockdown of STAT3 significantly decreased *EZH2* expression and increased VDR expression in CRC cells (Figures 5A and 5B). Moreover, *EZH2* expression was markedly increased and VDR expression was significantly decreased after overexpression of STAT3 in CRC cells (Figures 5C and 5D), suggesting that STAT3 may play an important role in *EZH2* up-regulation and VDR down-regulation. Knockdown of *EZH2* blocked STAT3-induced down-regulation of VDR expression levels in CRC cells (Figures 5E and 5F), indicating that STAT3-mediated VDR down-regulation depends on the activation of *EZH2*.

To explore the mechanism of STAT3-induced *EZH2* up-regulation in CRC cells and based on our preliminary luciferase reporter gene data (Supplementary Figure 3), we constructed a luciferase reporter plasmid containing part of the *EZH2* promoter region (from -420 to +80 nt). Overexpression of STAT3 dramatically increased the transcriptional activity of the *EZH2* promoter in HCT116 cells (Figure 6A). Moreover, DNA sequence analysis of the *EZH2* promoter region revealed six putative STAT3 binding sites. The

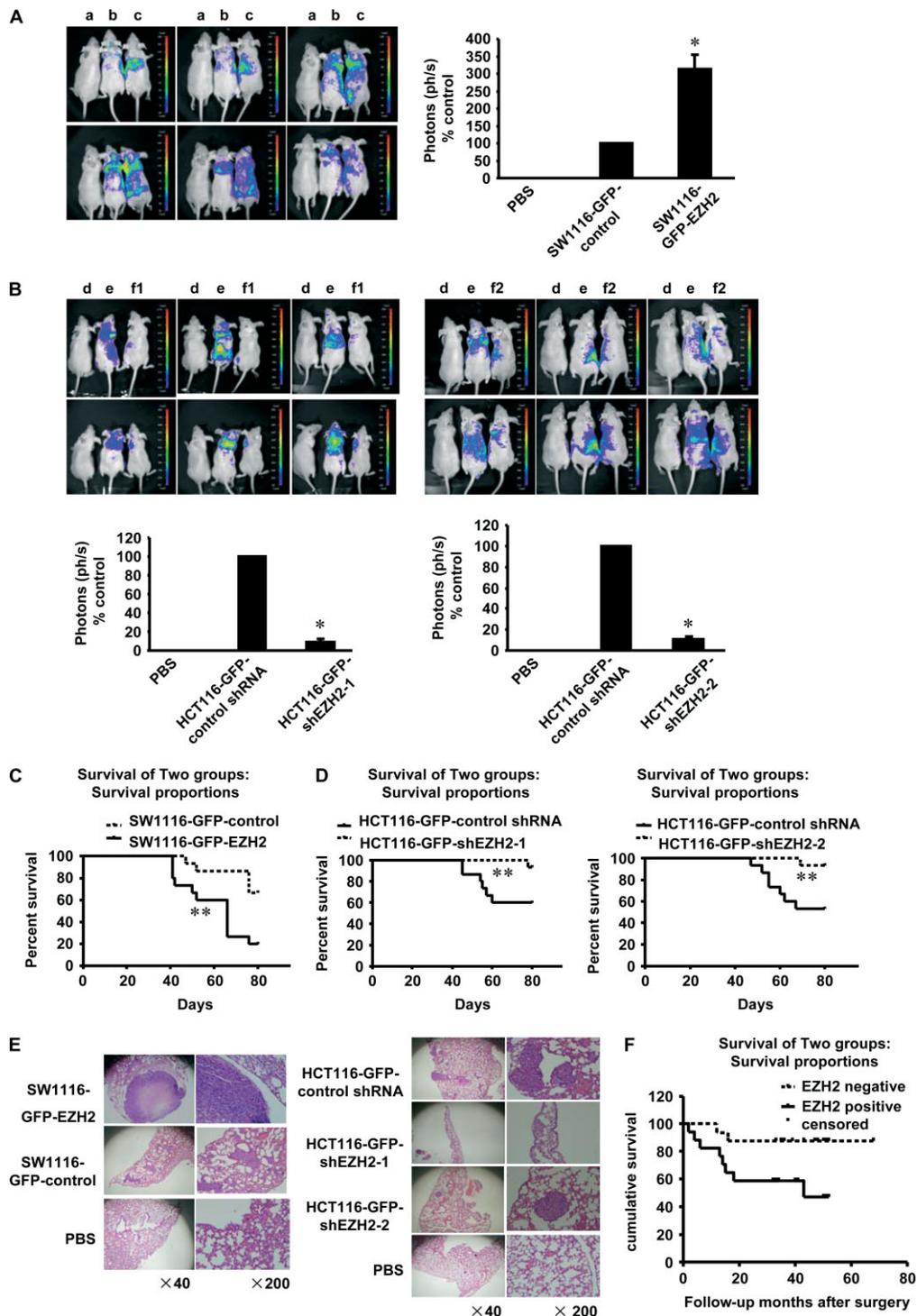


Figure 2. Functional impact of *EZH2* overexpression/knockdown on the metastatic potential of CRC cells *in vivo*. (A) Assessment of the subcutaneous metastatic capacity of 2.5×10^6 SW1116-GFP-control or SW1116-GFP-EZH2 cells after inoculating nude mice via tail vein injection. Biofluorescence images and summarized data show subcutaneous metastasis and total photon flux for each treatment group (a: mice injected with PBS; b: mice injected with SW1116-GFP-control stable cells; c: mice injected with SW1116-GFP-EZH2 stable cells). $n = 15$, ANOVA, $*p < 0.0001$. (B) Assessment of subcutaneous metastatic capacity of 2.5×10^6 HCT116-GFP-control shRNA or HCT116-GFP-shEZH2-1/2 tumour cells by inoculating nude mice via tail vein injection. Biofluorescence images and summarized data show subcutaneous metastasis and total photon flux for each treatment group (d: mice injected with PBS; e: mice injected with HCT116-GFP-control shRNA stable cells; f1: mice injected with HCT116-GFP-shEZH2-1 stable cells; f2: HCT116-GFP-shEZH2-2 stable cells). $n = 15$, ANOVA, $*p < 0.0001$. (C, D) Survival curves for mice. $n = 15$, $**p < 0.05$ (Student's *t*-test), compared with SW1116-GFP-control cells or HCT116-GFP-control shRNA cells. (E) Increased numbers of metastatic CRC cells were detected in the lungs of nude mice at 17 weeks after the injection of the SW1116-GFP-EZH2 group compared with the SW1116-GFP-control group. Fewer metastatic CRC cells were detected in the lungs of nude mice injected with HCT116-GFP-shEZH2-1/2 cells at 17 weeks compared with mice injected with HCT116-GFP control shRNA cells. (F) Survival analysis showed that *EZH2*-positive tumours have an unfavourable prognosis compared with *EZH2*-negative tumours in patients ($p < 0.05$, HR = 0.1973, 95% CI 0.06–0.66).

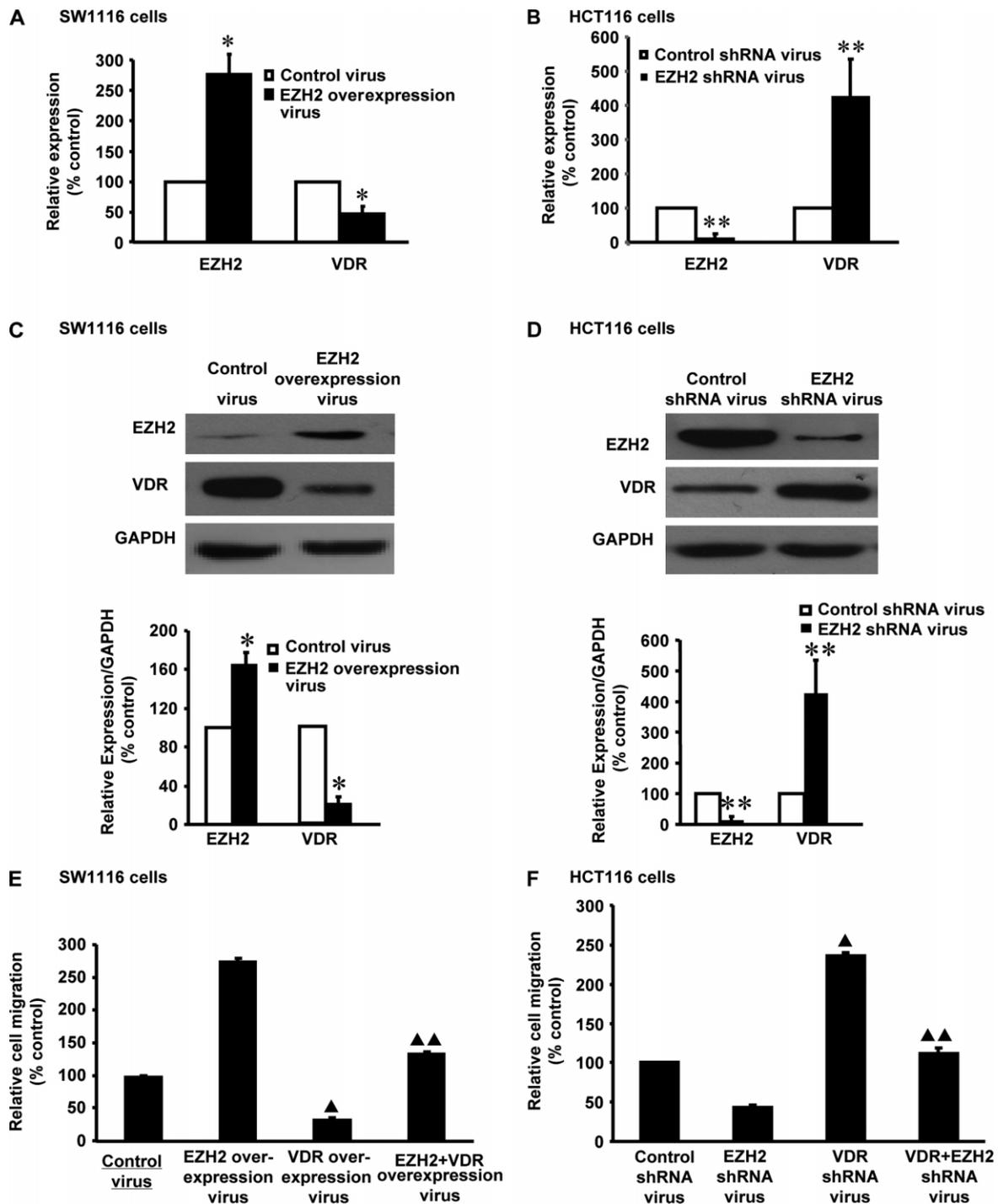


Figure 3. Role of *EZH2* in VDR regulation and effect of VDR on *EZH2*-induced CRC cell invasion. (A) Real-time PCR data show that infection of *EZH2* overexpression virus dramatically increased *EZH2* mRNA expression and decreased *VDR* mRNA expression in SW1116 cells. $n = 3$, $*p < 0.01$ (Student's t -test). (B) Real-time PCR show that *EZH2* shRNA virus significantly decreased the mRNA level of the *EZH2* gene and increased the mRNA level of the *VDR* gene in HCT116 CRC cells. $n = 3$, $**p < 0.01$ (Student's t -test). (C) Western blot analysis data show that overexpression of *EZH2* significantly decreased the expression of *VDR* in SW1116 cells. $n = 3$, $*p < 0.01$ (Student's t -test). (D) Knockdown of *EZH2* dramatically increased the expression of *VDR* in HCT116 cells. $n = 3$, $**p < 0.01$ (Student's t -test). (E, F) Transwell Matrigel invasion assays were performed in CRC cells infected with different viruses as indicated. Cells were observed under a light microscope and photographed. Cells were counted from five random microscopic fields (200 \times) per insert in triplicate. The migrated cell numbers were normalized to that of the control group. Data are shown as mean \pm SD from three separate experiments. $n = 3$, ANOVA, $p < 0.01$, compared with control; $p < 0.05$, compared with *EZH2* overexpression virus or *EZH2* shRNA virus.

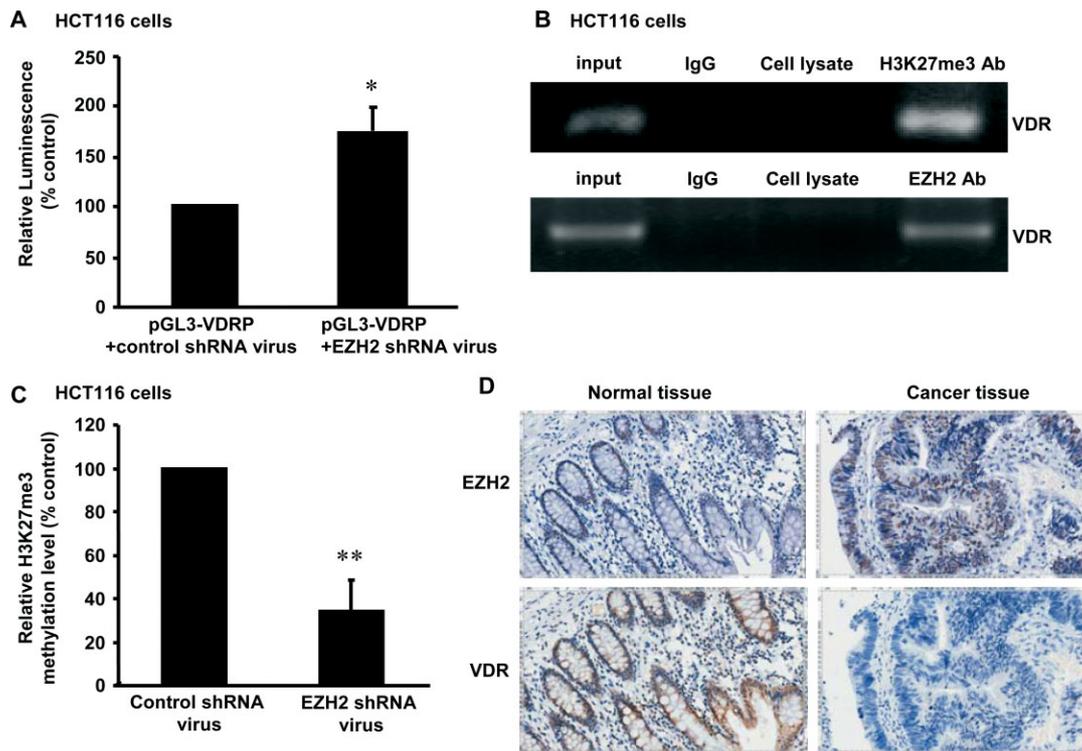


Figure 4. The mechanism of *EZH2*-mediated *VDR* down-regulation in CRC cells. (A) Knockdown of *EZH2* significantly increased the luciferase activity of the *VDR* gene promoter. $n = 3$, $*p = 0.0005$ (Student's *t*-test). (B) The band in the gel of the ChIP assay suggests that the H3K27me3 site exists in the *VDR* gene promoter. Input DNA was used as a positive control; cell lysates incubated with non-relevant rabbit IgG or cell lysate without antibody incubation were used as negative controls. (C) Real-time PCR of ChIP samples showed that knockdown of *EZH2* dramatically decreased the trimethylation level of H3K27 in the *VDR* promoter in HCT116 cells. $n = 3$, $**p = 0.0097$ (Student's *t*-test). (D) Immunohistochemical analysis of consecutive human CRC tissue sections showed that higher levels of *EZH2* nuclear staining were observed in CRC samples than in normal colonic epithelial samples, and the expression of *VDR* showed the opposite trend (original magnification $\times 400$).

transcriptional activity of the *EZH2* promoter was significantly decreased after mutation of STAT3 binding sites in the *EZH2* promoter (Figure 6B). The data suggest that STAT3 may activate the *EZH2* promoter. In a ChIP assay, we used a pair of primers covering a region from -141 to 48 nt, containing four putative STAT3 binding sites, in the *EZH2* promoter. Figure 6D shows that *EZH2* genomic DNA was detectable in the ChIP assay with STAT3 antibody incubated. ChIP real-time PCR analysis demonstrated that overexpression of STAT3 significantly increased the recruitment of STAT3 to the *EZH2* promoter (Figure 6E). The data indicate that STAT3 may bind to the *EZH2* promoter. In addition, down-regulation of *EZH2* blocked the STAT3-induced increase in cell invasion in HCT116 cells (Figure 6F), indicating that *EZH2* may participate in STAT3-induced CRC cell invasion.

Expression of pSTAT3, STAT3, *EZH2*, and *VDR* in CRC tissues

We also found an association between pSTAT3/*VDR* expression and several known clinicopathological features in CRC (Supplementary Tables 3 and 4). The results suggest that pSTAT3 expression was positively correlated with histological grade, vascular invasion, and lymph node metastasis, and *VDR* expression

was negatively correlated with TNM stage, vascular invasion, and lymph node metastasis. Furthermore, we used univariate and multivariate analyses to analyse whether the expression of *EZH2*, *VDR*, and pSTAT3 was associated with metastasis (vascular invasion and lymph node metastasis) in human CRC (Table 1). In univariate analysis, *EZH2*, *VDR*, and pSTAT3 were significantly associated with vascular invasion and lymph node metastasis, respectively; however, only *EZH2* was found to be significantly associated with vascular invasion and lymph node metastasis in CRC patients in multivariate analyses.

We found that 85.5% (71/83) of the CRC patients who had low levels of *VDR* expression in their tumour tissues exhibited up-regulation of *EZH2* ($p < 0.001$). The tumour tissues of 83.33% (65/78) of the patients displaying high levels of pSTAT3 also expressed high levels of *EZH2* ($p < 0.001$). Furthermore, 81.93% (68/83) of the patients with low levels of *VDR* in their tumour tissues exhibited up-regulation of pSTAT3 ($p < 0.001$) (Supplementary Table 5).

Supplementary Table 6 shows the frequencies of STAT3, pSTAT3, *EZH2*, and *VDR* expression levels in tumour tissues formed by CRC cells in which STAT3 was down-regulated or up-regulated, compared with control tumour tissues, in the nude mouse xenograft model. High levels of pSTAT3 were found

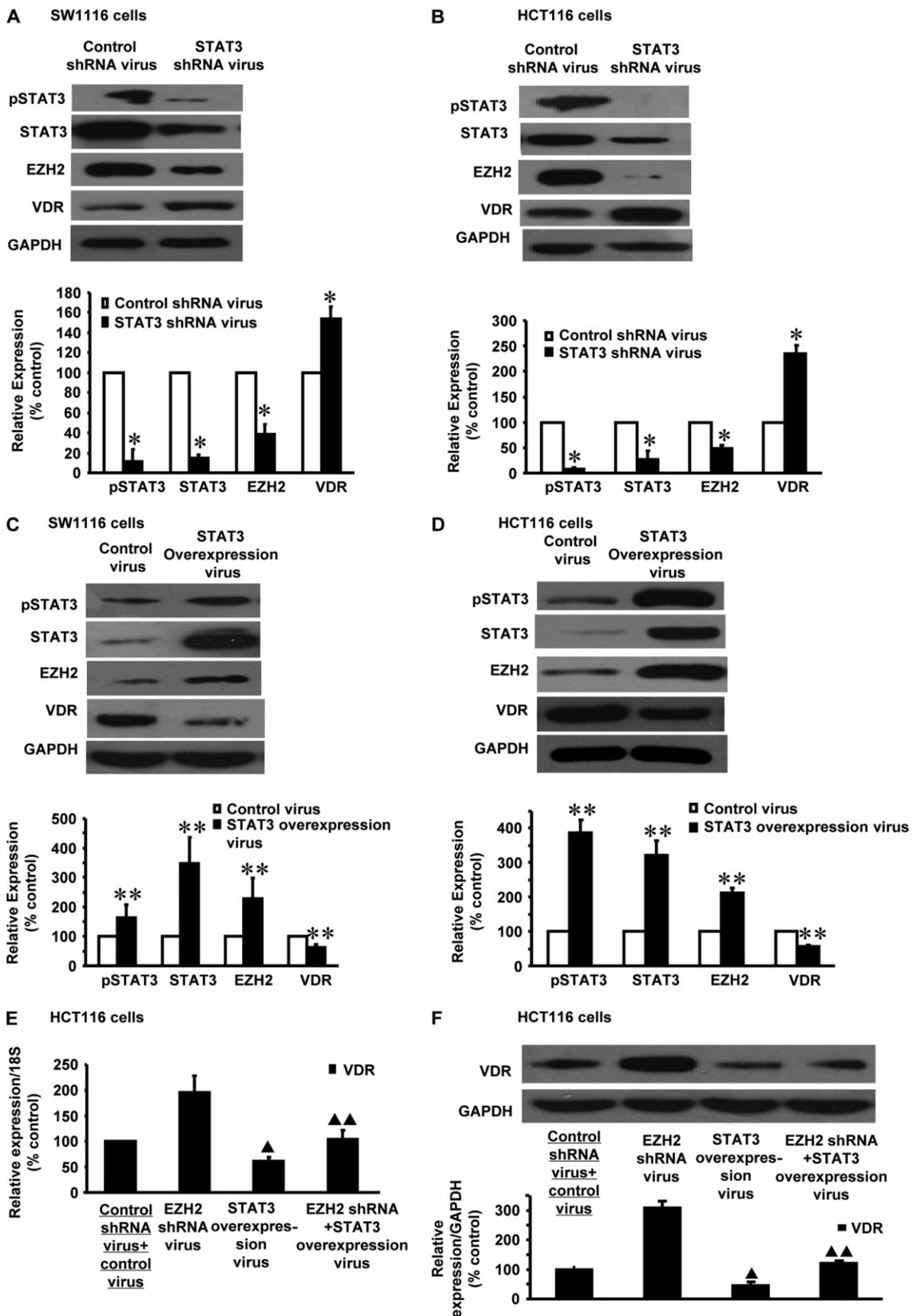


Figure 5. Effect of STAT3 on *EZH2* and VDR expression in CRC cells. Western blot analysis data show that infection of STAT3 shRNA virus dramatically inhibited the phosphorylation and expression of STAT3. STAT3 down-regulation significantly decreased the expression of *EZH2* and increased the expression of VDR in SW1116 (A) and HCT116 cells (B). * $p < 0.01$ (Student's *t*-test), compared with control shRNA virus. Western blot analysis data show that the phosphorylation and expression of STAT3 were successfully increased after introducing STAT3 overexpression virus. *EZH2* expression was significantly increased and VDR expression was decreased after the overexpression of STAT3 in SW1116 (C) and HCT116 cells (D). $n = 3$, ** $p < 0.01$ (Student's *t*-test), compared with control virus. Real-time PCR and western blot data show that introducing *EZH2* shRNA virus significantly blocked STAT3-mediated VDR down-regulation in CRC cells (E, F). ANOVA, $p < 0.05$, compared with control virus group; $p < 0.05$, compared with STAT3 overexpression virus group.

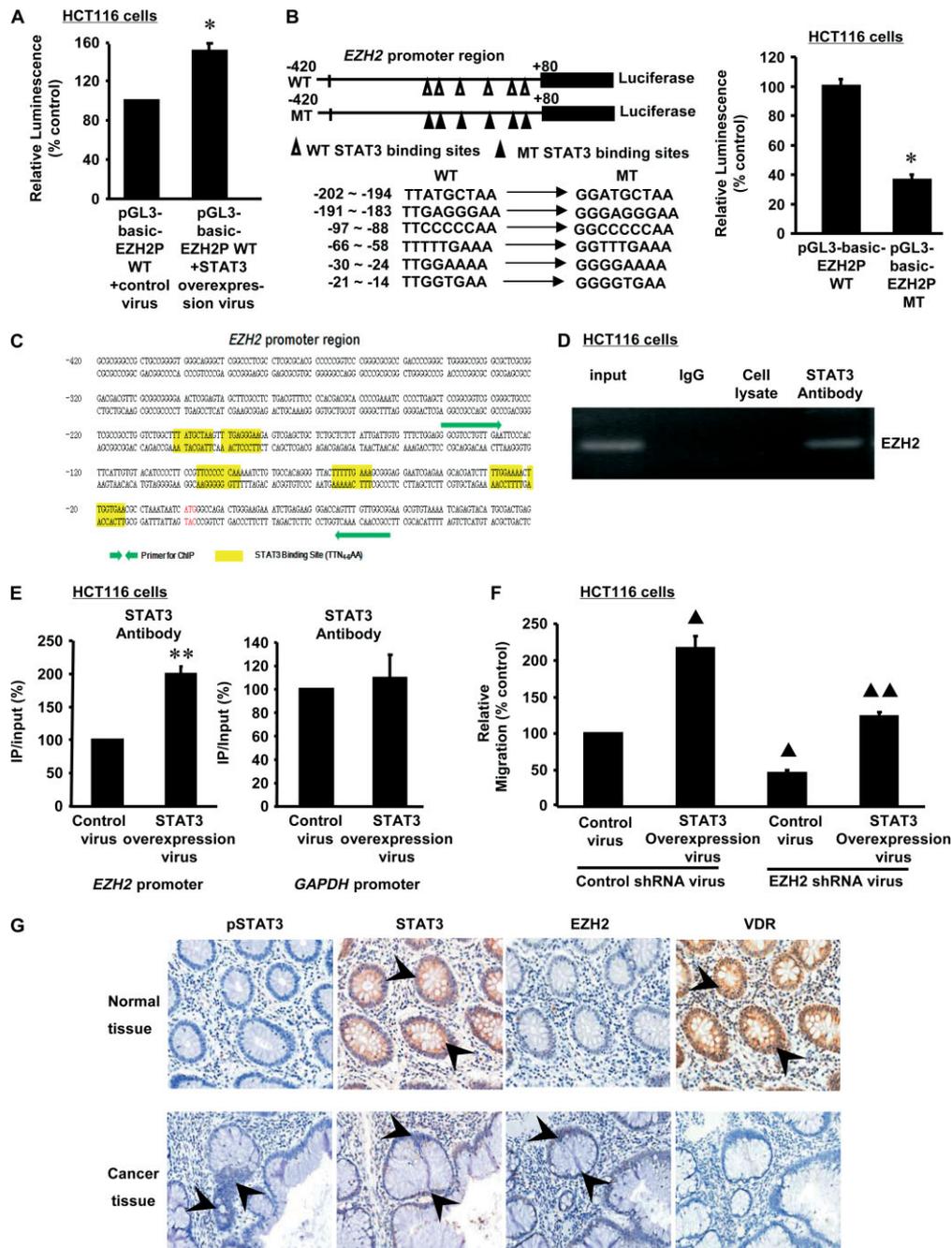


Figure 6. The mechanism of STAT3-mediated *EZH2* up-regulation in CRC cells. (A) STAT3 up-regulation dramatically increased the luciferase activity of the *EZH2* gene promoter, suggesting that STAT3 regulates the expression of *EZH2* by modulating the transcriptional activity of the *EZH2* gene. $n = 3$, $*p < 0.01$ (Student's t -test). (B) STAT3 binding sites in the *EZH2* gene promoter. White and black triangles indicate a wild-type or mutant sequence for STAT3 binding sites, respectively. WT = wild type; MT = mutant type of each STAT3 mutation binding site. Mutation of STAT3 binding sites significantly decreased the transcriptional activity of the *EZH2* promoter in the luciferase assay. $n = 3$, $*p < 0.01$ (Student's t -test). (C) Bioinformatic analysis of STAT3 transcriptional factor binding sites in part of the *EZH2* gene promoter region. Numbers on the left-hand side indicate the locations upstream of the first base of the initial translation site. STAT3 binding sites are highlighted, and the DNA sequence encompassed by two arrows was amplified in the ChIP assay. *EZH2* DNA was detected in the chromatin sample immunoprecipitated from HCT116 cells using an antibody against STAT3 (D), suggesting that STAT3 binds to the *EZH2* promoter. (E) Real-time PCR of the ChIP samples showed that overexpression of STAT3 dramatically increased the binding efficiency of STAT3 to the *EZH2* promoter in CRC cells. ChIP assay real-time PCR was performed using *GAPDH* as a negative control for STAT3 binding. $n = 3$, $**p < 0.01$ (Student's t -test). (F) Transwell Matrigel invasion assays were performed in CRC cells infected with different viruses as indicated. Cells were observed under a light microscope and photographed. Cells were counted from five random microscopic fields ($200\times$) per insert in triplicate. The migrated cell numbers were normalized to that of the control group. Data are shown as mean \pm SD of three separate experiments. $n = 3$, ANOVA, $p < 0.01$, compared with control virus plus control shRNA virus; $p < 0.05$, compared with STAT3 overexpression virus plus control shRNA virus. (G) Immunohistochemical analysis for pSTAT3 and *EZH2* in consecutive human CRC tissue sections. *EZH2* and pSTAT3 were expressed at higher levels in CRC than in normal colonic epithelial samples, and the expression of VDR showed the opposite trend. There was no significant difference in STAT3 expression in normal colonic epithelial samples or CRC tissues (original magnification $\times 400$).

Table 1. Univariate and multivariate analyses using the Cox proportional hazards model. *EZH2* expression is significantly associated with the metastasis (vascular invasion and lymph node metastasis) of patients

	Univariate analysis		Multivariate analysis		
	No of cases	<i>p</i> value	Odds ratio (95% CI)	<i>p</i> value	Odds ratio (95% CI)
pSTAT3 expression					
High	78	0.01*	4.43 (1.91–10.28)	0.083	2.43 (0.89–6.60)
Low	51				
<i>EZH2</i> expression					
High	86	0.01*	5.17 (2.06–12.99)	0.035*	3.09 (1.08–8.81)
Low	43				
VDR expression					
High	46	0.045*	0.45 (0.20–1.06)	0.629	0.79 (0.30–2.06)
Low	83				

CI = confidence interval.

*Statistically significant.

in STAT3-overexpressing tumours, and there was almost no pSTAT3 staining in the STAT3-knockdown tumours (Figure 7A). High levels of *EZH2* expression were found in 87% of the STAT3-overexpressing tumours and 7% of the STAT3-knockdown tumours (Figure 7C). VDR was highly expressed in 79% of the STAT3-knockdown tumours, and no VDR staining was detected in the STAT3-overexpressing tumours (Figure 7D). Our data suggest that the expression levels of pSTAT3 and *EZH2* were significantly up-regulated (chi-squared test, $p < 0.001$), while VDR expression was significantly decreased (chi-squared test, $p < 0.001$) in STAT3-overexpressing tumours. Conversely, the expression of pSTAT3 and *EZH2* was significantly decreased (chi-squared test, $p < 0.001$), while VDR expression was significantly increased in the STAT3-knockdown tumours.

Discussion

As a transcriptional repressor, *EZH2* is overexpressed and has been regarded as a novel candidate oncogene in several types of human cancer [8,38,39]. It has been reported that overexpression of *EZH2* correlates with increased invasion in several cancers [12,13]. However, the abnormalities of *EZH2* and its underlying mechanisms in the pathogenesis of CRC are not fully understood. Four lines of argument suggest that *EZH2* plays an important role in CRC cell motility and metastasis. First, the expression of *EZH2* was significantly increased in CRC cancer tissues and cell lines; second, overexpression of *EZH2* in CRC tissues may increase the aggressiveness of the tumour; third, down-regulation of *EZH2* significantly decreased the cell invasive ability and secretion of MMP2 and MMP9 in CRC cells; and finally, overexpression of *EZH2* markedly increased the metastatic ability of CRC cells

and decreased overall survival in an *in vivo* model of CRC metastasis. Conversely, down-regulation of *EZH2* significantly decreased the metastatic ability of CRC cells and increased overall survival *in vivo*.

Overexpression of *EZH2* has been shown to down-regulate the expression of E-cadherin by increasing the H3K27 trimethylation level at the *E-cadherin* promoter in prostate cancer cells [35]. An inverse correlation has been demonstrated between *EZH2* and runt-related transcription factor 3 (*RUNX3*) gene expression in gastric, breast, prostate, colon, and pancreatic cancer cell lines, and *EZH2* may bind to the *RUNX3* gene promoter and trimethylate the H3K27 mark [40]. *EZH2* has also been reported to regulate cell migration/invasion via the regulation of E-cadherin and transforming growth factor beta 1 (TGF- β 1) expression in ovarian carcinoma [33]. However, the molecular mechanisms by which *EZH2* regulates CRC cell migration/invasion remain unclear. Vitamin D and VDR appear to mediate the prevention of tumour development by inducing cellular differentiation and inhibiting proliferation [41–43]. The expression of VDR is repressed in high-grade and metastatic colorectal cancer [44]. We found that the mRNA and protein levels of VDR were significantly up-regulated by knock-down of *EZH2* expression in CRC cells (Figure 3). In luciferase assays, the transcriptional activity of the VDR promoter was dramatically increased by down-regulating *EZH2* (Figures 4A–4C). These data indicate that *EZH2* may mediate VDR expression by regulating the transcriptional activity of the VDR promoter in CRC cells. Our result is consistent with the report that *EZH2* was predicted to regulate VDR gene expression in germinal centre B cells [29]. Previous immunohistochemical studies have shown a reduction in the level of VDR in CRC samples [14,20,45]. We found that high *EZH2* expression in human clinical specimens correlated with low expression of VDR (Spearman's correlation coefficient $r = -0.65720$; $p < 0.0001$; Figure 4D). Furthermore, *EZH2*-mediated CRC cell invasion depended on the down-regulation of VDR (Figures 3E and 3F). Down-regulation of VDR due to overexpression of *EZH2* may present one pathway whereby *EZH2* affects CRC cell invasion.

Previously we reported that the activation of STAT3 may mediate human CRC tumorigenesis and invasion [27], and STAT3 may directly induce cell invasion and participate in the development of resistance to chemotherapy and apoptosis during the epithelial–mesenchymal transition (EMT) stage of CRC progression [46]. As *EZH2* was identified to participate in the invasion of CRC cells in the present study, we further examined whether STAT3 mediates the expression of *EZH2* in CRC cells. We found that inhibition of STAT3 expression dramatically decreased *EZH2* expression and increased VDR expression in CRC cells (Figures 5A and 5B). In addition, overexpression of STAT3 significantly increased *EZH2* expression and decreased VDR expression in CRC cells (Figures 5C and 5D). These data demonstrate

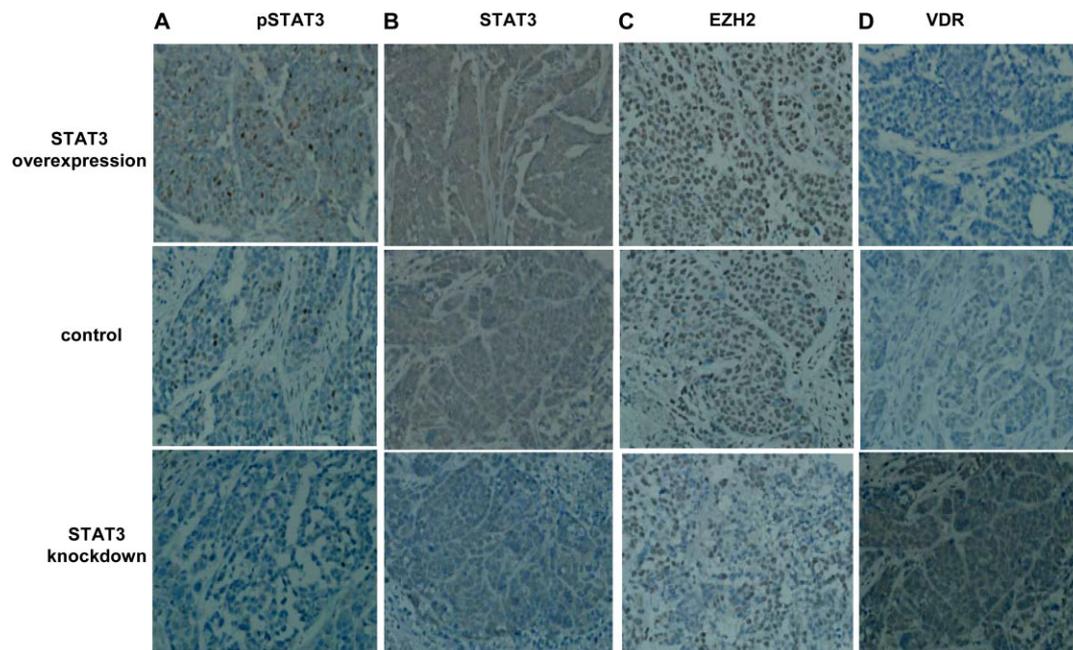


Figure 7. Expression levels of pSTAT3, STAT3, *EZH2*, and VDR in a nude mouse xenograft model of CRC. Immunohistochemical analysis of pSTAT3, STAT3, *EZH2*, and VDR in xenograft tissue sections. STAT3 was successfully up-regulated or down-regulated after introducing STAT3 shRNA and the STAT3 overexpression virus into nude mouse CRC cell model, respectively (A, B). The level of pSTAT3 and the expression of *EZH2* were significantly up-regulated in tumour samples with STAT3 overexpression (C), while the levels of pSTAT3 and *EZH2* expression were significantly down-regulated in STAT3-knockdown tumour samples. The expression of VDR displayed an opposite trend (D). (A–D) Original magnification: $\times 400$.

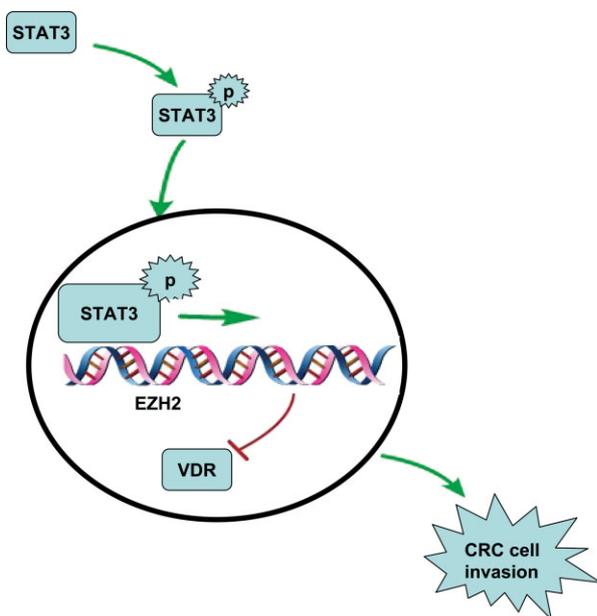


Figure 8. Model for the possible mechanism of *EZH2*-induced invasion in CRC and the regulatory mechanism of *EZH2*. Activation of STAT3 in CRC cells leads to overexpression of *EZH2*, which results in decreased expression of VDR and increased metastatic ability in CRC cells.

that STAT3 may regulate *EZH2* and VDR expression in CRC cells. Moreover, *EZH2* may play an important role in STAT3-induced VDR down-regulation and invasion in CRC cells (Figures 5E, 5F, and 6F). This finding is consistent with bioinformatics predictions that STAT3 may regulate *EZH2* expression in prostate

cancer cells [28]. Furthermore, a positive transcriptional effect of STAT3 binding the *EZH2* promoter was shown in this study via luciferase and ChIP assays (Figures 6A–6E). However, VDR DNA was not detected in the ChIP assay using an antibody against STAT3 (data not shown), which suggests that STAT3 may directly bind to the *EZH2* promoter, but not to the VDR promoter.

Our immunohistochemical data showed that pSTAT3 and *EZH2* expression correlated positively with metastasis in human CRC, compared with control (Figure 6G and Table 1). In contrast, VDR expression was negatively associated with metastasis in human CRC (Figure 6G and Table 1). Moreover, a strong correlation between human CRC metastasis and *EZH2* expression was illustrated in the multivariate analyses. Highly inverse correlations between the expression of *EZH2* and pSTAT3 and that of VDR were also revealed in the animal model.

In conclusion, we found that *EZH2* may play an important role in the process of tumour invasion in CRC. Overexpression of *EZH2* may result in H3K27 trimethylation of the VDR gene promoter, indicating that VDR is a novel *EZH2* target gene. *EZH2* contributes to CRC cell invasion by suppressing VDR expression. Moreover, *EZH2* overexpression can be induced by activated STAT3. *EZH2* may mediate the STAT3-induced down-regulation of VDR and promote cell invasion in CRC (Figure 8). These findings may provide a potential target for treating aggressive colorectal cancers by inhibiting *EZH2*.

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Author contribution statement

Y-W Lin and L-L Ren performed all the experiments, analysed data, and wrote the manuscript. H Xiong, W Du, D-F Sun, Y-N Yu, T-T Sun, Y-R Weng, Y-C Wang, Y Cui, J-L Wang, and J Xu performed the experiments. Z-h Wang and H-y Chen analysed data. Z-G Han and N Shen provided technical support in the *in vivo* experiments. W Zou designed the experiments. J Hong and W Cao conceived and supervised the study, and wrote the manuscript. J-Y Fang designed and supervised this project and revised the manuscript.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary methods.

Figure S1. (A) Western blot analysis data show that introduction of lenti-EZH2 shRNA-1/2 virus successfully decreased EZH2 expression in HCT116 CRC cells. (B) Western blot analysis data show that transduction of lenti-EZH2 overexpression virus remarkably increased the expression of EZH2 in SW1116 CRC cells.

Figure S2. The concentrations of MMP2 and MMP9 were tested by ELISA in control or EZH2 shRNA virus-infected CRC cells.

Figure S3. Deletion variants of the EZH2 promoter pGL3-1220, 1020, 820, 620, 420, 220, and 20 were derived from pGL3-1220.

Table S1. Clinicopathological characteristics of EZH2 expression in patients with CRC.

Table S2. Haematoxylin and eosin staining of lungs to evaluate the presence of lung metastasis in nude mice.

Table S3. Clinicopathological characteristics of pSTAT3 expression in patients with CRC.

Table S4. Clinicopathological characteristics of VDR expression in patients with CRC.

Table S5. Correlation among the expression of EZH2, pSTAT3 or VDR in colorectal cancers.

Table S6. Immunohistochemical staining of 27 cases of subcutaneously transplanted tumour in nude mice to evaluate the expression of pSTAT3, STAT3, EZH2, and VDR.