

# Long noncoding RNA *CRNDE* functions as a competing endogenous RNA to promote metastasis and oxaliplatin resistance by sponging miR-136 in colorectal cancer

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**Abstract:** Colorectal neoplasia differentially expressed (*CRNDE*) is a novel gene recognized as a long noncoding RNA (lncRNA) that is highly elevated in colorectal cancer and many other solid tumors but its functions on metastasis and oxaliplatin (OXA) resistance are unknown. In our study, we confirmed the upregulation of *CRNDE* in both primary specimens from colorectal cancer patients and colorectal cancer cell lines. Knockdown of *CRNDE* expression inhibited the migration and invasion potency of colorectal cancer cells with no effect on cell apoptosis. Overexpression of *CRNDE* promoted the migration and invasion potency of colorectal cancer cells. Furthermore, we found that *CRNDE* conferred chemoresistance in colorectal cancer cells. Knockdown of *CRNDE* with OXA treatment decreased cell viability and promoted DNA damage and cell apoptosis, while the overexpression of *CRNDE* with OXA treatment reduced DNA damage and cell apoptosis. Further in-depth mechanistic studies revealed that *CRNDE* functioned as a competing endogenous RNA for miR-136, led to the de-repression of its endogenous target, E2F transcription factor 1 (E2F1). Overall, our findings demonstrate that *CRNDE* functions as a competing endogenous RNA to promote metastasis and OXA resistance by sponging miR-136 in colorectal cancer.

**Keywords:** *CRNDE*, colorectal cancer, metastasis, oxaliplatin resistance, miR-136, E2F1

## Introduction

Colorectal cancer is still one of the most common cancers in the world, with high metastasis and recurrence rate being the most critical concerns.<sup>1,2</sup> Although encouraging progress in diagnosis and cancer therapy has been achieved in the past decade, the 5-year overall survival rate is less than 10% in advanced disease and chemotherapy treatment remains essential for these patients. The activation of survival signaling pathways such as EGFR, PI3K/Akt, MAPK and STAT3 plays key roles in colon cancer initiation, development, progression and drug resistance, and therapies targeting those signaling axes are beneficial.<sup>3-6</sup> The chemotherapy drugs such as 5-fluorouracil, oxaliplatin (OXA), irinotecan, cetuximab and bevacizumab are the first-line options for treatment of metastatic colorectal cancer.<sup>7-9</sup> Increasing data have implicated the molecular mechanisms underlying resistance to OXA. The understanding of the molecular mechanisms underlying resistance to OXA is important to improve prediction of treatment response and guide treatment decisions in patients with metastatic colorectal cancer.

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The novel gene colorectal neoplasia differentially expressed (*CRNDE*) transcripts are recognized as long noncoding RNAs (lncRNA) and located on chromosome 16 of the human genome. The expression of *CRNDE* is highly elevated in colorectal adenomas and carcinomas. *CRNDE* is the gene symbol of colorectal neoplasia differentially expressed, a novel gene the expression of which is highly elevated in colorectal adenomas and carcinomas (data from UCSC Genome Browser).<sup>10</sup> Growing evidence has indicated that *CRNDE* acts through epigenetic mechanisms to regulate cell differentiation/pluripotency, which may relate to its deregulation in cancer. *CRNDE* expression is significantly upregulated in many cancers, including colorectal cancer and gliomas. Elevation in *CRNDE* expression has been shown to promote cell proliferation, migration and invasion while inhibiting apoptosis of glioma cells.<sup>10</sup> Collective evidence has indicated that *CRNDE* is associated with adverse clinical characteristics and poor prognosis by regulating miRNAs in many solid tumors.<sup>11,12</sup> However, the role of *CRNDE* in metastasis and OXA resistance of colorectal cancer and the depth mechanism are largely unknown.

In this study, we confirmed the upregulation of *CRNDE* in both primary specimens from colorectal cancer patients and colorectal cancer cell lines. *CRNDE* was knocked down by *CRNDE* siRNAs, and cell viability, migration and invasion potency of colorectal cancer cells were assessed. Our results showed that the knockdown of *CRNDE* inhibited the migration and invasion potency of colorectal cancer cells with no effect on cell apoptosis. We constructed plasmid pcDNA-*CRNDE* and pcDNA-Vector to ectopically express *CRNDE* and found that the overexpression of *CRNDE* promoted the migration and invasion potency of colorectal cancer cells. To determine whether *CRNDE* confers chemoresistance in colorectal cancer cells, the *CRNDE* knockdown and overexpressed HCT116 cells were treated with OXA. We found that the knockdown of *CRNDE* with OXA treatment decreased cell viability and promoted DNA damage and cell apoptosis, while the overexpression of *CRNDE* with OXA treatment reduced DNA damage and cell apoptosis.

Accumulated evidence showed that a range of lncRNA-sharing miRNA response elements (MREs) may act as a decoy to sequester miRNAs to prevent them from binding to targets and hence modulate many downstream target gene through translation. To gain insight into the possible mechanism, we used the bioinformatics databases to predict the potential lncRNA-miRNA interactions and found that miR-136 is a putative *CRNDE*-binding miRNA.

Previous studies reported that miR-136 plays a key role in temozolomide resistance by targeting the AEG-1 protein in glioma cell lines and miR-136 modulates the tumor sensitivity response to cisplatin by targeting E2F transcription factor 1 (E2F1) in glioma cells.<sup>13,14</sup> In addition, the level of *CRNDE* and miR-136 in colorectal cancer tissues is inversely correlated by linear regression analysis. RNA immunoprecipitation assay and luciferase activity assays confirmed that *CRNDE* is the target of miR-136. We confirmed that E2F1 is a target of miR-136 in colorectal cancer cells and found that both gene and protein levels of E2F1 increased significantly in the *CRNDE*-overexpressed HCT116 cells. These observations indicate that *CRNDE* is a target of miR-136 and modulates the expression of E2F1.

## Materials and methods

### Cell culture and treatment

Human colorectal adenocarcinoma cell lines (SW480, HCT116 and HT-29) were obtained from American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco Modified Eagle Medium (DMEM) (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA). Human normal colon epithelial cell lines (HcoEpic and NCM460) were obtained from Xiehe Cell Bank of the Chinese Academy of Medical Sciences (Beijing, People's Republic of China) and were cultured in McCoy's 5A medium (Invitrogen, Life Technologies). Cells were maintained at 37°C in a water-saturated atmosphere with 5% CO<sub>2</sub>.

### Clinical samples

The tumor tissues were collected from 10 colorectal cancer patients during surgery at Baoji City First People's Hospital (Nanjing, People's Republic of China). The study and the study protocol were approved by the institutional ethics committee of Baoji City First People's Hospital. The tumor tissues were immediately frozen and kept at -80°C until assay. Written informed consent was obtained from all patients.

### RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted from colorectal cancer tissues and cells using TRIzol reagent (Invitrogen). In all, 1 µg RNA was used as the template for single-strand cDNA synthesis utilizing random primers and the PrimeScript reverse transcriptase (M-MLV; Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Quantitative polymerase chain reaction (Q-PCR) was performed for *CRNDE*, GAPDH, E2F1, miR-136-5p and U6. The cDNA was amplified by an

Applied Biosystems step-one plus sequence detection system (Applied Biosystems, Foster City, CA, USA). The relative expressions were normalized to endogenous controls using the comparative cycle threshold method, and fold change was calculated as  $2^{-\Delta\Delta Ct}$  in gene expression.

### Cell viability assay

Cells were seeded in 96-well microtiter plates at a density of 3,000 cells/well. The cells were treated with OXA for 24 hours after transfected. The media was removed and fresh media was added to each well. Then, 10  $\mu$ L of CCK-8 solution (CCK-8; Dojin, Kumamoto, Japan) was added into each well and incubated for 2 hours at 37°C. The absorbance was measured at 450 nm using the SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### Apoptosis assay

Cell apoptosis was evaluated by annexin V-fluorescein isothiocyanate (FITC/PI) staining. Cells were collected and double stained with annexin V-FITC/PI according to the manufacturer's instructions. Then, the cells were analyzed by an EPICS XL-MCL FAC scan (Becton-Dickinson, Mountain View, CA, USA) and the data were analyzed by CELL Quest 3.0 software (BD Biosciences, San Jose, CA, USA).

### Cell migration assay

Wound healing assay was performed by measuring the movement of cells in a scraped, acellular area to assess cell motility. The wound closure was observed and photos were taken to assess the level of migration after 0 and 48 hours.

### Matrigel invasion assay

Cell invasion through the Matrigel membrane was quantitated using Matrigel-coated transwell chambers (BD Biosciences) according to the manufacturer's instructions. Cells ( $1 \times 10^4$ ) were seeded into the upper Matrigel-coated chambers with DMEM containing 10% FBS in the lower chamber. After 36 hours, non-invading cells in the upper chamber were removed by scrubbing with a cotton-tipped swab. Afterwards, the cells were fixed with 4% paraformaldehyde for 20 minutes and stained with 0.2% crystal violet (Sigma-Aldrich, St Louis, MO, USA) for 10 minutes. Six fields for each chamber were photographed using an inverted microscope and camera, and invading cells counted in each field.

### Western blot analysis

Cells were homogenized in radio immunoprecipitation assay (RIPA) plus buffer in a buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02%  $\text{NaN}_3$ , 0.1% sodium dodecyl sulfate [SDS],

100  $\mu$ g/mL phenylmethylsulfonyl fluoride, 1  $\mu$ g/mL aprotinin, 1% Triton). After centrifugation, cell lysates (100  $\mu$ g/lane) were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Antibodies against E2F1 (Santa Cruz, 1:1,000), GAPDH (CST, 1:1,000),  $\gamma$ H2AX (CST, 1:2,000) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Promab, 1:1,000) were used. Protein bands were detected by the enhanced chemiluminescence reaction, and blot film was scanned.

### Cell transfection

siRNA oligonucleotides and negative control were designed from the ~350 base pair highly conserved gVC-In4 region within the *CRNDE* locus target sequence and synthesized by RiboBio Co Ltd (Guangzhou, People's Republic of China). The pre-miR-136 (miRNA mimic), or its pre-control (scrambled negative controls), was designed and synthesized by RiboBio Co Ltd. The *CRNDE* full-length sequence was synthesized and subcloned into a pcDNA 3.1 vector (Invitrogen, Shanghai, People's Republic of China). The cells were transfected with the aforementioned siRNA1, siRNA2, si-control, pre-miR-136, pre-control, pCDNA-*CRNDE* and vectors for 48 hours using Lipofectamine™ 3000 (Invitrogen, Shanghai, People's Republic of China) following the manufacturer's protocol. Cells were harvested after 48 hours for quantitative real-time polymerase chain reaction (qRT-PCR) to assess the efficiency of knockdown and overexpression.

### RNA immunoprecipitation

The cells were rinsed with cold phosphate-buffered saline and lysed by a complete RNA lysis buffer with protease inhibitor and RNase inhibitor from an EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore) according to the manufacturer's protocol. The cell lysates were stored at  $-80^\circ\text{C}$  before use and supernatant from cell lysates was collected by high-speed centrifugation. The cell lysate was incubated with RIP immunoprecipitation buffer containing magnetic beads conjugated with human anti-Argonaute2 (Ago2) antibody (Abcam, Bristol, UK) and negative control normal mouse immunoglobulin G (IgG) (Sigma-Aldrich) overnight. The beads were rinsed with cold NT2 buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM  $\text{MgCl}_2$ , 0.5% NP-40), followed by incubation with 10 mg/mL proteinase K buffer. The RNA bound to Ago2 antibody was extracted with TRIzol reagent. Then, the concentration and quality of RNA were measured. Furthermore, purified RNA was analyzed by qRT-PCR. RNA levels of *CRNDE* were presented as fold enrichment in Ago2 relative to IgG immunoprecipitates.

## Luciferase reporter assay

Luciferase reporter plasmids (*CRNDE*-Wt and *CRNDE*-mut) were designed and constructed by Generay (Shanghai, People's Republic of China). The HEK293T cells cultured in 24-well plates were co-transfected with luciferase reporter plasmids and miRNA mimics by Lipofectamine™ 3000 transfection reagent to analyze the interaction between *CRNDE* and miR-136. At 48 hours after transfection, luciferase activity assays were performed with the dual-luciferase reporter assay system.

## Statistical analysis

All results are presented as mean  $\pm$  standard error of mean of at least three independent experiments. Student's *t*-test was used to assess differences between two groups, and one-way analysis of variance was used for multiple comparisons. A value of  $P < 0.05$  was considered to be statistically significant.

## Results

### *CRNDE* was increased in colorectal cancer tissues and cells

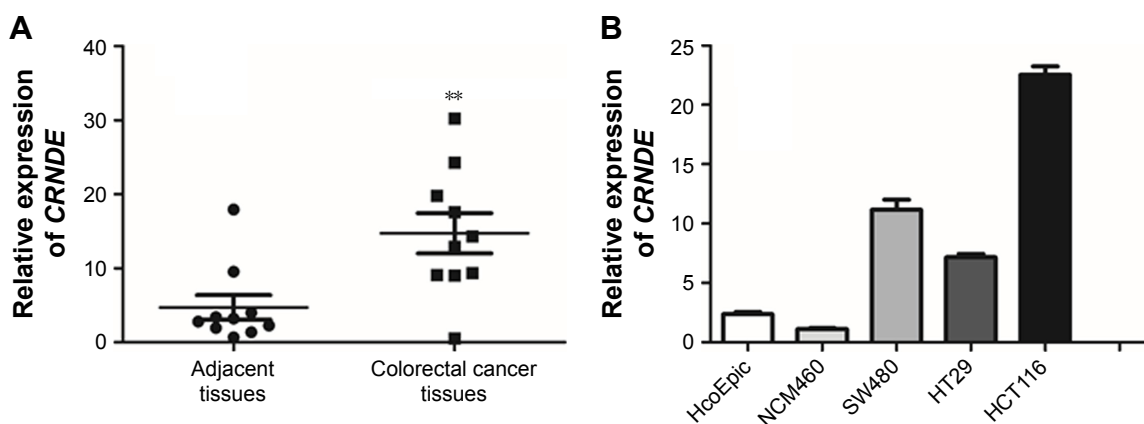
The relative expression levels of *CRNDE* were first assessed in colorectal cancer tissues and *CRNDE* expression was dramatically upregulated (Figure 1A). The expression levels of *CRNDE* in colorectal cancer cell lines and human normal colon cell lines were further measured. Notably, three cell lines (SW480, HCT116 and HT29) showed higher levels of *CRNDE* than normal cell lines (HcoEpic and NCM460) (Figure 1B). SW480 and HCT116 cell lines expressed relatively high *CRNDE* levels and they were selected for further study to assess the potential functional role of *CRNDE*.

## Knockdown of *CRNDE* inhibited the migration and invasion of colorectal cancer cells

To investigate the biological role of *CRNDE* in colorectal cancer cells, the SW480 and HCT116 cells were transfected with *CRNDE* siRNAs or si-control to knockdown *CRNDE* in SW480 and HCT116 cells (Figure 2A). CCK8 assays showed that cell proliferation was decreased in SW480 and HCT116 cells transfected with *CRNDE* siRNA2 after 48 hours (Figure 2B). Wound healing assay revealed that cell migration potency of HCT116 and SW480 cells was inhibited by *CRNDE* knockdown (Figures 2C and S1A). Matrigel invasion assay revealed that the invasion potency of SW480 and HCT116 cells was also inhibited by *CRNDE* knockdown (Figure 2D and E). Cell apoptosis was evaluated and no significant change of cell apoptosis was found by *CRNDE* knockdown (Figure 2F and G).

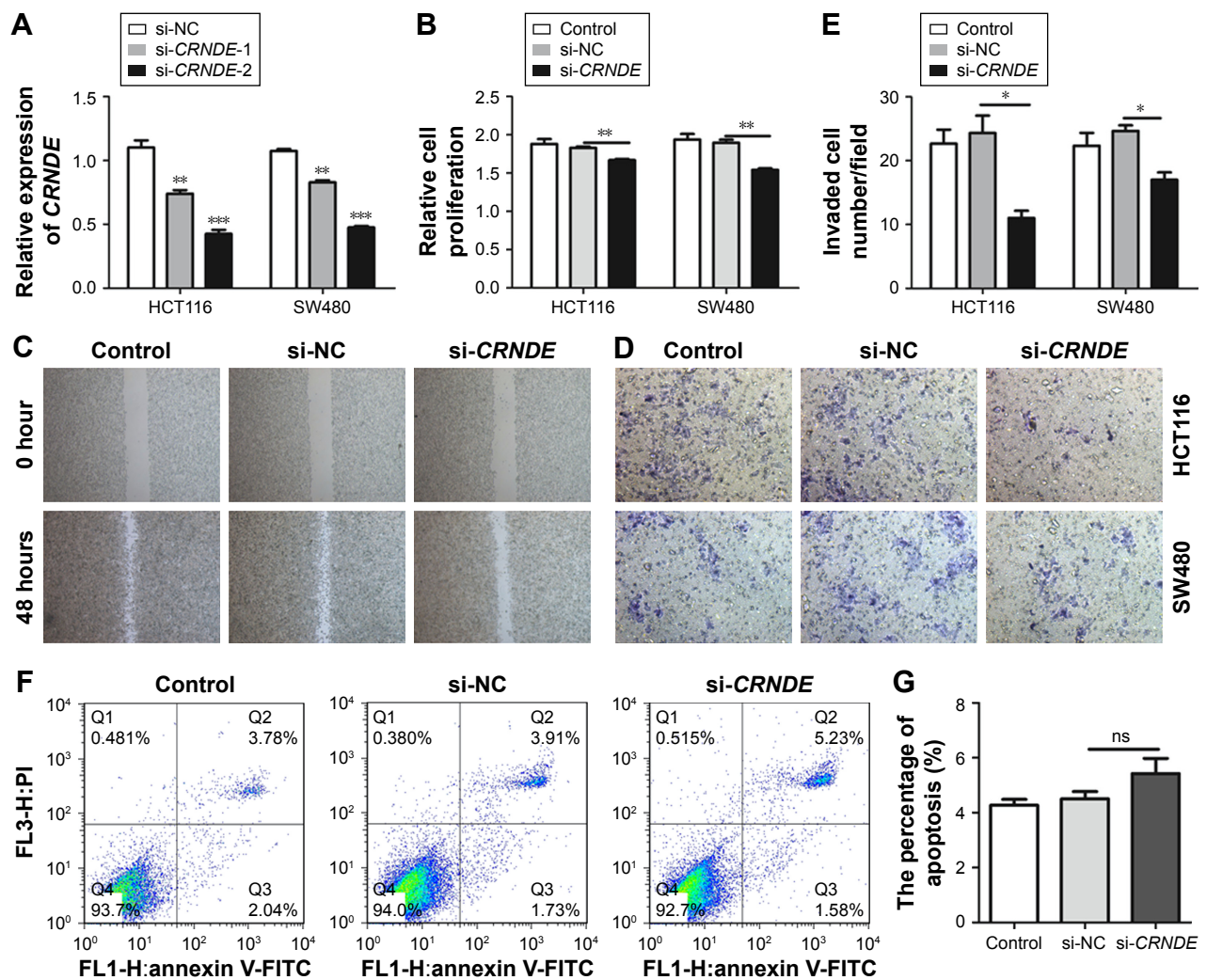
## Overexpression of *CRNDE* enhanced the migration and invasion of colorectal cancer cells

To further ascertain the role of *CRNDE* in proliferation, migration, invasion and apoptosis of colorectal cancer cells, we constructed plasmid pcDNA-*CRNDE* and pcDNA-Vector. The ectopic-expressed *CRNDE* in SW480 cells increased cell viability compared with transfection with pcDNA-Vector. However, there was no difference in HCT116 cells (Figure 3A). Wound healing assay revealed that cell migration potency of HCT116 and SW480 cells was enhanced by *CRNDE* overexpression (Figure 3B and Figure S1B). *CRNDE* overexpression enhanced the invasion potency of SW480 and HCT116 cells (Figure 3C and D).



**Figure 1** Upregulation of *CRNDE* was observed in both colorectal cancer tissues and cell lines. **(A)** *CRNDE* was significantly upregulated in colorectal cancer tissues in tumor tissues relative to adjacent normal tissues ( $n=10$ ). **(B)** Expression of *CRNDE* in colorectal cancer cell lines (SW480, HCT116 and HT-29) and normal human colon cell lines (HcoEpic and NCM460). Data represent three independent experiments (average and sem of triplicate samples).  $**P < 0.01$ .

**Abbreviations:** *CRNDE*, colorectal neoplasia differentially expressed; sem, standard error of mean.



**Figure 2** Knockdown of *CRNDE* inhibited the proliferation, migration and invasion of colorectal cancer cells. (A) The *CRNDE* level of SW480 and HCT116 cells was decreased after being transfected with *CRNDE* siRNAs or si-control control. (B) Cell proliferation analysis using CCK-8 assay after being transfected with *CRNDE* siRNAs or si-control. (C) Scratch-wound healing assay was used to assess the migration potency of HCT116 cells after being transfected with *CRNDE* siRNAs or si-control control. The wound closure was calculated at 48 hours. (D, E) Matrigel invasion assay was performed to test the change of invasion potency after being transfected with *CRNDE* siRNAs or si-control control. Representative images of the invaded cells are shown. Six fields for each chamber were photographed using an inverted microscope and camera, and invading cells counted in each field. The average number was shown. (F, G) Flow cytometry was used to evaluate the cell apoptosis of HCT116 cells with knockdown of *CRNDE*. Data represent three independent experiments (average and sem of triplicate samples). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

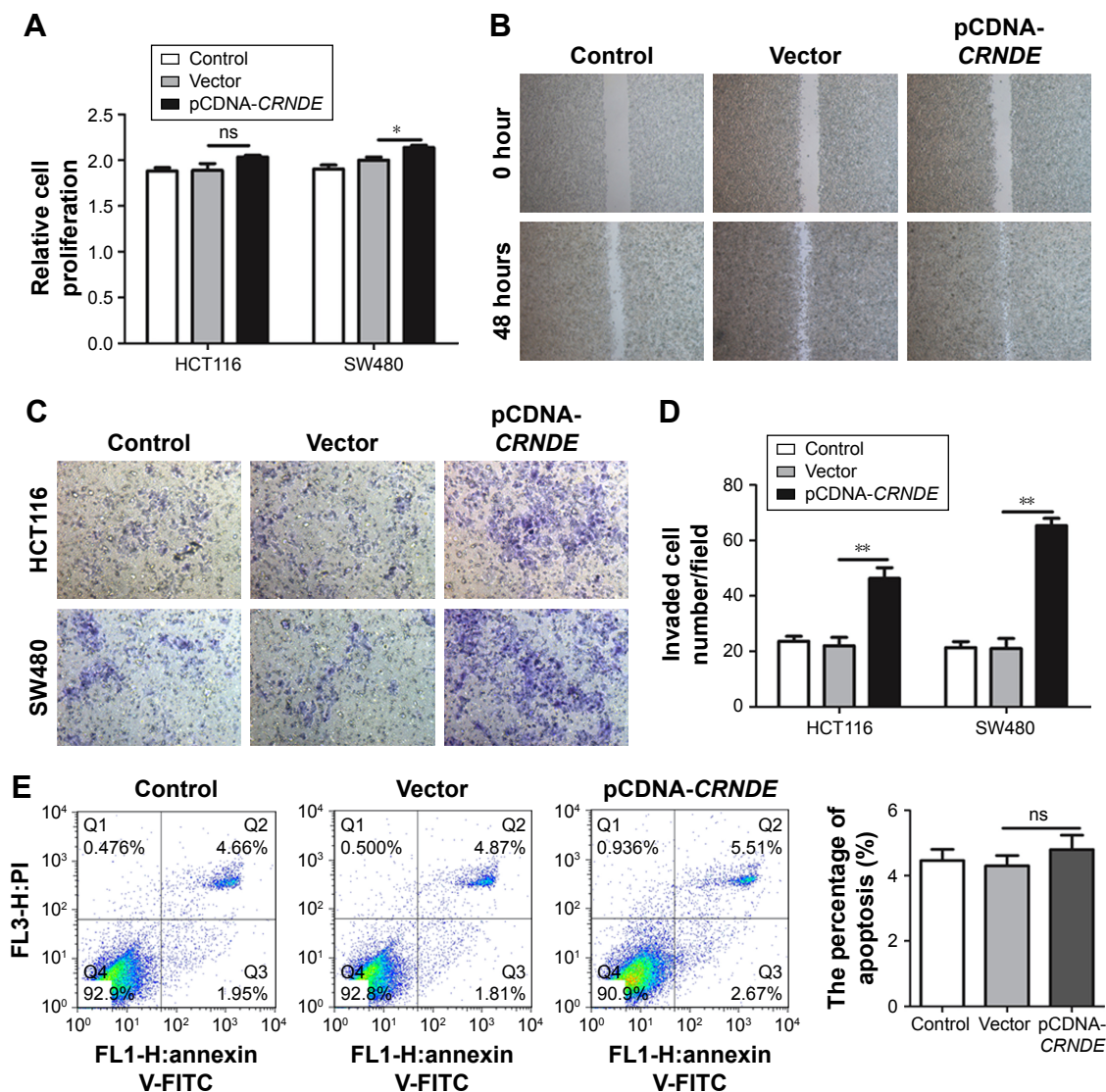
**Abbreviations:** *CRNDE*, colorectal neoplasia differentially expressed; sem, standard error of mean; ns, not significant.

Furthermore, there is no significant change of cell apoptosis in *CRNDE*-overexpressed cells (Figure 3E).

## Knockdown of *CRNDE* enhances colorectal cancer cell sensitivity to OXA

To determine whether *CRNDE* confers chemoresistance in colorectal cancer cells, we transfected HCT116 cells with *CRNDE* siRNA or si-control and treated with OXA. As shown in Figure 4A, the knockdown of *CRNDE* alone had little effect on cell proliferation than control. However, the knockdown of *CRNDE* with OXA treatment had a strong effect on cell proliferation than control with OXA treatment. We also found the OXA drug resistance of HCT116 cells

conferred by *CRNDE* was dose dependent (Figure 4B). OXA causes DNA crosslinking and stimulates H2AX phosphorylation at serine 139 to generate  $\gamma$ H2AX as a major marker for DNA damage signaling in response to DNA double-strand break.<sup>15</sup> OXA-triggered  $\gamma$ H2AX in HCT116 cells transfected with *CRNDE* siRNA was elevated significantly, suggesting that *CRNDE* may control OXA resistance in colorectal cancer cells at the DNA damage induction level. (Figure 4C and D). OXA could bind to double-stranded DNA and form DNA adducts, interfering with DNA replication and ultimately triggering apoptosis. Hence, we examined whether *CRNDE* is involved in OXA-induced chemoresistance by affecting apoptosis. As shown in Figure 4E and F,



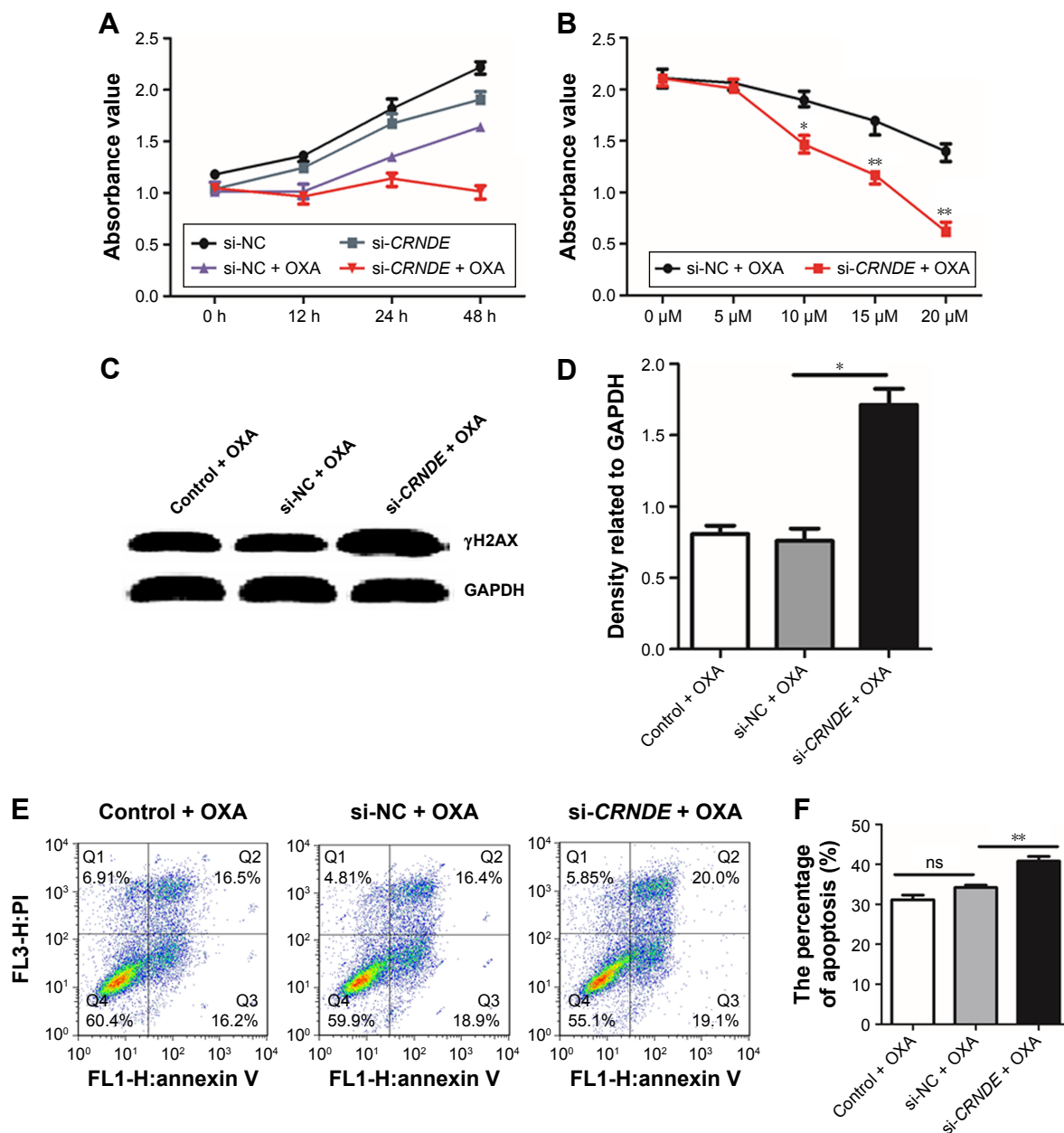
**Figure 3** Overexpression of *CRNDE* enhances the proliferation, migration and invasion of colorectal cancer cells. HCT116 cells were transfected with pcDNA-*CRNDE* or pcDNA-Vector. (A) CCK-8 assay was performed to assess cell viability. (B) Scratch-wound healing assay was used to assess the migration potency of HCT116 cells after being transfected. (C, D) Matrigel invasion assay was performed to test the change of invasion potency after being transfected. Representative images of the invaded cells are shown. Six fields for each chamber were photographed using an inverted microscope and camera, and invading cells counted in each field. The average number is shown. (E) Flow cytometry was used to evaluate the cell apoptosis. Data represent three independent experiments (average and sem of triplicate samples). \* $P < 0.05$ , \*\* $P < 0.01$ . **Abbreviations:** *CRNDE*, colorectal neoplasia differentially expressed; sem, standard error of mean; ns, not significant.

the knockdown of *CRNDE* with OXA treatment induced the increased proportion of apoptosis cells. We have shown that the knockdown of *CRNDE* alone in HCT116 cells has no effect on apoptosis (Figure 2F). The results provided evidence that *CRNDE* enhances chemoresistance in colorectal cancer cells.

### Overexpression of *CRNDE* enhances OXA chemoresistance of colorectal cancer cells

In order to further investigate the role of *CRNDE* in OXA resistance of colorectal cancer cells, the HCT116 cells

were transfected with pcDNA-*CRNDE* or pcDNA-Vector. 48 hours later, cells were treated with OXA for 24 hours or 48 hours. The *CRNDE* ectopic-expressed HCT116 cells were treated with OXA, and reduction of cell viability was less evident compared with cells transfected with pcDNA-Vector (Figure 5A). The level of a major marker for DNA damage,  $\gamma$ H2AX, was decreased when *CRNDE* ectopic-expressed HCT116 cells were treated with OXA compared with the control cells (Figure 5B). In addition, overexpression of *CRNDE* decreased the cell apoptosis proportion of HCT116 cells under the OXA treatment (Figure 5C and D). No impact of overexpression of *CRNDE* without OXA treatment on cell



**Figure 4** Knockdown of *CRNDE* enhances colorectal cancer cell sensitivity to OXA. HCT116 cells were transfected with *CRNDE* siRNAs or si-control control. After 48 hours, cells were treated with OXA. (A) The transfected HCT116 cells were treated with 15  $\mu$ M (50% inhibitory concentration values) OXA, and CCK-8 assay was performed to assess cell viability after 0, 12, 24, and 48 hours. (B) The transfected HCT116 cells were treated with different doses of OXA, and CCK-8 assay was performed to assess cell viability after 48 hours. (C) Western blot was used to analyze the protein expression of  $\gamma$ -H2AX in HCT116 cells after 48 hours of OXA treatment. (D) Densitometry plot of results from Figure 4C. The relative expression levels were normalized to GAPDH. (E, F) Flow cytometric analysis was used to evaluate the apoptosis of HCT116 cells after 48 hours of OXA treatment. Data represent three independent experiments (average and sem of triplicate samples). \* $P < 0.05$ , \*\* $P < 0.01$ .

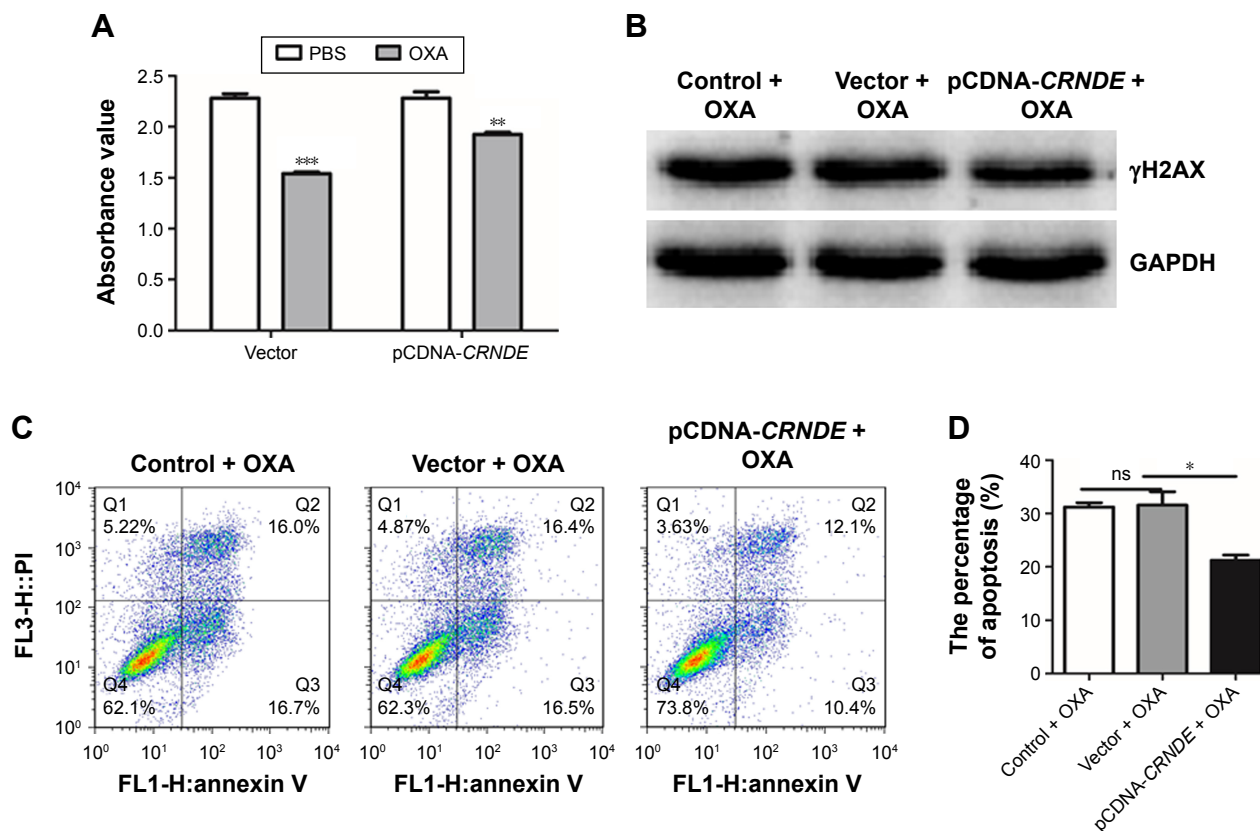
**Abbreviations:** *CRNDE*, colorectal neoplasia differentially expressed; OXA, oxaliplatin; sem, standard error of mean; ns, not significant.

apoptosis was demonstrated (Figure 3E). Together, our findings demonstrated that *CRNDE* enhances colorectal cancer cell resistance to OXA.

### *CRNDE* is a target of miR-136 and modulates its target E2F1

Further in-depth study is required for a better understanding of the role of *CRNDE* in colorectal cancer cell metastasis

and OXA resistance. Analysis with bioinformatics databases (Starbase, RNAhybrid) that predict potential lncRNA-miRNA interactions revealed that miR-136 is a putative *CRNDE*-binding miRNA (Figure 6A). We calculated the miR-136 levels in the colorectal cancer tissues and found that *CRNDE* and miR-136 level in the colorectal cancer tissue is inversely correlated by linear regression analysis (Figure 6B).



**Figure 5** Overexpression of *CRNDE* enhances the OXA chemoresistance of colorectal cancer cells. HCT116 cells were transfected with pcDNA-*CRNDE* or pcDNA-Vector. After 48 hours, cells were treated with 15  $\mu$ M (50% inhibitory concentration values) OXA for 24 hours or 48 hours. (A) CCK-8 assay was performed after 24 hours of OXA treatment. (B) Western blot was used to analyze the protein expression of  $\gamma$ H2AX in HCT116 cells after 48 hours of OXA treatment. (C, D) Flow cytometric analysis was used to evaluate the apoptosis of HCT116 cells after 48 hours of OXA treatment. Data represent three independent experiments (average and sem of triplicate samples). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

**Abbreviations:** *CRNDE*, colorectal neoplasia differentially expressed; OXA, oxaliplatin; sem, standard error of mean; PBS, phosphate-buffered saline; ns, not significant.

We supposed that *CRNDE* might harbor one miR-136 binding site and hijacking miR-136 by serving as a miRNA sponge. It has been shown that miRNA exerts its function by binding to Ago2, a core component of the RNA-induced silencing complex regulating miRNA-related gene expression changes. To assess our prediction, RNA-binding protein immunoprecipitation assay was performed. The results of RNA immunoprecipitation assay showed that *CRNDE* was preferentially enriched in Ago2-containing beads compared to the beads harboring control IgG antibody (Figure 6C). To further quantify our prediction that *CRNDE* could harbor one miR-136 binding site, wild-type and mutant *CRNDE* sequence containing the putative miR-136 recognition site was cloned downstream of the luciferase gene and co-transfected into HEK293T cells with pre-miR-136 or pre-control. Luciferase activity assays showed that miR-136 suppressed the activity of luciferase reporter harboring wild-type *CRNDE* but not the mutant *CRNDE* (Figure 6D). Collective data suggest that *CRNDE* acts as a miRNA decoy for miR-136.

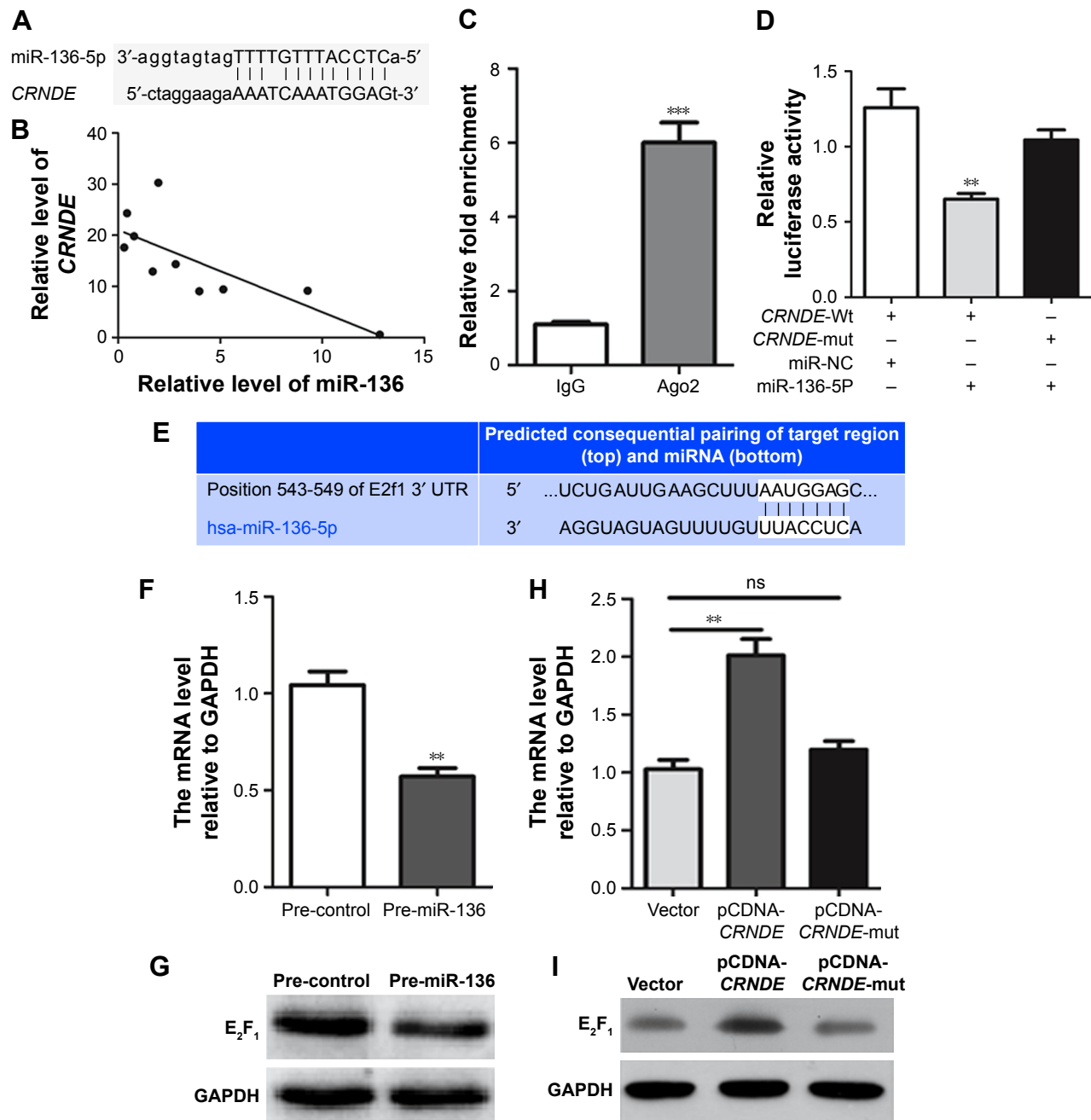
It has been reported that E2F1 is a direct target of miR-136 in glioma cells.<sup>13</sup> We performed a bioinformatics

analysis by TargetScan algorithm ([www.targetscan.org](http://www.targetscan.org)) and identified E2F1 as a predicted target of miR-136 (Figure 6E). Both gene and protein levels of E2F1 decreased in the HCT116 cells transfected by pre-miR-136 relative to pre-control (Figure 6F and G). We confirmed that E2F1 was a target of miR-136 in colorectal cancer cells. To determine whether *CRNDE* affects E2F1 expression, the HCT116 cells were transfected with pcDNA-Vector, pcDNA-*CRNDE* or pcDNA-*CRNDE*-mut (miR-136 and *CRNDE* binding sites were mutated) plasmid and gene and protein levels of E2F1 were tested. As shown in Figure 6H and I, both gene and protein levels of E2F1 increased significantly in *CRNDE*-overexpressed HCT116 cells compared with vector group. However, no change was found in HCT116 cells transfected with pcDNA-*CRNDE*-mut compared with vector plasmid.

## Discussion

OXA is a platinum analog and the third-generation platinum drug used as adjuvant or first-line drug for treatment of metastatic colorectal cancer.<sup>16</sup> OXA induces cytotoxicity mainly through the formation of platinum-DNA adducts resulting





**Figure 6** CRNDE is a target of miR-136 and modulates its target E2F1. (A) Schematic diagrams of the mutual interactions between miR-136 and CRNDE. (B) The inverse correlation of the expression of CRNDE and miR-136 among the tissue samples was measured by linear regression analysis. (C) CRNDE levels in the immunoprecipitates were measured by Q-PCR and presented as fold enrichment in Ago2 relative to IgG immunoprecipitates. (D) The luciferase reporter plasmid containing wild-type or mutant CRNDE was co-transfected into HEK293T cells with pre-miR-136 mimics or pre-NC. Luciferase reporter genes assay was performed to determine if the luciferase activity was normalized to Renilla activity. Three independent experiments were performed using one-way ANOVA analysis. (E) The predicted miR-136 binding sites in the 3-UTR of E2F1 are showed in schematic diagrams. (F) The gene level of E2F1 in HCT116 cells transfected with pre-miR-136 and pre-control. (G) The protein level of E2F1 in HCT116 cells transfected with pre-miR-136 and pre-control. (H) The HCT116 cells were transfected with pCDNA-Vector, pCDNA-CRNDE or pCDNA-CRNDE-mut (miR-136 and CRNDE binding sites were mutated) plasmid and the gene of E2F1 was tested. (I) The HCT116 cells were transfected with pCDNA-Vector, pCDNA-CRNDE or pCDNA-CRNDE-mut (miR-136 and CRNDE binding sites were mutated) plasmid and protein levels of E2F1 were tested. Data represent three independent experiments (average and sem of triplicate samples). \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

**Abbreviations:** CRNDE, colorectal neoplasia differentially expressed; Q-PCR, quantitative polymerase chain reaction; Ago2, anti-Argonaute2; IgG, immunoglobulin G; ANOVA, analysis of variance; sem, standard error of mean; ns, not significant.

in DNA transcription and replication blockade and induces DNA damage-mediated cell apoptosis. OXA activates DNA damage repair and/or cell death signaling pathways.<sup>17</sup> The molecular mechanisms of OXA chemoresistance are complex

and multifactorial processes, such as drug influx/efflux modifications, DNA damage repair alterations, decrease of cell apoptosis and autophagy.<sup>18,19</sup> The activation of survival signaling pathways is a major mechanism for OXA resistance.

Previous studies reported that the expression of EGFR increased proportionally in relation to the level of acquired resistance to OXA in the tumor cell lines that are resistant to OXA.<sup>20</sup> PI3K/Akt pathway activation was involved in OXA chemoresistance in human colon cancer.<sup>21,22</sup>

LncRNAs, more than 200 nucleotides in length, have emerged as critical regulators of human disease and prognostic markers in several cancers. LncRNAs are involved in tumor proliferation, metastasis and multiple cellular processes such as proliferation, migration, invasion, apoptosis and chemoresistance.<sup>16,18,23–26</sup> Growing evidence has documented that miRNAs have important regulatory functions in biological processes that represent the hallmarks of cancer, such as proliferation, apoptosis, invasion and metastasis by targeting gene for deregulation or translational repression. What is more, collective data suggest that miRNAs are closely associated with the acquired chemoresistance in human carcinoma. In vitro, overexpression of miR-153, -203 and -143 has been associated with acquired resistance to OXA through modulation of FOXO3a, ATM kinase and IGF-1R, respectively.<sup>27–29</sup> We predicted the potential lncRNA–miRNA interactions by bioinformatics databases and found that miR-136 is a putative *CRNDE* binding miRNA. The *CRNDE*–miR-136 interactions were confirmed by RNA immunoprecipitation assay and luciferase activity assays. E2F1 has been reported to be a direct target of miR-136 and involved in sensitization to chemotherapy in glioma cells.

E2F1 is a family member of eight (E2F1-8) transcription factors that regulate the cell cycle, migration, apoptosis and chemoresistance.<sup>30–32</sup> The E2F1 expression was inhibited by pRb and released from phosphorylated pRB complexes leads to recruits coactivators to *E2F*-responsive genes in G1 phase transcription, DNA synthesis and DNA repair. E2F1 is aberrantly expressed in many malignancies, including melanoma, bladder tumor, lung tumor, prostate tumor, colorectal cancer and cisplatin-resistant ovarian cancer cell lines.<sup>33–38</sup> E2F1 overexpression may promote proliferation or regulate cell apoptosis.<sup>39,40</sup> It has been shown that miR-302b could enhance breast cancer cell sensitivity to cisplatin by targeting E2F1 and ATM. By this way, the cellular DNA damage response was enhanced.<sup>32</sup> We confirmed that E2F1 is a target of miR-136 in colorectal cancer cells. To determine whether *CRNDE* affects E2F1 expression, we assessed the gene and protein levels of E2F1 in *CRNDE*-overexpressed HCT116 cells. Our results showed that both gene and protein levels of E2F1 increased significantly in the *CRNDE*-overexpressed HCT116 cells. Together, the collecting data

indicate that *CRNDE* is a target of miR-136 and modulates the expression of E2F1.

*CRNDE* promotes metastasis and OXA resistance of colorectal cancer by functioning as a miR-136 sponge and modulating the expression of E2F1. Thus, our results shed light on utilizing *CRNDE* as a potential novel therapeutic target for the treatment of colorectal cancer.

## Conclusion

In conclusion, *CRNDE* is involved in the migration and invasion of colorectal cancer cells. Furthermore, *CRNDE* conferred chemoresistance in colorectal cancer cells. Overexpression of *CRNDE* with OXA treatment reduced DNA damage and cell apoptosis. Further in-depth mechanistic studies revealed that *CRNDE* functions as a competing endogenous RNA to promote metastasis and OXA resistance by sponging miR-136 in colorectal cancer.

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## Disclosure

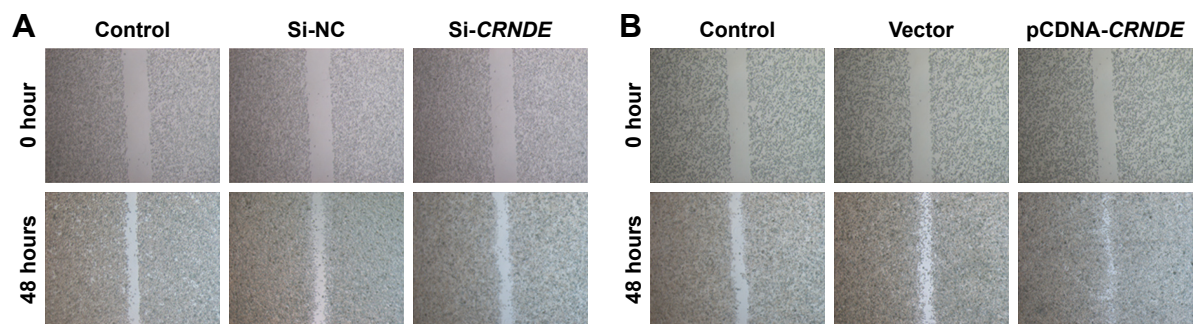
The authors report no conflicts of interest in this work.

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## Supplementary material



**Figure S1** *CRNDE* was involved in cell migration of colorectal cancer cells. **(A)** Scratch-wound healing assay was used to assess the migration potency of SW480 cells after being transfected with *CRNDE* siRNAs or si-control control. The wound closure was calculated at 48 hours. **(B)** Scratch-wound healing assay was used to assess the migration potency of SW480 cells after being transfected with pcDNA-*CRNDE* or pcDNA-Vector. The wound closure was calculated at 48 hours.

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