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Original Article

ZEB1 promotes DNA homologous recombination repair and contributes to the 5-Fluorouracil resistance in colorectal cancer

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Abstract: Chemotherapy resistance represents a significant obstacle in clinical practice of colorectal cancer (CRC). In this study we aim to clarify the underlying mechanism of chemotherapy resistance mediated by ZEB1 in CRC. shRNA-mediated repression of ZEB1 induced DNA damage in SW480 and RKO cells. Ectopic expression of ZEB1 suppressed the DNA damage caused by *ZEB1* knocking down in SW480 and RKO cells. In addition, ZEB1 directly targeted several DNA damage response (DDR) factors including NBS1, RNF8 and RNF168, and thereby the homologous recombination (HR) repair is mediated by ZEB1 via NBS1, RNF8 and RNF168 in CRC cells. Furthermore, ZEB1 maintained chromosome stability in CRC cells. By inducing NBS1, RNF8 and RNF168, ZEB1 is capable of promoting the 5-Fluorouracil (5-FU) resistance in CRC cells via enhancing the DDR signaling and DNA repair. The high expression of *ZEB1*, *NBS1*, *RNF8* and *RNF168* is associated with chemotherapy resistance in primary CRC patients. In conclusion, ZEB1 directly induces the expression of NBS1, RNF8 and RNF168, and thereby enhances DNA HR repair in CRC. The ZEB1-mediated DNA repair contributes to the 5-FU resistance in CRC.

Keywords: ZEB1, NBS1, RNF8, RNF168, colorectal cancer, DNA repair, chemotherapy resistance

Introduction

Colorectal cancer (CRC) remains a major contributor to global mortality rates related to cancers [1]. The 5-year survival rate in patients diagnosed with early CRC is over 90%, but the rate drops below 53% in patients with locally advanced CRC (stage IIIC) and to 12% in patients diagnosed with metastatic CRC (mCRC) or stage IV CRC [2]. Unfortunately, most CRC patients are diagnosed at advanced stages, which largely limits treatment options. Recently, chemotherapy, molecularly targeted therapy and immunotherapy have shown considerable promise to improve CRC treatment outcomes. However, drug resistance represents a significant obstacle in clinical practice [3]. Some patients are unresponsive to treatments or develop primary/secondary resistance, which poses significant challenges for effective cancer management.

Epithelial-to-mesenchymal transition (EMT) is a critical determinant of the chemotherapy response in cancer cells [4, 5]. Zinc finger E-box binding homeobox 1 (ZEB1) is a key transcription factor that promotes EMT. ZEB1 is involved in the regulation of DNA damage response (DDR), cancer cell differentiation, and metastasis [6]. Recent researches have highlighted the upregulation of ZEB1 as a potential mediator of chemoresistance [7, 8]. It has been postulated that cancer cells undergoing EMT acquire properties similar to those of cancer stem cells (CSCs), such as enhanced DDR and DNA repair capabilities, self-renewal, and chemoresistance [6]. Functional studies have demonstrated that ZEB1 can induce chemoresistance irrespective

of other EMT-related changes [6]. Additionally, ZEB1 is known to regulate DDR by forming a complex with p300/PCAF and through direct interaction with ATM [9-11], a kinase associated with radio-resistance. ATM can also phosphorylate ZEB1, and thereby enhances ZEB1 stability [12]. Furthermore, downregulation of ZEB1 reduces the abundance of ATR-activated CHK1 [12], an effector kinase of DDR.

However, it is still unclear the molecular mechanism that ZEB1 regulates the DNA damage response and subsequent DNA repair. In this study, we could show that ZEB1 enhances homologous recombination (HR) repair by inducing the expression of DDR regulators/factors, and thereby ZEB1 contributes to the chemoresistance in colorectal cancer cells.

Methods and materials

Cells and treatments

The CRC cell lines SW480 and RKO were incubated in Dulbecco's Modified Eagle's High Glucose Medium (DMEM, Gibco, USA) with 5% fetal bovine serum (FBS, Gibco, USA). All cells grew as monolayers at 37°C with 5% CO_2. Cells were transfected with vectors or siRNAs by using Lipofectamine 2000 (Invitrogen, USA) according to the protocol provided by the manufacturer. The final concentration of transfection for vectors was 1 μg/ml and the final concentration of siRNAs was 12.5 nM. The ectopic ZEB1 was achieved by introducing cells with a pcDNA3.1 vector containing the CDS region of *ZEB1* (pcD-ZEB1). The pcD-ZEB1 vector was obtained from KamBio (Shanghai, China). The specific siRNA pools targeting *NBS1*, *RNF8* and *RNF168* were obtained from Qiagen. Each siRNA pools contained four siRNAs targeting different sites to avoid off-target effect. For 5-Fluorouracil (5-FU, Sigma) treatment, cells were treated with different concentrations ranging from 0 μM to 15 μM.

Generation of ZEB1 knockdown CRC cells

To achieve ZEB1 suppression in SW480 and RKO cells, we transfected a specific ZEB1 shRNA vector into cells and obtained cells stably expressing low ZEB1 as previously described [13], thereafter the ZEB1-knocking down cells were named as $SW480^{KD}$ and RKO^{KD}. In general, the shZEB1 vector and packaging plasmid mix were co-transfected into HEK-293T cells by using Lipofectamine 2000. After 48 hours, the lentiviral particles were harvested. SW480 and RKO cells were infected with the virus at the recommended amount. Polybrene (Merck, TR-1003-G, final concentration was 10 μg/ml) was used to improve the efficiency of the lentiviral transduction. After 48 hours, stable cells were selected by treating infected cells with G418 (Sigma, PP2374-1KT). Western blot was used to validate the efficiency of ZEB1 knocking down in $SW480^{KD}$ and RKO^{KD} cells.

RNA isolation and quantitative PCR (q-PCR) reaction

Following different treatments, the total RNAs of treated cells were isolated and purified by using QIAzol Lysis Reagent (Qiagen) according to the provided instructions. For each sample, 500 ng of RNA were used for the first strand cDNA synthesis by using IScript cDNA Synthesis Kit (Bio-Rad) with Oligo (dT) primers. The relative gene expression was quantified by q-PCR performed on the platform of ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). *GAPDH* served as an internal control. The relative gene expression was calculated by 2^(ΔΔCT) as described previously [14]. The primers used in q-PCR were listed in Table S1.

Protein isolation and western blot analysis

The protein of cells was harvested in 80 μl of RIPA lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM phenylmethyl sulfonyl fluoride) following the different treatments. The lysate was centrifuged at 12,000 g for 20 min at 4°C after sonicated 2 times for 5 seconds each. The protein in the supernatant was collected and subjected to quantification by a BCA kit (ThermoFisher, USA) according to the provided protocol. For each sample, 30 μg of protein were loaded and subjected to the separation in 10% SDS-PAGE. The separated protein was transferred to 0.45 μm PVDF membrane (Millipore, USA). The membrane was blocked by 5% skim milk dissolved in TBST buffer (20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20, pH 7.5) for 30 min at room tempera-

ture. After blocking, membrane was incubated with the primary antibody at 4°C overnight. Next, membrane was incubated with the secondary antibody at room temperature for 1 hour. The protein on the membrane was imaged by using the enhanced chemo luminescence (ECL) reagents. The information of antibodies was listed in the Table S2.

Immunofluorescence

For immunofluorescence, a round glass was inserted into the 6-well plate. Next, cells were seeded into the round glass-coated 6-well plate at the density of 2×10^5 cells/well for incubation. After different treatments, the medium was discarded and cells were washed twice with PBS buffer. Then cells were fixed by 800 μl of methanol for 20 min at room temperature. After fixation, cells were penetrated by 0.1% Triton X-100 dissolved in PBS for 5 min at room temperature. Cells were subsequently blocked by 1% BSA PBS buffer for 30 min at room temperature, and then subjected to the incubation of primary antibody at 4°C overnight in the dark. Then cells were incubated with the secondary antibody for 1 hour at room temperature in the dark. Finally, cells were incubated with 1 μg/ml of DAPI for 1 min at room temperature in the dark. After incubation. The glass containing cells were embedded on a decker glass with antifade reagent (Invitrogen, Prolong Gold). The immunofluorescence was observed and recorded by Olympus IX70 system with 63 × oil lance. The information of antibodies was listed in the Table S3.

Comet assay

The DNA damage was evaluated by comet assay as described previously [15]. Briefly, after different treatments, cells were collected and seeded on the agarose-coated slide. Cells were then treated with the provided lysis buffer, and cells were subjected to the electrophoresis in alkaline buffer (pH = 10.0) for 30 min at 24 V voltage. After electrophoresis, the slide was neutralized with a neutralization buffer and then the cells were stained by fluorescent dye binding to DNA.

Homologous recombination (HR) repair assay

The dual HR reporter assay was conducted according to the previously published method [16]. In brief, the specified cells were seeded in 6-well plates at 50% confluence one day prior to transfection. Transfection was performed using 500 ng of pCBA I-SceI plasmid (Addgene) and 500 ng of pDR-GFP plasmid (Addgene). After 72-hour incubation, the transfected cells were harvested and the expression of GFP was measured using the BD FACSCanto II Flow Cytometer.

Chromosome spreading and enumeration

Mitotic chromosome spreads were prepared following the protocol described elsewhere [17], and 100 spreads per experimental condition were meticulously examined to enumerate the chromosomes manually.

q-ChIP

SW480 cells were subjected to qChIP analysis following the protocol described previously. To initiate cross-linking, formaldehyde (37%) was added to the culture medium. The final concentration of formaldehyde in the medium was 1%. Cross-linking was proceed at room temperature for 10 minutes and was subsequently quenched by the addition of glycine (0.125 M final concentration). The fixed cells were then washed twice with PBS and collected in SDS buffer (50 mM Tris at pH 8.1, 0.5% SDS, 100 mM NaCl, 5 mM EDTA, and protease inhibitors). After centrifugation, the cells were resuspended in 3 ml of IP buffer (100 mM Tris at pH 8.6, 0.3% SDS, 1.7% Triton X-100, and 5 mM EDTA). Cell disruption was achieved by subjecting the cells to 5-7 cycles of sonication (30 seconds each) using a tapered 1/4-microtip (6.5 mm) in a Branson digital sonifier 250 D, operating at 30% power. This yielded genomic DNA fragments ranging in size from 300 to 500 bp. For each immunoprecipitation, 1 ml of diluted lysate (5×10^7 cells/ml) was precleared by adding 300 μl of blocked protein A or G beads (50% slurry of protein A-Sepharose from Amersham or Rec-protein G-Sepharose 4B ZYMED from Invitrogen) in the presence of 0.5 mg/ml fatty acid-free BSA (Sigma) and 0.2 mg/ml salmon sperm DNA in TE. The samples were subjected to overnight immunoprecipitation at 4°C using antibodies specific to the target factors or modified histones. The immune complexes were recovered by adding 50 μl of blocked beads and incubating for 2 hours at 4°C. The beads were then washed, and cross-links were

reversed using 1% SDS and 0.1 M $NaHCO_3$. The eluted material was purified using the QIAquick PCR purification Kit (Qiagen). The resulting DNA was resuspended in 1 ml of T buffer (10 mM Tris-HCl at pH 8) and directly used for qPCR. The information of primers used in q-ChIP was listed in Table S3.

Cell proliferation assay

Cell proliferation was evaluated in triplicate by using a CCK8 kit (Bio-Rad Laboratories, Inc.) following the instructions provided by the manufacturer. Cells (2×10^3) suspended in 200 μl of medium were seeded into a 96-well plate and cultured at 37°C. After incubation at 37°C for 2 hours, the original medium was replaced with medium containing 10% CCK8 solution. The absorbance was measured at 450 nm using a microplate reader.

Bioinformatics analysis

The clinical data was obtained from TCGA data portal (tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp) and NCBI GEO (GSE17538). The ChIP-Seq data was obtained from Cistrome DB (http://cistrome.org/db/#/) and presented with UCSC genome browser.

Statistics

Data analysis was performed using GraphPad Prism 9. The t-test and one-way ANOVA were employed to compare quantitative data for group pairs and multiple groups, respectively. In situations where unequal variance was observed, the rank-sum test was utilized. The chi-square test was used to assess categorical data. A *p*-value of less than 0.05 was considered statistically significant.

Results

Stable repression of ZEB1 induces DNA damage in colorectal cancer cells

In order to characterize the function of ZEB1 in DNA damage, we stably knocked down *ZEB1* in SW480 and RKO cells by introducing a specific *ZEB1* shRNA vector. The efficiency of *ZEB1* knockdown was validated by western blot (**Figure 1A**), and the *ZEB1* knockdown cells were named as $SW480^{KD}$ and RKO^{KD} cells. The spontaneous DNA damage was induced in the $SW480^{KD}$ cells compared to the *ZEB1* proficient SW480 cells, which was represented by the extended DNA tail in $SW480^{KD}$ cells (**Figure 1B**) and the increased frequency of γH2AX foci in $SW480^{KD}$ cells (**Figure 1C**). Similar results were obtained in RKO^{KD} cells (**Figure 1B** and **1C**). Therefore, the ZEB1-repression results in promoted DNA damage in CRC cell lines. Interestingly, significant less γH2AX foci were observed when ZEB1 expression was rescued in $SW480^{KD}$ and RKO^{KD} cells (**Figure 1D**), indicating that ectopic ZEB1 prevents the DNA damage. Since the proliferation repression is a known result of DNA damage [18], here we evaluated the proliferation capability of CRC cell lines with different ZEB1 expression status. Loss of *ZEB1* suppressed the proliferation of SW480 and RKO cells (**Figure 1E**). Conversely, ectopic ZEB1 induced the proliferation capability of $SW480^{KD}$ and RKO^{KD} cells (**Figure 1E**). Taken together, stable repression of ZEB1 by shRNA induces DNA damage and suppresses the proliferation of CRC cell lines.

ZEB1 enhances HR by inducing DNA repair factors

Since *ZEB1*-repression caused the increased γH2AX foci, indicating accumulated DNA double strand breaks (DSBs) in cells [19], next we asked whether ZEB1 regulates HR repair that is responsible for the DSBs [20]. To this end, we evaluated the efficiency of HR via a dual reporter assay. Knocking down of *ZEB1* repressed the HR-mediated DNA repair, which was evidenced by the decreased number of GFP positive cells (**Figure 2A**). Conversely, ectopic ZEB1 enhanced the HR-mediated DNA repair in $SW480^{KD}$ and RKO^{KD} cells (**Figure 2B**). Therefore, ZEB1 represses DNA damage via enhancing HR-mediated DNA repair.

Next, we demonstrated the mechanism underlying the ZEB1-associated DNA damage response regulation. To determine the potential ZEB1-associated regulators of DNA damage response, we screened the regulators by ZEB1 ChIP-Seq analysis via online bioinformatics tool. *NBS1*, a component of MRN complex [21], appeared to be a direct target of ZEB1 (**Figure 2C**). In addition, *RNF8* and *RNF168*, two components of ATM pathway that amplifies the DNA damage response signal [22], were also potential targets of ZEB1 (**Figure 2C**). Indeed, q-ChIP

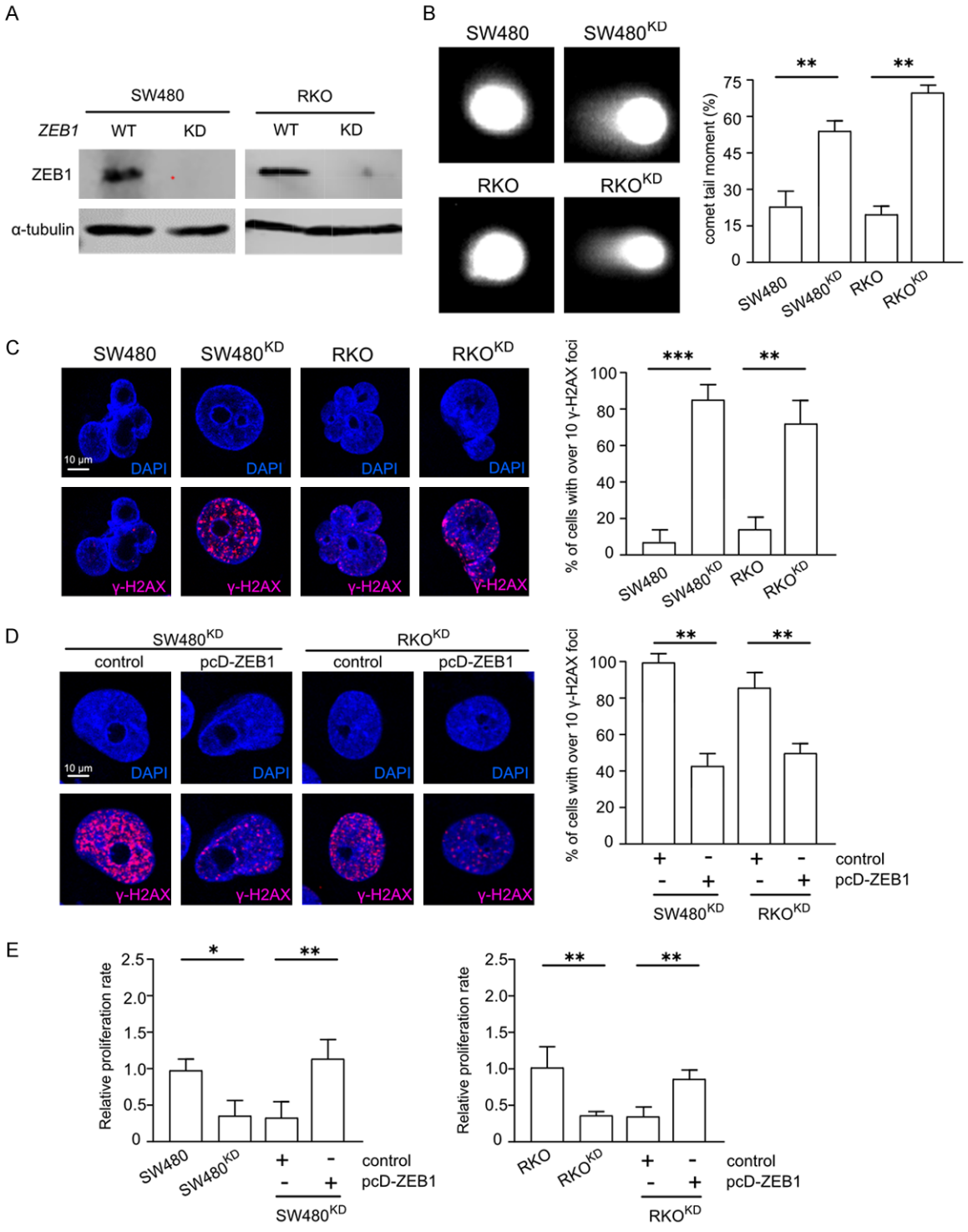

Figure 1. Repression of ZEB1 induces DNA damage in colorectal cancer cells. A. The efficiency of ZEB1 shRNA was validated by western blot analysis (n = 3). B. DNA damage was evaluated by comet assay in SW480, RKO, SW480^{KD} and RKO^{KD} cells (n = 10). C. DNA damage was evaluated by immunofluorescence on γ-H2AX in SW480, RKO, SW480^{KD} and RKO^{KD} cells. The cells with over 10 γ-H2AX foci were considered as positive (n = 100), scale bar: 10 μm. D. DNA damage was evaluated by immunofluorescence on γ-H2AX in SW480^{KD} and RKO^{KD} cells following the ZEB1 ectopic expression by pcD-ZEB1 vector transfection 48 hours. The cells with over 10 γ-H2AX foci were considered as positive (n = 100), scale bar: 10 μm. E. The proliferation was determined by CCK8 assay in the indicated cells and in the cells with the indicated treatments (n = 3). The proliferation rates were normalized to the result of SW480 or RKO group respectively. *P < 0.05, **P < 0.01, ***P < 0.001.

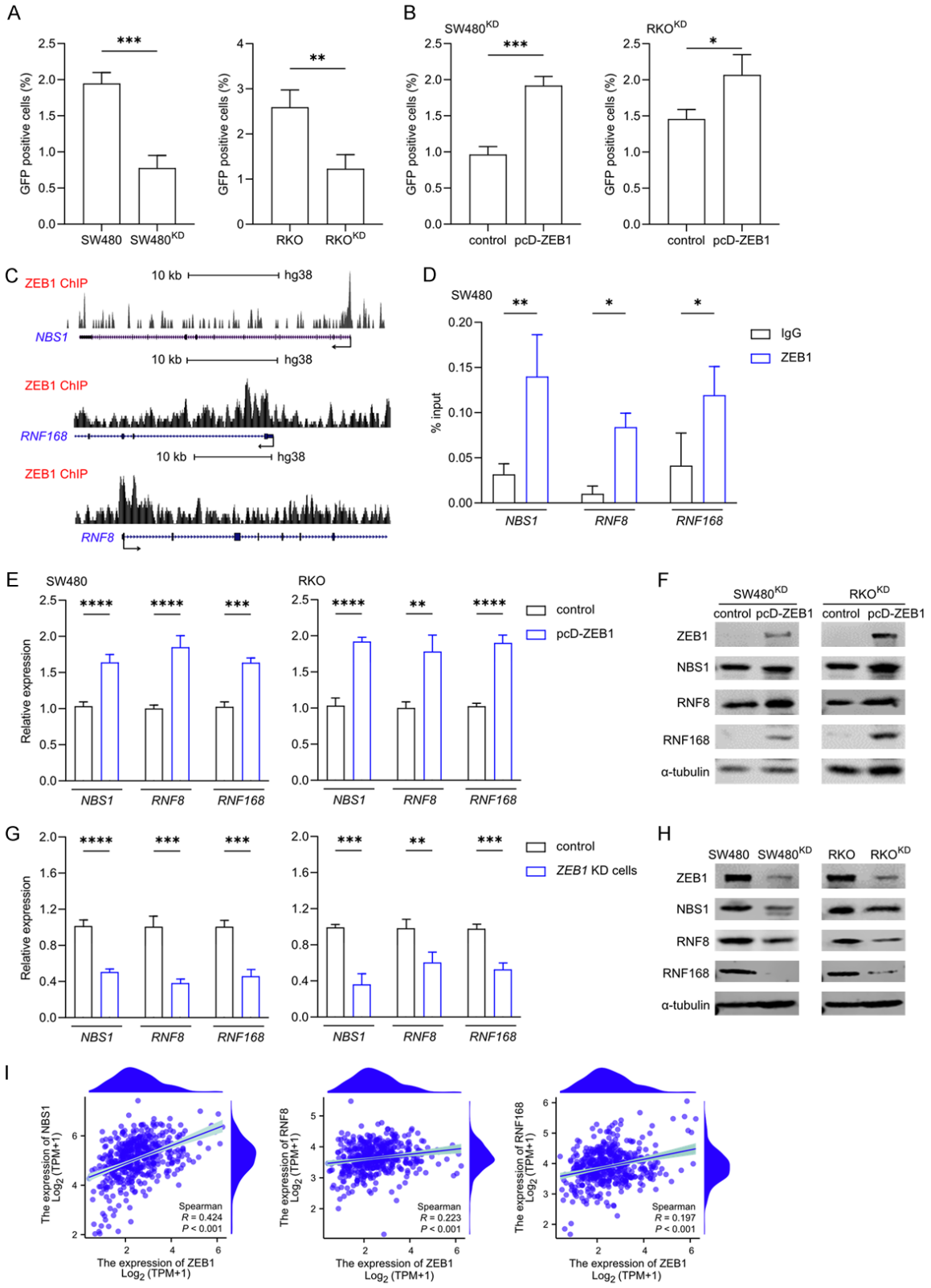

Figure 2. ZEB1 enhances DNA repair. A. The efficiency of HR was determined with the percentage of GFP positive cells by flow cytometry in SW480/SW480^{KD} cells (left panel) and RKO/RKO^{KD} cells (right panel) (n = 3). B. The efficiency of HR was determined with the percentage of GFP positive cells by flow cytometry in SW480^{KD} cells and RKO^{KD} cells following the transfection of pcD-ZEB1 for 48 hours (n = 3). C. The scheme of the indicated genes in the

ZEB1 ChIP-Seq analysis. There are ZEB1 ChIP peaks located in the region of transcription start site. D. The targets of ZEB1 were validated by q-ChIP analysis performed in SW480 cells (n = 3). E. The expression of NBS1, RNF8 and RNF168 was determined by q-PCR following the ectopic expression of ZEB1 for 48 hours in SW480 cells (left panel) and RKO cells (right panel) (n = 3). F. The expression of NBS1, RNF8 and RNF168 was determined by western blot following the ectopic expression of ZEB1 for 48 hours in $SW480^{KD}$ cells and RKO^{KD} cells (n = 3). G. The expression of NBS1, RNF8 and RNF168 was determined by q-PCR in SW480/$SW480^{KD}$ cells (left panel) and RKO/RKO^{KD} cells (right panel) (n = 3). H. The expression of NBS1, RNF8 and RNF168 was determined by western blot in SW480/$SW480^{KD}$ cells and RKO/RKO^{KD} cells (n = 3). I. The expression correlation between ZEB1 and its target genes was analyzed in TCGA-COAD patient cohorts. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

analysis validated that *NBS1*, *RNF8* and *RNF168* are direct targets of ZEB1 (**Figure 2D**). Ectopic ZEB1 induced the expression of *NBS1*, *RNF8* and *RNF168* at mRNA level in SW480 and RKO cells (**Figure 2E**). Rescuing of ZEB1 expression also induced NBS1, RNF8 and RNF168 in $SW480^{KD}$ and RKO^{KD} cells (**Figure 2F**). The basal expression of NBS1, RNF8 and RNF168 were repressed in $SW480^{KD}$ and RKO^{KD} cells (**Figure 2G** and **2H**). Furthermore, the expression of ZEB1 is positively associated with the expression of NBS1, RNF8 and RNF168 in colorectal cancer patient cohorts (**Figure 2I**). Therefore, ZEB1 functions as a transcription factor and induces the expression of NBS1, RNF8 and RNF168, which contributes to the transduction of DNA damage response signals.

NBS1, RNF8 and RNF168 mediate the function of ZEB1

Since we had identified that *NBS1*, *RNF8* and *RNF168* are direct targets of ZEB1, next we determined how these regulators of DNA damage response mediate the function of ZEB1 in DNA repair. To this end, we employed specific siRNA pools against *NBS1*, *RNF8* or *RNF168* to repress their expressions in SW480 and RKO cells (**Figure 3A**). The ZEB1-induced HR was repressed by silencing NBS1, RNF8 or RNF168 in SW480 and RKO cells (**Figure 3B**). Interestingly, repression of *ZEB1* increased the frequency of abnormal chromosome in SW480 and RKO cells compared to *ZEB1* proficient cells (**Figure 3C**). Ectopic expression of ZEB1 inhibited the DNA damage in $SW480^{KD}$ and RKO^{KD} cells. However, the effect of ectopic ZEB1 was largely abrogated by silencing NBS1, RNF8 or RNF168 in $SW480^{KD}$ and RKO^{KD} cells (**Figure 3D**). The above results indicate that ZEB1 maintains genome stability. NBS1, RNF8 and RNF168 mediate the function of ZEB1 in inducing DNA repair. The proliferation of SW480 and RKO cells was significantly induced by ectopic ZEB1. However, the effect of ZEB1 was repressed by silencing NBS1, RNF8 or RNF168 (**Figure 3E**). Taken together, ZEB1 enhances HR via inducing NBS1, RNF8 and RNF168.

Regulation of NBS1, RNF8/168 by ZEB1 in mice

Next, we validated the conservation of the ZEB1-associated regulation in mice. The previous ChIP-Seq data performed in mice indicated that *Nbs1*, *Rnf8* and *Rnf168* may serve as conserved targets of Zeb1 in mice (**Figure 4A**). To confirm the targets in mice, we performed qChIP analysis in primary colonic fibroblasts derived from C57BL/6 mice. Indeed, *Nbs1*, *Rnf8* and *Rnf168* were enriched in Zeb1 ChIP in primary colonic fibroblasts (**Figure 4B**). In addition, the expression of *Nbs1*, *Rnf8* and *Rnf168* was induced by ectopic Zeb1 in primary colonic fibroblasts (**Figure 4C**), whereas silencing of Zeb1 repressed the expression of *Nbs1*, *Rnf8* and *Rnf168* (**Figure 4D**). Therefore, *Nbs1*, *Rnf8* and *Rnf168* are conserved ZEB1 targets in mice. Furthermore, we evaluated the effect of Zeb1 on DNA damage in the mouse primary colonic fibroblasts. The DNA damage was induced by the irradiation (IR) with 10 Gy for 3 hours in primary colonic fibroblasts. Ectopic expression of Zeb1 significantly reduced the DNA damage induced by IR (**Figure 4E**). However, silencing Nbs1, Rnf8 or Rnf168 largely abrogated the effect of Zeb1 (**Figure 4E**), indicating that the ZEB1-induced DNA repair is conserved in mice, which is mediated by Nbs1, Rnf8 and Rnf168.

ZEB1 modulates the 5-FU sensitivity in CRC cells

Previous studies have shown that the enhanced DNA damage response contributes to the chemotherapy resistance in colorectal cancer via keeping genome more tolerant to the chemi-

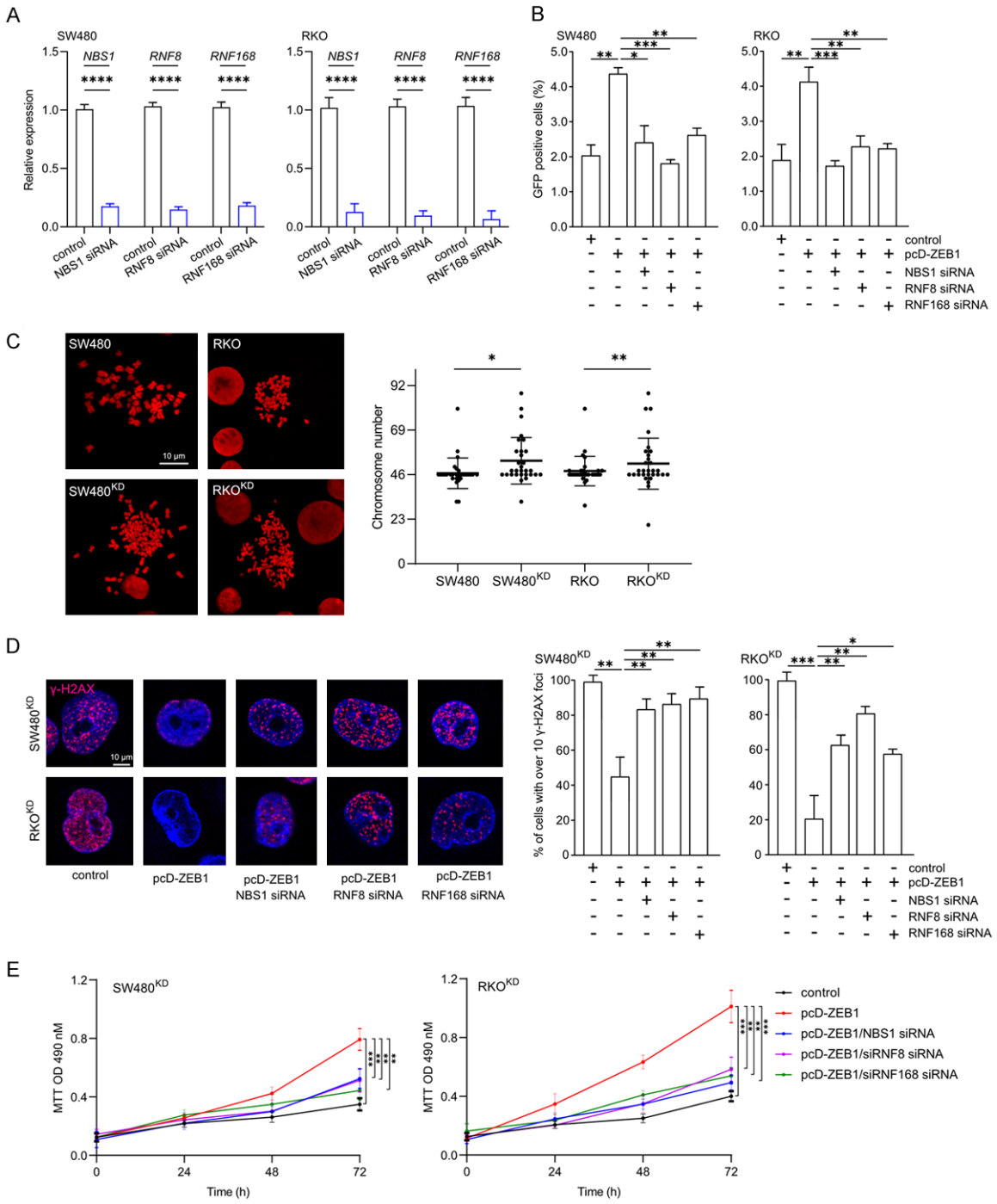

Figure 3. NBS1, RNF8 and RNF168 mediate the function of ZEB1. A. The efficiency of specific siRNAs of NBS1, RNF8 and RNF168 was evaluated by q-PCR in SW480 (left panel) and RKO cells (right panel) (n = 3). B. The efficiency of HR was determined with the percentage of GFP positive cells by flow cytometry in SW480 (left panel) and RKO cells (right panel) following the indicated transfections for 48 hours (n = 3). C. The chromosome enumeration in SW480/SW480^{KD} cells and RKO/RKO^{KD} cells. Total 30 of nuclei were counted for each group (n = 30), scale bar: 10 μm. D. DNA damage was evaluated by immunofluorescence on γ-H2AX in SW480^{KD} and RKO^{KD} cells following the ZEB1 ectopic expression and the indicated siRNAs transfection (n = 100), scale bar: 10 μm. E. The proliferation was determined by CCK8 assay in SW480^{KD} and RKO^{KD} cells with the indicated transfections (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

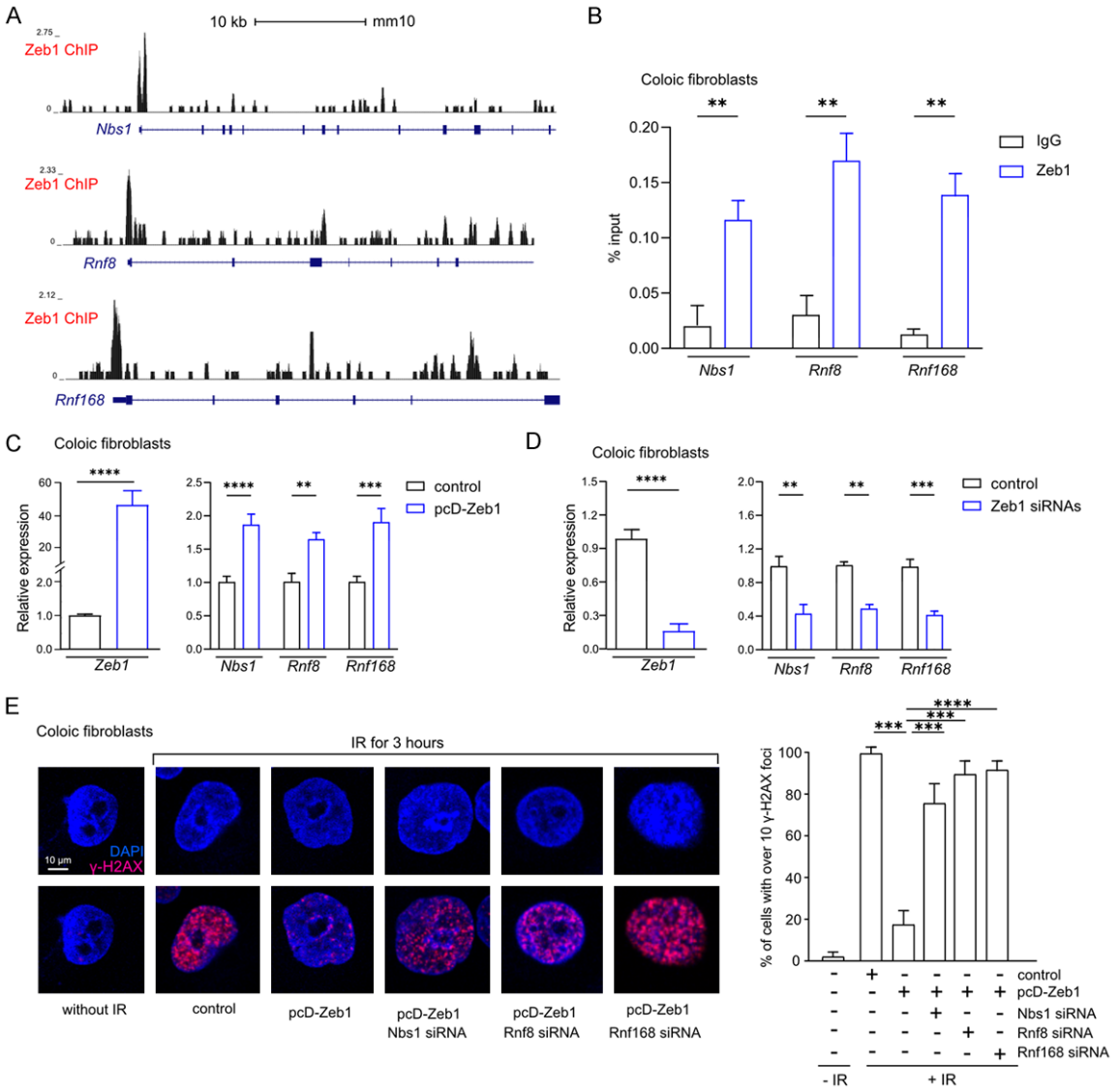

Figure 4. Regulation of Nbs1, Rnf8/168 by Zeb1 in colonic fibroblasts from mice. A. The scheme of the indicated genes in the Zeb1 ChIP-Seq analysis. There are Zeb1 ChIP peaks located in the region of transcription start site. B. The targets of Zeb1 were validated by q-ChIP analysis performed in C57BL/6 mouse primary colonic fibroblasts (n = 3). C. Left: the efficiency of pcD-Zeb1 transfection determined by q-PCR. Right: the expression of Nbs1, Rnf8 and Rnf168 was determined by q-PCR following the ectopic expression of Zeb1 for 48 hours in C57BL/6 mouse primary colonic fibroblasts (n = 3). D. Left: the efficiency of Zeb1 siRNA pools determined by qPCR. Right: the expression of Nbs1, Rnf8 and Rnf168 was determined by q-PCR following the repression of Zeb1 for 48 hours in C57BL/6 mouse primary colonic fibroblasts (n = 3). E. DNA damage was evaluated by immunofluorescence on γ-H2AX in C57BL/6 mouse primary colonic fibroblasts following the 10 Gy irradiation (IR) for 3 hours and the indicated transfections for 48 hours (n = 100), scale bar: 10 μm. **P < 0.01, ***P < 0.001, ****P < 0.0001.

cals such as 5-FU [23]. To determine the effect of ZEB1 on chemoresistance, we treated cells with 5-FU. The IC50 of 5-FU was higher in the *ZEB1* proficient SW480 and RKO cells compared to SW480^{KD} and RKO^{KD} cells (**Figure 5A**). Ectopic expression of ZEB1 induced the 5-FU resistance in SW480^{KD} and RKO^{KD} cells (**Figure 5B**). Therefore, ZEB1 is associated with the 5-FU resistance in colorectal cancer cells. Interestingly, silencing *NBS1*, *RNF8* or *RNF168* repressed the 5-FU resistance caused by ectopic ZEB1 in SW480^{KD} and RKO^{KD} cells (**Figure 5C**). The above results indicate that ZEB1 induces the 5-FU resistance via promoting the expression of NBS1, RNF8 and RNF168. In addition, ectopic expression of ZEB1 promoted

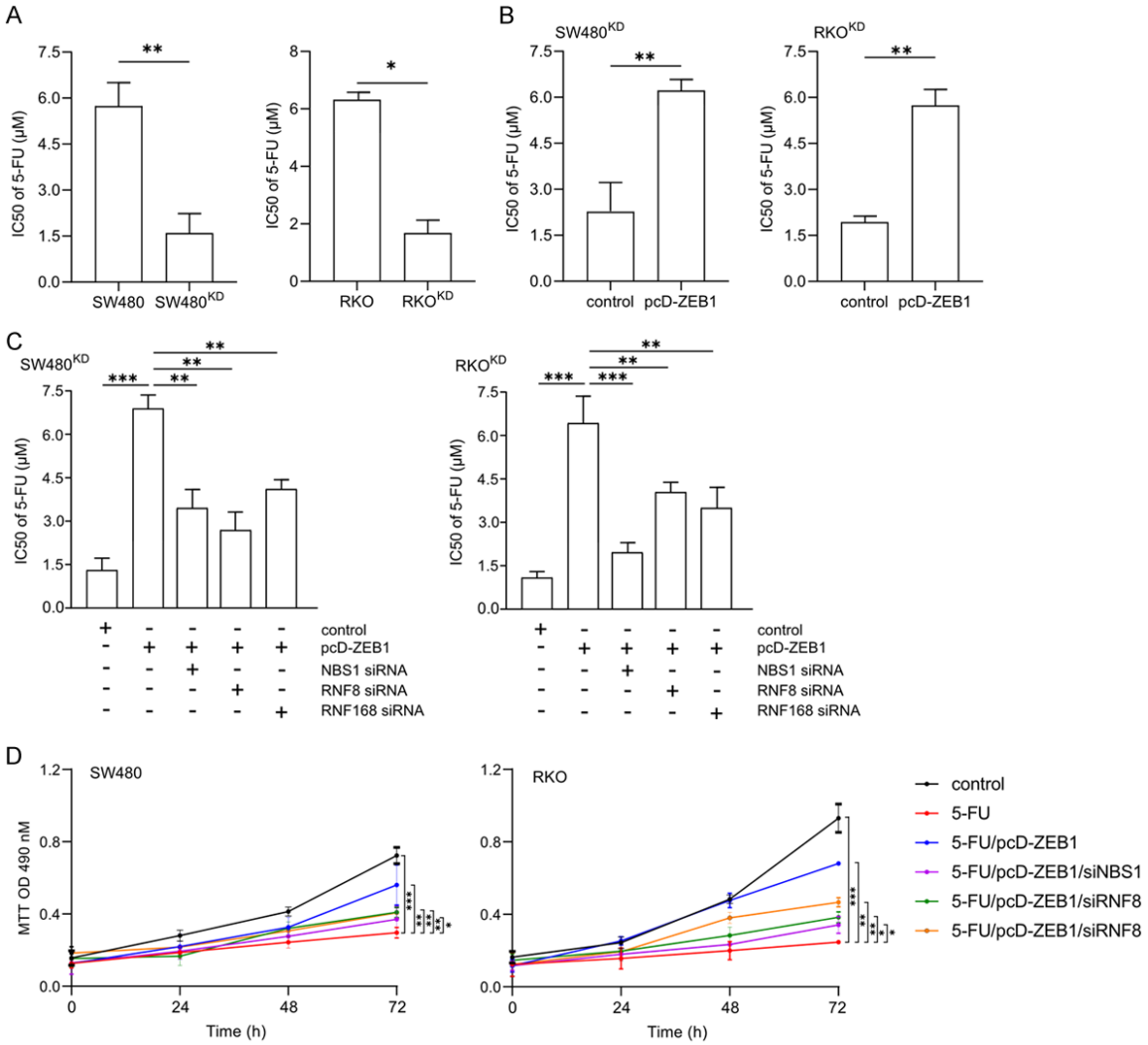

Figure 5. ZEB1 modulates the chemotherapy sensitivity. A. The IC50 of 5-FU was determined by CCK8 assay in SW480/SW480^{KD} cells (left panel) and RKO/RKO^{KD} cells (right panel) (n = 3). B. The IC50 of 5-FU was determined by CCK8 assay in SW480^{KD} cells (left panel) and RKO^{KD} cells (right panel) following the ectopic expression of ZEB1 for 48 hours (n = 3). C. The IC50 of 5-FU was determined by CCK8 assay in SW480^{KD} cells (left panel) and RKO^{KD} cells (right panel) following the ectopic expression of ZEB1 and the indicated siRNA transfections for 48 hours (n = 3). D. The proliferation was determined by CCK8 assay in SW480 (left panel) and RKO cells (right panel) with the indicated treatments (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001.

the proliferation of SW480 and RKO cells treated with 5-FU, whereas silencing of NBS1, RNF8 or RNF168 suppressed the proliferation induced by ZEB1 in the condition of 5-FU treatment (**Figure 5D**). In summary, ZEB1 constitutes the 5-FU resistance via enhancing DNA damage response, which is mediated by NBS1, RNF8 and RNF168.

Association of ZEB1, NBS1, RNF8 and RNF168 with chemotherapy effectiveness in CRC patients

Since we observed that ZEB1 is associated with 5-FU resistance in CRC cell lines, next we analyzed clinical data of CRC patients to reveal the associations of ZEB1, NBS1, RNF8 and RNF168 with the effectiveness of chemotherapy. By analyzing the data from TCGA-COAD, the high expression of *ZEB1* and *RNF168* indicated a tendency of poor overall survival rate in CRC patients (**Figure 6A**). However, the expression level of *NBS1* and *RNF8* is indifferent for the overall survival rate of CRC patients (**Figure 6A**). We also employed a patient cohort (GSE17538) containing 244 CRC patients to determine the association between the interested genes and the overall survival rate. In GSE17538, the high expression of *NBS1*, *RNF8*

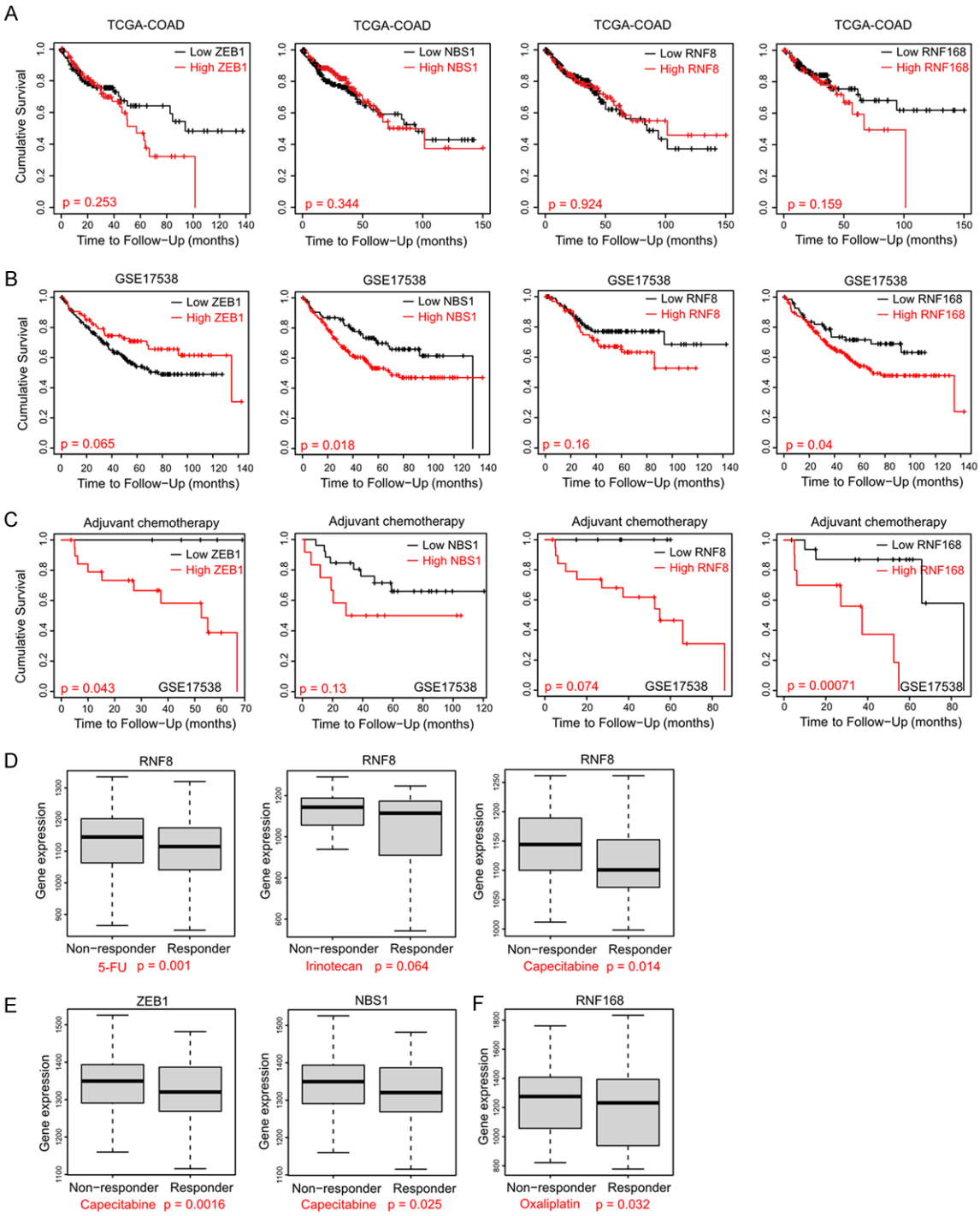

Figure 6. Association of *ZEB1*, *NBS1*, *RNF8* and *RNF168* with chemotherapy effectiveness in CRC patients. A. Associations of *ZEB1*, *NBS1*, *RNF8* and *RNF168* with survival in TCGA-COAD. B. Associations of *ZEB1*, *NBS1*, *RNF8* and *RNF168* with survival in GSE17538 datasets. C. Associations of *ZEB1*, *NBS1*, *RNF8* and *RNF168* with survival of patients received adjuvant chemotherapy in GSE17538 datasets. D. The expression of *RNF8* in non-responder and responder groups treated by 5-FU (n = 279 vs. 379), Irinotecan (n = 110 vs. 111) and Capecitabine (n = 112 vs. 110) respectively. E. The expression of *ZEB1* (n = 62 vs. 47) and *NBS1* (n = 62 vs. 47) in non-responder and responder groups treated by Capecitabine. F. The expression of RNF168 in non-responder (n = 173) and responder (n = 265) groups treated by Oxaliplatin.

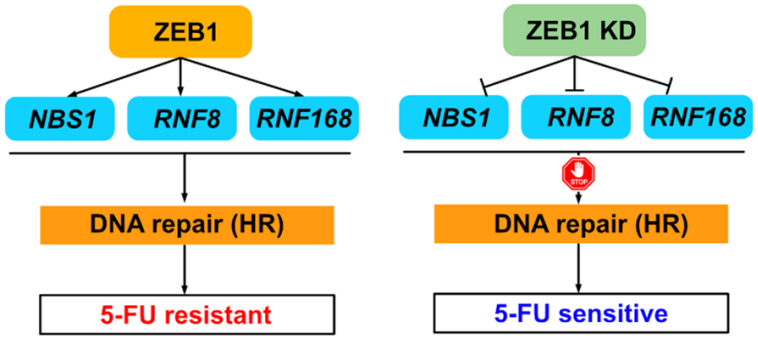

Figure 7. The regulation model of ZEB1 in the chemotherapy resistance of CRC. ZEB1 functions as a transcription factor that induces the expression of NBS1, RNF8 and RNF168, which subsequently promotes the HR and results in the 5-FU resistance in colorectal cancer.

and *RNF168* is associated with the poor overall survival rate (**Figure 6B**). Interestingly, low expression of ZEB1 is associated with poor overall survival rate in GSE17538 (**Figure 6B**). Next, we clarified the association of these genes with overall survival rate in the patients received adjuvant chemotherapy. High expression of *ZEB1*, *NBS1*, *RNF8* and *RNF168* is positively associated with the poor overall survival rate in patients received chemotherapy in GSE17538 (**Figure 6C**). The above results suggest that ZEB1, NBS1, RNF8 and RNF168 are potential biomarkers of the chemotherapy effectiveness/resistance in CRC. In addition, we explored the association of these genes with the responsibility of individual chemotherapy drugs used in CRC [24]. The expression of *RNF8* is up-regulated in the patients who are resistant to 5-FU, Irinotecan or Capecitabine (**Figure 6D**). High expression of *ZEB1* and *NBS1* was observed in Capecitabine-resistant patients (**Figure 6E**). The Oxaliplatin-resistant patients display a higher expression level of *RNF168* (**Figure 6F**). Therefore, the high expression of *ZEB1*, *NBS1*, *RNF8* and *RNF168* are associated with chemoresistance in CRC patients.

Discussion

In this study, we show that the EMT-TF ZEB1 is a regulator of DNA damage response (DDR). ZEB1 enhances the HR by inducing the expression of NBS1, RNF8 and RNF168, which subsequently contributes to the 5-FU resistance in CRC cells (summarized in **Figure 7**). Previous studies indicated that ZEB1 is required for DNA repair, and thereby restores the DNA integrity and maintains the genome stability [6]. Indeed, the frequency of abnormal chromosome was increased in SW480^{KD} and RKO^{KD} cells. ZEB1 is able to induce HR repair by stimulating the phosphorylation of CHK1 [12], a critical regulator and kinase in DDR. In line with the previous studies, the HR efficiency was elevated by ectopic ZEB1 in CRC cells in our study. In addition, the DDR factors including NBS1, RNF8 and RNF168 mediate the function of ZEB1 in HR. Therefore, ZEB1 controls DNA repair by activating the DNA damage response.

NBS1 is a member of MRN complex that is recruited to the DNA breaks flanked by γH2AX. MRN is capable of activating ATM via the direct interaction between ATM and NBS1 [25]. Previous study indicated that ATM stabilizes ZEB1 via repressing the ubiquitination of CHK1 [12]. ZEB1 maintains the activation of ATM by recruiting the transcriptional coactivator p300/PCAF to the ATM promoter [26]. Here, we showed that ZEB1, as transcription factor, directly induced the expression of NBS1. Therefore, overexpression of ZEB1 may constantly keep ATM activated during the DDR through the potential ZEB1-ATM positive feedback loop mediated by NBS1.

Following the recognition of γH2AX by MRN, RNF8 and RNF168 function as E3 ubiquitin ligases to ubiquitylate H2A and H2AX, and DDR factors, such as BRCA1, 53BP1 and MDC1, are subsequently recruited to MRN to amplify DDR signals [27-29]. In our study, *RNF8* and *RNF168* were clarified as direct targets of ZEB1, which implies that ZEB1 may induce the DDR signaling via promoting the recruitment of DDR factors to DSB sites. Indeed, silencing of RNF8 and RNF168 maintained the high frequency of γH2AX following IR treatments. The frequency of γH2AX foci was reduced rapidly with ectopic ZEB1 expression, suggesting that ZEB1 could activate DDR signaling, and thereby trigger DNA repair.

DDR and DNA repair maintain genome stability and integrity via correcting the impaired DNA [30]. Defection of DNA repair pathway contributes to carcinogenesis. However, DNA repair can lead to the resistance of radiation therapy and chemotherapy [31]. Previous studies

showed that HR inhibitors sensitize cancer cells to ionizing radiation or/and chemo-potentiation [32, 33]. Here ectopic ZEB1 enhanced the 5-FU resistance in CRC cells. Since ZEB1 promoted HR via inducing NBS1, RNF8 and RNF168, silencing ZEB1 sensitizes CRC cells to 5-FU treatment via repressing DNA repair. Our study indicates that ZEB1, as a transcription factor, induces DNA repair via promoting the expression of NBS1, RNF8 and RNF168, which confers the resistance to 5-FU treatment in CRC cell lines. Here we also analyzed patient cohort to support our results obtained in cell lines. For patients received adjuvant chemotherapy, high expression of *ZEB1*, *NBS1*, *RNF8* and *RNF168* indicates a tendency towards poor survival, which implies that the expression level of these genes is involved in the chemotherapy effectiveness, and thereby affects the outcome of chemotherapy. Interestingly, the effect of ZEB1, NBS1, RNF8 and RNF168 varies in the treatments with different chemotherapy drugs, indicating that different strategies should be considered to take these genes as biomarkers of chemoresistance in CRC.

In conclusion, ZEB1 functions as a transcription factor inducing the expression of NBS1, RNF8 and RNF168, and thereby ZEB1 enhances DNA repair in colorectal cancer. The ZEB1-mediated DNA repair contributes to the 5-FU resistance in colorectal cancer cells.

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Disclosure of conflict of interest

None.

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Table S1. Primers used in q-PCR analysis

Primers	5'-3'
NBS1 For	TCTGTCAGGACGGCAGGAAAGA
NBS1 Rev	CACCTCCAAAGACAACTGCGGA
RNF8 For	GGAGAAAAGGACCTGAAGCAACA
RNF8 Rev	GCTTCAAAGTCCTTCTTGCTGCG
RNF168 For	CTCTCGTCAACGTGGAACTGTG
RNF168 Rev	GAGCAGACGAACTGGCTGATAG
GAPDH For	GTCTCCTCTGACTTCAACAGCG
GAPDH Rev	ACCACCCTGTTGCTGTAGCCAA
Nbs1 mmu For	CAGACCTATTCCTGAAGCGGAG
Nbs1 mmu Rev	AAGGCTTGGTCCTGGAGTCGTT
Rnf8 mmu For	AGGACAAGGTGCAAGCGCAGAA
Rnf8 mmu Rev	GAAGGAGCAGAAACTGTGGGCA
Rnf168 mmu For	CCTTGGCATGTAAGGCTGAGCT
Rnf168 mmu Rev	AGTTTCCAGGCAGGTCTGAGGA
Gapdh mmu For	CATCACTGCCACCCAGAAGACTG
Gapdh mmu Rev	ATGCCAGTGAGCTTCCCGTTCAG

Table S2. Antibodies used in western blot analysis

Antibody	Company	Dilution	Application	Host species
NBS1	Abcam; ab175800	1:1000	WB	Rabbit
RNF8	Abcam; ab128872	1:1000	WB	Rabbit
RNF168	Abcam; ab271099	1:1000	WB	Rabbit
ZEB1	CST; 70512	1:1000/1:50	WB/ChIP	Rabbit
α-tubulin	Novus; DM1A	1:10000	WB	Mouse
Phospho-Histone H2A.X	CST; 20E3	1:100	IF	Rabbit

Table S3. Primers used in qChIP analysis

Primers	5'-3'
NBS1 For	AGGTGTCGCTGAATGTAAGGT
NBS1 Rev	AACTGCAAACGCACGAAACT
RNF8 For	AGCAACAGACTAGGCAGACTTA
RNF8 Rev	TCCAGAACCATCACCCCAGT
RNF168 For	GGACGCGGCTCCGGGAGG
RNF168 Rev	GCAGTTTTCTGGGTGGGAAGT
NBS1 mmu For	TCAACAGCACTTTGATACATCTAGTTTAA
NBS1 mmu Rev	CGGGGGCGCCACCTAGTG
RNF8 mmu For	GCTTGCCAGCAAGTAGCTTTACT
RNF8 mmu Rev	GGGAGGCGGGCGGGAAGT
RNF168 mmu For	ACTTTAGAAAGCTTTGTTAGGAGAAGG
RNF168 mmu Rev	GTCTGCGTTCCCGCCTAGC