CRNDE acts as an epigenetic modulator of the p300/YY1 complex to promote HCC progression and therapeutic resistance

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the most common primary liver malignancies worldwide. The long-term prognosis for HCC remains extremely poor, with drug resistance being the major underlying cause of recurrence and mortality. The IncRNA colorectal neoplasia differentially expressed (CRNDE) is an epigenetic mediator and plays an important role to drive proliferation and drug resistance in HCC. However, CRNDE as an epigenetic regulator with influences sorafenib resistance in HCC is unclear. Thus, we explore the potential of targeting the CRNDE/p300/YY1 axis as a novel therapeutic strategy to overcome sorafenib resistance of HCC.

Method: Detection of the expression level of CRNDE and EGFR in clinical specimens of HCC. CRNDE, EGFR, p300, and YY1 expression were altered in HCC cells through transfection with different plasmids, and cell proliferation, migration, invasion, and sorafenib resistance were subsequently observed. Immunoprecipitation, chromatin immunoprecipitation, re-chromatin immunoprecipitation, site-directed mutagenesis, RNA Immunoprecipitation, immune fluorescence, qRT-PCR, and western blotting were performed to uncover the mechanisms of CRNDE regulation. The xenograft nude mice model was used to investigate the tumor growth and sorafenib resistance.

Results: In this study, we showed that CRNDE expression is significantly positively correlated with that of epidermal growth factor receptor (EGFR) in clinical specimens of HCC and induces proliferation and sorafenib resistance of HCC via EGFR-mediated signaling. Mechanistically, CRNDE stabilized the p300/YY1 complex at the EGFR promoter and simultaneously enhanced histone H3K9 and H3K27 acetylation, which serve as markers of relaxed chromatin. EGFR was positively upregulated by the epigenetic complex, p300/YY1, in a manner dependent on CRNDE expression, leading to enhanced tumor cell proliferation and sorafenib resistance. Furthermore, C646, a p300 inhibitor, suppressed EGFR transcriptional activity by decreasing chromatin relaxation and YY1 binding, which effectively reduced proliferation/sorafenib resistance and prolonged overall survival.

Conclusion: Our collective findings support the potential of targeting the CRNDE/p300/YY1 axis as a novel therapeutic strategy to overcome sorafenib resistance of HCC.

Keywords: CRNDE, EGFR, Hepatocellular carcinoma (HCC), H3K9Ac, H3K27Ac, p300, C646, Sorafenib

Introduction

Hepatocellular carcinoma (HCC), the predominant primary liver cancer and sixth most common cancer type worldwide [1], is the third leading cause of cancer-related death. High mortality, poor prognosis, and high-level
recurrence of HCC are significantly associated with ineffective therapeutic management [2]. Sorafenib, a multi-kinase inhibitor, serves as effective first-line therapy that has successfully been used to extend the survival of advanced-stage HCC patients. However, the clinical benefits of sorafenib remain modest and most often consist of temporary tumor stabilization, thereby extending the overall median survival of patients [3]. To facilitate the development of effective therapeutic strategies, the mechanisms underlying sorafenib resistance require extensive clarification.

Dysregulation of IncRNAs in HCC can lead to sorafenib resistance [4]. Research to date has predominantly focused on the functions of IncRNAs as miRNA sponges regulating HCC target gene expression and mediating sorafenib resistance [5–7]. IncRNAs are involved in a number of other mechanisms, such as forming complexes regulating gene transcription by recruiting chromatin-modifying proteins to influence the epigenetic state, coordinating interactions between distal regulatory elements [8]. At present, the pathways by which IncRNAs influence sorafenib resistance are unclear. The IncRNA CRNDE was initially identified in colorectal cancer. CRNDE has been shown to act as an oncogene promoting tumor proliferation, invasion, migration, and drug resistance in multiple cancer types, including HCC [9–12]. A recent study showed that CRNDE induces sorafenib resistance in HCC by triggering autophagy via upregulation of ATG4B [13]. Additionally, CRNDE is reported to inhibit CELF2 and LAT52 expression and promote HCC proliferation, migration, and chemoresistance through epigenetic interactions with EZH2, a key component of the PRC2 complex. While accumulating evidence clearly indicates a role of CRNDE in drug resistance, the epigenetic molecular mechanisms by which CRNDE promotes resistance of HCC to sorafenib remain to be established. Accordingly, we focused on the mechanisms of action of CRNDE in sorafenib resistance of HCC and further evaluated the potential efficacy of epigenetic inhibitors in combating resistance.

The epidermal growth factor receptor EGFR is frequently overexpressed in the majority of HCC clinical samples and correlated with carcinogenesis [14] and therapeutic resistance to drugs (including sorafenib) [14, 15]. Notably, a monoclonal antibody directed against EGFR or RNA interference knockdown of EGFR has been shown to enhance the sensitivity of sorafenib-resistant HCC cell lines [3]. Clarification of the mechanisms affecting EGFR expression may therefore aid in effective management of sorafenib resistance in HCC.

Data from the current study confirmed dysregulation of CRNDE, with overexpression in tumors of HCC patients. CRNDE promoted HCC proliferation and sorafenib resistance through associations with the epigenetic regulator protein, p300, and transcription factor, YY1, and further regulation of chromatin relaxation to mediate EGFR expression and activity. Notably, treatment of HCC cells with a p300 inhibitor induced a significant decrease in proliferation and sorafenib resistance of HCC cells overexpressing CRNDE, supporting the involvement of epigenetic chromatin relaxation in CRNDE-mediated tumor growth and sorafenib resistance. In summary, we have uncovered a novel mechanism by which CRNDE mediates tumor growth and sorafenib resistance via interactions with p300 that may offer effective treatment avenues to combat sorafenib resistance in HCC.

Materials and methods

Human HCC specimens

Paired human HCC specimens were obtained from the Taiwan Liver Cancer Network (TLCN). Total RNA was reverse transcribed into cDNA and mRNA expression analyzed using qRT-PCR. The protocol was approved by the Medical Ethics and Human Clinical Trial Committee at Chang-Gung Memorial Hospital (IRB: 202000434B0).

Cell lines and chemical reagents

Human hepatoma cell lines (HepG2, Mahlavu, Huh7, J7, Hep3B, and SkHep1) were routinely grown in DMEM supplemented with 10% fetal bovine serum. Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Sorafenib was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and C636 and histone acetyltransferase inhibitor (HATi II) from Sigma-Aldrich (St Louis, MO, USA). All reagents were stored and used according to the manufacturer’s instructions.

Gene knockdown using short-hairpin RNA and siRNA

Lentivirus-based pLKO.1 plasmids expressing short-hairpin RNA (shRNA) targeting CRNDE were constructed following the guidelines of the RNAi Consortium (TRC) shRNA Design Process from the National RNAi Core Facility (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). The shRNA sequence obtained was cloned into pLKO-TRC001. The oligonucleotides used for shRNA construction, shRNA clone list, and siRNA sequences are presented in Additional file 10: Table S1. Stable clones expressing shRNA plasmids via lentivirus were established in HCC cells.

Generation of overexpression plasmids

The pcDNA3-CRNDE expression plasmid was generated from the pcDNA3 vector using EcoRI and XhoI restriction sites. pLAS5w.PeGFP-I2-Bsd and pcDNA3.1/Myc-His A vectors were used to generate YY1-expressing pLAS5w.PeGFP-I2-Bsd YY1 and pcDNA3.1/Myc-His...
A-YY1, respectively, followed by 48 h of incubation with DNA and TurboFect Reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). All plasmids were prepared using an EasyPrep EndoFree Maxi Plasmid Extraction kit (DPT-BA17; Biotools Co., Ltd., Taipei, Taiwan).

**Quantitative RT-PCR**
Quantitative reverse transcription PCR (qRT-PCR) was performed on total cellular RNA extracted with TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the supplier’s protocol (MAN0016385). The GenBank sequences and primer sequences are listed in Additional file 11: Table S2. Primers were designed with Primer3 Input (Life Technologies, Carlsbad, CA, USA).

**Chromatin immunoprecipitation (ChIP) and re-ChIP assay**
Formaldehyde cross-linking and ChIP and re-ChIP assays were performed using a commercial kit [16] (Upstate Biotechnology, Lake Placid, NY). Chromatin was sonicated on ice to obtain DNA fragments of 0.3–1.5 kb (averaging 500 bp). A proportion of supernatant (20%) was used as total input control. To isolate a specific complex of interest, suitable antibodies were used to immunoprecipitate the chromatin components. Following removal of bound proteins, immunoprecipitated DNA was subjected to quantitative or regular PCR (35 cycles). Products amplified via regular PCR were separated on a 1.5% agarose gel and visualized via ethidium bromide staining. YY1 binding sites on the endogenous EGFR promoter were predicted using UCSC (https://genome.ucsc.edu/index.html; USA) and PROMO research (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promominit.cgi?dirDB=TF_8.3). The primer sequences are listed in Additional file 12: Table S3. ChIP-quantitative PCR (qPCR) was performed using a specific kit and the same primer pairs. Relative quantification of IP products was performed using the ΔΔCt method with normalization to control IgG. ΔCt for each IP gene was calculated as $2^{-\Delta\Delta C_{t_{(IP)}}} = 2^{-(C_{t_{(IP)}}-C_{t_{(control\, IgG)})}} - 2^{-(C_{t_{(control\, IgG)}}-C_{t_{(input)}})}$, with normalization of the relative level of DNA (in relation to input) specifically immunoprecipitated with YY1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (sc-7341), p300 (61401), and H3K9Ac (61663) (Active Motif, Carlsbad, CA, USA) antibodies to that immunoprecipitated with control IgG. The primer sequences are listed in Additional file 12: Table S3.

**Cell death and proliferation analysis**
Cells (3000 cells/dish) were grown in 10 mm culture dishes ($5 \times 10^6$ cells/dish). Cells were transfected with pcDNA3.1/Myc-His A-YY1 expression plasmid prior to harvesting in lysis buffer. IP experiments were performed with antibodies against Myc (Abcam, Cambridge, MA, USA). Immunoprecipitated complexes were separated via 10% SDS-PAGE and subjected to western blot with the indicated antibodies.

**Generation of luciferase-linked wild-type and mutant EGFR promoter constructs**
The Renilla luciferase plasmid pGL3TK (2 μg; originally from Promega) kindly provided by our colleague, Professor J. Horng was co-transfected with each reporter plasmid for normalization. Transcriptional activity was monitored using a dual luciferase reporter assay (Promega) and a luminescence reader (LMaxII384, Molecular Devices, Sunnyvale, CA) according to the manufacturer’s instructions. For each experiment, luciferase activity was normalized to that of Renilla luciferase. To generate transcription factor-binding mutants, putative binding sites were altered via site-directed mutagenesis (SDM) [17]. The primers used for the construction of mutant fragments are listed in Additional file 13: Table S4.

**Western blot analysis**
Protein expression was determined as outlined previously [18]. Following electrophoresis and transfer, membranes were incubated with the appropriate primary antibodies (Additional file 14: Table S5) followed by goat anti-mouse or goat anti-rabbit horseradish peroxidase secondary antibodies (Amersham, Buckinghamshire, UK). Signals were visualized using enhanced chemiluminescence (ECL, Amersham).

**Co-immunoprecipitation (co-IP) assay**
CRNDE-silenced and CRNDE overexpressing HCC cells were grown in 10 mm culture dishes ($5 \times 10^6$ cells/dish). Cells were transfected with pcDNA3.1/Myc-His A-YY1 expression plasmid prior to harvesting in lysis buffer. IP experiments were performed with antibodies against Myc (Abcam, Cambridge, MA, USA). Immunoprecipitated complexes were separated via 10% SDS-PAGE and subjected to western blot with the indicated antibodies.

**RNA immunoprecipitation (RIP)**
The RIP assay was performed as described previously [19] using an antibody against p300 protein (Active Motif, Carlsbad, CA, USA).

**Immunohistochemistry**
IHC staining was performed with specific primary antibodies (Additional file 14: Table S5) overnight at 4 °C as described previously [20]. Ki67 and EGFR were detected the next day using mouse antibody IgG conjugated to horseradish peroxidase (HRP; Santa Cruz Biotechnology) and dianaminobenzidine (DAB). All sections
Fractionation of nuclear and cytoplasmic RNA

Cells were washed twice with ice-cold PBS, followed by centrifugation for 5 min at 290 g and 4 °C. Cell pellets were resuspended in 1 mL RSB buffer (10 mM Tris–Cl (pH 7.4), 10 mM NaCl, and 3 mM MgCl₂) for 3 min on ice. After further centrifugation at 1500 g for 3 min at 4 °C, the supernatant was discarded. Subsequently, cells were lysed via gentle resuspension in 150 μL RSBG40 (10 mM Tris–Cl (pH 7.4), 10 mM NaCl, and 3 mM MgCl₂, 10% glycerol, and 0.5% Nonidet P-40) and samples centrifuged at 4500 g for 3 min at 4 °C. The supernatant (cytoplasmic) fraction was collected in a new Eppendorf tube and 1 mL TRIZol reagent added to each sample (both the nuclear pellet and cytoplasmic supernatant) for extraction of RNA.

Immunofluorescence staining

IF staining was performed according to a previous report [20]. Incubations were performed with primary rabbit anti-EGFR antibody (Cell Signaling Technology, Beverly, MA, USA) (1 μg/mL) overnight at 4 °C and secondary antibodies conjugated with Alexa-488 (1:200; Invitrogen) in PBS for 1 h at room temperature. Confocal laser scanning immunofluorescence microscopy (CLSM) was conducted using a Zeiss LSM510 META microscope (Carl Zeiss, Oberkochen, Germany). Images were analyzed using SM510 META Software and Adobe Photoshop 6.0.

Animal models

In model I, nude mice were subcutaneously injected with pcDNA3-control or CRNDE overexpressing Huh7 (5 × 10⁵) cells. Tumor sizes were measured as described in a previous study [21]. In model II, nude mice were subcutaneously injected with pcDNA3-control or CRNDE overexpressing Huh7 (1 × 10⁷) cells and treated with different drugs (DMSO, C646, sorafenib and sorafenib plus C646). At a tumor volume of 60 mm³, tumors were intraperitoneally injected (IP) three times a week with DMSO or C646 (1 mg/kg) followed by oral administration of DMSO or sorafenib (30 mg/kg) twice a week for 30 days. Representative tumors were excised 43 days after the treatment regimen and sizes measured as described previously t [21]. Animal experiments were conducted according to the guidelines of United States National Institutes of Health and the Chang Gang Institutional Animal Care and Use Committee Guide for the Care and Use of Laboratory animals (Approval No.CGU109-021).

Statistical analysis

Results were expressed as means ± s.d. of three independent experiments. Fisher’s exact or Mann–Whitney U test was used for between-group comparisons, where appropriate, and correlations between the results obtained with the two different methods analyzed with the Spearman’s test. Kaplan–Meier curves were constructed to analyze survival outcomes. Overall survival (OS) with death as an event was analyzed using the log-rank test. Statistical significance (p value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. p values < 0.05 were considered significant.

Results

CRNDE is highly expressed in human HCC and positively correlated with tumor size, pathological stage, and poor survival

CRNDE was upregulated in 240 paired in-house HCC specimens via qRT-PCR, compared with adjacent non-cancerous tissue, CRNDE was significantly upregulated (68%) in HCC tumor tissues (cohort 1, Fig. 1A). Similarly, CRNDE expression in HCC tumor tissue specimens was markedly higher relative to normal counterparts (cohort 1, Fig. 1B). These results were consistent with gene expression data from other publicly available datasets comprising several human hepatoma samples (cohort 2, The Cancer Genome Atlas data set; cohort 3, The Oncomine database from Wurmbach Liver Statistics), both showing high expression of CRNDE in HCC tumor tissues (Fig. 1C, D). Overall survival analysis of HCC patients further supported...
Fig. 1 (See legend on previous page.)
an association of high expression of CRNDE with shorter survival compared with low CRNDE expression (Fig. 1E). To further assess the significance of CRNDE expression in HCC, clinicopathological features were examined. Notably, CRNDE was increased at advanced pathological stage (cohort 1, Fig. 1F; \( p = 0.0019 \)), larger tumors (cohort 1, Fig. 1G; \( p = 0.0032 \)), virus infection (cohort 1, Additional file 1: Fig. S1A, HCV: \( p = 0.0124 \), HBV + HCV: \( p = 0.0008 \)), and cirrhosis (cohort 1, Additional file 1: Fig. S1B, \( p = 0.0470 \)). Our collective results support an oncogenic role of CRNDE in the biological progression of HCC.

**CRNDE promotes HCC tumorigenesis and sorafenib resistance**

We subsequently examined CRNDE expression in HCC cell lines. To this end, Hep3B and SkHep1 cells highly expressing CRNDE were selected for shRNA-based knockdown analysis (Fig. 2A) and CRNDE expression was induced in Huh7 and J7 cells expressing low levels of CRNDE via plasmid transfection. Knockdown of CRNDE significantly suppressed proliferation of both Hep3B and SkHep1 cells (Additional file 2: Fig. S2A, B). Conversely, CRNDE overexpression promoted Huh7 and J7 cell proliferation relative to cells expressing pcDNA3-control (Additional file 2: Fig. S2C, D). Simultaneously, in vivo experiments revealed that the final tumor volume was significantly greater in the CRNDE overexpression than the pcDNA3-control cell group (Additional file 2: Fig. S2E, F). Moreover, survival benefits were reduced under conditions of CRNDE overexpression, supporting an oncogenic role in HCC (Additional file 2: Fig. S2G). Additionally, CRNDE clearly suppressed migration and invasion of Hep3B and SkHep1 (Additional file 2: Fig. S2H, I, L, M). Conversely, migration and invasion abilities were enhanced in Huh7 and J7 cells overexpressing CRNDE (Additional file 2: Fig. S2J, K, N, O). In vivo suggested that the number of metastatic nodules in lungs was reduced in mice injected with CRNDE-silenced Hep3B (Additional file 2: Fig. S2P) and increased in livers of mice administered Huh7 overexpressing CRNDE (Additional file 2: Fig. S2Q). Silencing of CRNDE in Hep3B and SkHep1 cells promoted sensitization to sorafenib relative to the control shLuc group (Additional file 2: Fig. S2R, S) while CRNDE overexpression in Huh7 and J7 cells induced greater resistance to sorafenib relative to the corresponding pcDNA3-control groups (Additional file 2: Fig. S2T, U). These data are consistent with previous findings [11, 22, 23] that CRNDE is not only highly expressed but also markedly promotes tumorigenesis and sorafenib resistance in HCC specimens.

**CRNDE is positively correlated with upregulation of EGFR, thereby increasing proliferation and sorafenib resistance of HCC**

Recent findings support an oncogenic role of CRNDE in glioma, whereby its upregulation is positively correlated with activation of EGFR signaling [24]. In our experiments, EGFR downstream signaling molecules (such as p-EGFR and p-STAT3) were reduced upon CRNDE silencing in Hep3B and SkHep1 cells (Figs. 2B, Additional file 3: Fig. S3A, and conversely, enhanced upon CRNDE overexpression in Huh7 and J7 cells (Figs. 2C, Additional file 3: Fig. S3B). Notably, suppression of CRNDE in HCC cells markedly reduced EGFR at both mRNA and protein levels while its overexpression exerted the opposite effect (Fig. 2B–E, Additional file 3: Fig. S3A–D). qRT-PCR analysis was further conducted to confirm the effects of CRNDE silencing and overexpression (Additional file 3: Fig. S3E–H). Compared with the pcDNA3-control group, higher green fluorescence intensity of EGFR was detected in the CRNDE overexpressing group via immunofluorescence staining (Additional file 3: Fig. S3J). Compared with adjacent non-cancerous tissue (N), CRNDE was significantly upregulated in HCC tumor tissues (T) (cohort 1, Fig. 2F). To validate upregulation of EGFR in HCC specimens, EGFR expression patterns were examined in 240 paired HCC specimens. Our qRT-PCR findings revealed significant expression of EGFR in HCC tumor tissues (T) (Fig. 2G). Additionally, the available online dataset suggested that the EGFR expression was no different between normal (N) and tumor (T) in cohort 2 and cohort 3 (Additional file 4: Fig. S3J and S3K). However, EGFR expression in HCC tumor tissue specimens was markedly higher relative to normal counterparts in our 240 paired HCC specimens (Fig. 2G), and EGFR was significantly increased at advanced pathological stage (cohort 1, Additional file 4: Fig. S3L; \( p = 0.0319 \)), larger tumors (cohort 1, Additional file 4: Fig. S3M; \( p = 0.0346 \)), and virus infection (cohort 1, Additional file 4: Fig. S3N; HBV: \( p = 0.0118 \), HCV: \( p < 0.0001 \), HBV + HCV: \( p < 0.0001 \)). The Spearman correlation coefficient analysis confirmed a significant positive association of CRNDE with EGFR (Fig. 2H, Spearman \( r = 0.4239, p < 0.0001 \)). Simultaneous in vivo experiments disclosed that both the number of Ki67-positive cells and EGFR expression were increased in CRNDE overexpression relative to pcDNA3-control-transfected J7 cells, as determined via IHC staining (Fig. 2I). We further assessed whether EGFR affects HCC cell proliferation and sorafenib resistance under CRNDE overexpression conditions. In both pcDNA3-control and CRNDE overexpressing Huh7 and J7 cell lines, knockdown of EGFR led to a significant reduction in cell proliferation (Fig. 2J, Additional file 4: Fig. S3O) and suppression of CRNDE-induced sorafenib resistance.
Western blot experiments were conducted to ascertain siRNA-mediated inhibition of EGFR protein in pcDNA3-control and CRNDE overexpressing Huh7 and J7 cells (Fig. 2L, Additional file 4: Fig. S3Q). Our results indicate that CRNDE-mediated enhancement of EGFR expression is involved in proliferation and sorafenib resistance of HCC cells.

**CRNDE upregulates EGFR through mediating chromatin relaxation in HCC**

To establish the mechanisms by which CRNDE promotes EGFR mRNA transcription, the promoter assay was performed. The activity of the region 600 bp upstream of the promoter (Fig. 3A) was significantly reduced upon CRNDE silencing in Hep3B and SkHep1 cells (Fig. 3B, Additional file 4: Fig. S3P). Western blot analysis of EGFR and other proteins in Hep3B and SkHep1 cells revealed decreased expression of these proteins upon CRNDE silencing compared to control cells.

**Fig. 2** CRNDE is positively correlated with EGFR expression, thereby increasing proliferation and sorafenib resistance of HCC. A qRT-PCR analysis of CRNDE in various malignant compared with benign HCC cells (HepG2). B, C Western blot analysis of p-EGFR, EGFR, p-STAT3, and STAT3 protein levels in Hep3B cells (control and CRNDE depletion groups) and Huh7 cells (pcDNA3 and CRNDE overexpression groups). D, E qRT-PCR analysis of EGFR mRNA in Hep3B (control and CRNDE depletion groups) and Huh7 cells (pcDNA3 and CRNDE overexpression groups). F qRT-PCR analysis of CRNDE expression in 240 pairs of human HCC tissues (T) and adjacent normal (N) tissues. G qRT-PCR analysis of EGFR expression in 240 pairs of human HCC tissues (T) and adjacent normal (N) tissues. H Spearman correlation analysis of the clinical correlations between CRNDE and EGFR in HCC. I Representative IHC images of tumor xenografts stained with anti-Ki67 and anti-EGFR antibodies. Scale bar = 100 μm. J Huh7 cells were transfected with pcDNA3-control or CRNDE plasmids with siRNA-induced silencing of EGFR. Cell numbers were assessed after 48 h via the proliferation assay relative to control cells. K Huh7 cells transfected with pcDNA3-control and CRNDE overexpression plasmids with siRNA-induced silencing of EGFR were incubated with or without sorafenib and viability relative to control cells was assessed by MTT assay. L Western blot analysis of EGFR protein levels in Huh7 cells from pcDNA3-control and CRNDE overexpression groups with siRNA-induced silencing of EGFR. Statistical significance (p value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001)
Additional file 5: Fig. S4A), and conversely, enhanced with CRNDE overexpression in Huh7 and J7 cells (Fig. 3C, Additional file 5: Fig. S4B). Subcellular fractionation analysis using GAPDH and U1I as cytoplasmic and nuclear localization markers, respectively, showed that CRNDE primarily localizes in the nucleus of HCC cells (Additional file 5: Fig. S4C, D). Acetylated H3K9 and H3K27 are associated with alterations in transcription regulation [25, 26]. High H3K27Ac expression at the endogenous EGFR promoter was predicted using the UCSC Genome Browser (https://genome.ucsc.edu/). To further ascertain whether CRNDE is involved in the regulation of EGFR gene expression via chromatin relaxation, we examined the status of representative histone modifications. Acetylated H3K9 and H3K27 signals were reduced upon CRNDE silencing in Hep3B and SkHep1 cells (Fig. 3D, Additional file 5: Fig. S4E). Conversely, CRNDE overexpression enhanced acetylated H3K9 and H3K27 signals relative to control Huh7 and J7 cells (Fig. 3E, Additional file 5: Fig. S4F). Recent studies have shown that H3K9 and H3K27 are acetylated by p300, a member of the histone acetyltransferase family [27]. Accordingly, we investigated whether p300 is involved in the mediation of EGFR expression by CRNDE. Interestingly, knockdown of p300 inhibited EGFR protein and mRNA expression under CRNDE overexpression conditions in Huh7 cells (Fig. 3F, G) but had no effect in the corresponding pcDNA3-control group. Additionally, suppression of p300 reduced EGFR promoter activity in CRNDE overexpressing HCC cells (Fig. 3H, Additional file 5: Fig. S4G). To determine whether EGFR is transcriptionally activated by chromatin relaxation via p300 acetyltransferase activity mediated by CRNDE, the effect of C646 (a p300 acetyltransferase inhibitor [28, 29]) on EGFR expression was evaluated under CRNDE overexpression conditions. EGFR, H3K9Ac, and H3K27Ac protein signals were significantly induced in CRNDE overexpressing relative to the control cell groups. Notably, treatment with C646 led to a marked reduction in expression levels of these proteins (Fig. 3I, Additional file 5: Fig. S4H). Furthermore, C646 suppressed EGFR mRNA levels to a significant extent in Huh7 and J7 cells overexpressing CRNDE (Fig. 3J, Additional file 5: Fig S4I). In the luciferase reporter assay, treatment with C646 reduced EGFR promoter activity compared to that in DMSO under CRNDE overexpression (Fig. 3K, Additional file 5: Fig. S4J). These results suggest that CRNDE induces EGFR upregulation by increasing histone acetylation through the regulation of p300.

YY1 promotes HCC tumorigenesis via induction of EGFR transcription activity under conditions of CRNDE overexpression

We identified a series of transcription factors with binding sequences in the 600-bp region upstream of the promoter via the PROMO database (http://alggen.lsi.upc.edu/cgi-bin/promo_v3/promo/promo?g=TF_83) prediction, including C/EBPβ [30], c-myc [31], STAT3 [32], and YY1 [33], which could also interact with p300. To establish whether these transcription factors are involved in the regulation of EGFR in response to CRNDE, specific shRNAs were used for their knockdown in Huh7 cells overexpressing CRNDE. Interestingly, only the knockdown of YY1 inhibited the CRNDE-induced increase in EGFR mRNA (data not shown). Consistently, CRNDE-induced EGFR protein was inhibited upon YY1 silencing (Fig. 4A, B; Additional file 6: Fig. S5A, B), along with EGFR transcription activity (Fig. 4C; Additional file 6: Fig. S5C). The PROMO database predicts that the 600-bp region upstream of the promoter potentially contains two YY1 binding sites. Next, we designed variants of the YY1 binding sites in the EGFR promoter via site-directed mutagenesis (SDM), designated M1, M2, and M3 (Fig. 4D). EGFR promoter activity was inhibited in M2 and M3 variants under conditions of CRNDE overexpression (Fig. 4E, Additional file 6: Fig. S5D), supporting the involvement of YY1 in the transcriptional activation of EGFR by CRNDE. The ChIP assay was conducted to confirm YY1 binding to the promoter region of EGFR, ranging from 464 to 476 bp upstream of the transcription start site. A 249-bp PCR product containing the YY1-binding site on the EGFR promoter was obtained. The
Fig. 3 (See legend on previous page.)
Fig. 4 YY1 promotes HCC tumorigenesis via induction of EGFR transcription activity under conditions of CRNDE overexpression. A, B Western blot and qRT-PCR analysis of YY1 and EGFR protein and EGFR mRNA expression in Huh7 cells under conditions of CRNDE overexpression and silencing of YY1, respectively. C Relative luciferase activities of the EGFR promoter in Huh7 cells with CRNDE overexpression and shRNA-silenced YY1 were determined using the reporter assay. D Schematic representation of YY1 binding sites in the 0.6 kb EGFR promoter region. Partial wild-type (WT) and mutant YY1 binding sequences are listed below, including mutant YY1 at binding site 1 (M1), mutant YY1 at binding site 2 (M2), and double mutant YY1 at binding site 1 and 2 (M3). E Reporter assay of the relative luciferase activity of each promoter in Huh7 cells transfected with pcDNA3-control or CRNDE overexpression constructs. Luciferase activities of the EGFR promoter with mutant YY1 binding site 1 (M1), YY1 binding site 2 (M2), and YY1 binding site 1 and 2 (M3), taking wild-type (WT) EGFR promoter activity as 100%. F Schematic diagram of the promoter and other regions of the EGFR gene. The YY1 binding site and transcriptional initiation site (+1) are indicated. YY1 binding to the promoter region of EGFR, ranging from 464 to 476 bp upstream of the transcription start site, and a 249-bp PCR product containing the YY1-binding site on the EGFR promoter are shown. The positions of PCR primers and elements relative to the transcription initiation site are numbered. G-J YY1 binding to the EGFR promoter in Hep3B (control and CRNDE depletion) and Huh7 (pcDNA3 and CRNDE overexpression) cells was determined via ChIP PCR or ChIP-q-PCR. Images of representative gels of ChIP PCR products. The chromatin of cells subjected to the indicated treatments was immunoprecipitated with YY1 antibody or IgG control. Co-IP DNA for the YY1 binding region (EGFR) or negative control region (GAPDH) was detected using PCR. Statistical significance (p-value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (*p < 0.05; **p < 0.01; ***p < 0.001)
locations and sequences of PCR primers are depicted in Fig. 4F. YY1 binding was reduced upon CRNDE silencing in Hep3B and SkHep1 cells (Fig. 4G, H). CRNDE was used as a negative control (Additional file 6: Fig. S5G). GADPH was used as a negative control (Additional file 6: Fig. S5E). Conversely, CRNDE overexpression in Huh7 and J7 (Fig. 4I, J; Additional file 6: Fig. S5F) cells enhanced YY1 binding to the EGF promoter region. Based on these findings, we conclude that EGF transcriptional activity is induced by enhancing specific YY1 binding to its promoter region following CRNDE expression.

### CRNDE regulates H3K9Ac/p300/YY1 complex binding on the EGF promoter

p300 can act as both a coactivator and corepressor through interactions with YY1 and modulation of YY1-mediated transcriptional regulation [33]. Here, we further examined the hypothesis that p300 associates with YY1 as a transcriptional complex that regulates EGF under conditions of CRNDE overexpression. Data from the co-immunoprecipitation (co-IP) assay revealed decreased association of p300 with YY1 following CRNDE silencing in Hep3B (Fig. 5A). Conversely, CRNDE overexpression in Huh7 promoted the binding of p300 and YY1 (Fig. 5B). The RNA immunoprecipitation (RIP) assay confirmed the association of p300 with CRNDE in HCC (Fig. 5C, D; Additional file 7: Figs. S6A, B). These results suggest that p300 is not only associated with CRNDE in HCC but also further stimulates associations with YY1 under conditions of CRNDE overexpression. Furthermore, we observed decreased binding of p300/YY1 to the EGF promoter under CRNDE silencing in the re-ChIP assay (Fig. 5E, Additional file 7: Fig. S6C; negative control GADPH, Additional file 7: Fig. S6D, E). Conversely, CRNDE overexpression led to elevated binding to the EGF promoter (Fig. 5F, Additional file 7: Fig. S6F; negative control GADPH, Additional file 7: Fig. S6G, H). To further determine whether CRNDE influences p300/YY1 binding to the EGF promoter through chromatin relaxation, histone markers were assayed. Initially, H3K9Ac binding to the EGF promoter was markedly reduced upon CRNDE silencing (Fig. 5G, Additional file 7: Fig. S6I; negative control GADPH, Additional file 7: Fig. S6J, K) while CRNDE overexpression led to increased binding to the EGF promoter (Fig. 5H, Additional file 6: Fig. S5I; negative control GADPH, Additional file 7: Fig. S6M, N). Data from the further re-ChIP analysis revealed reduced H3K9Ac/YY1 binding to the EGF promoter upon CRNDE silencing (Fig. 5I, Additional file 7: Fig. S6O; negative control GADPH, Additional file 7: Fig. S6P, Q). Conversely, overexpression of CRNDE dramatically stimulated these interactions (Fig. 5J, Additional file 7: Fig. S6R; negative control GADPH, Additional file 7: Fig. S6S, T). However, no changes in the binding ability of H3K27Ac/YY1 to the EGF promoter under conditions of CRNDE expression were observed in the re-ChIP assay (data not shown). Finally, interactions of both H3K9Ac and YY1/H3K9Ac to the EGF promoter were enhanced under CRNDE overexpression, as determined from ChIP and re-ChIP analyses, and reduced following C646 treatment (Fig. 5K, L; negative control GADPH, Additional file 7: Fig. S6U, V). The collective results suggest that CRNDE mediates both chromatin relaxation and H3K9Ac/p300/YY1 complex binding at the EGF promoter region to regulate EGF transcriptional activity.

### CRNDE promotes CDX tumor growth in mice via p300/YY1/ H3K9Ac regulation of EGF

In HCC specimens, p300 enhances proliferation via epigenetic regulation of gene transcription [34–36]. Here, we assessed whether the promontory effects of p300 on HCC cell proliferation are related to CRNDE. Proliferation was significantly reduced in CRNDE overexpressing Huh7 and J7 cells and conversely, increased in pcDNA3-control cells with knockdown of p300 (Fig. 6A, B). Additionally, both migration and invasion abilities were increased in Huh7 cells with CRNDE overexpression and inhibited followed by knockdown of p300 or YY1 (Additional file 8: Fig. S7A, B). Quantification of Huh7 cell numbers using the transwell assay is presented in the right panel (Additional file 8: Fig. S7C, D). The collective results suggest
that CRNDE influences migration, invasion, and proliferation of HCC cells through p300 and YY1-mediated. Recent studies have shown that C646 effectively reduces the proliferation of HCC cells [35, 37, 38]. CRNDE overexpressing Huh7 and J7 cells exposed to C646 for 72 h displayed decreased viability in a dose-dependent manner compared to the pcDNA3-control groups (Fig. 6C, D). We further examined the potential therapeutic effect of C646 on HCC xenografts in nude mice. To this end, two groups of mice were subcutaneously injected with
Fig. 6 CRNDE promotes CDX tumor growth in mice via p300/YY1/H3K9Ac regulation of EGFR. A, B Huh7 and J7 cells were transfected with pcDNA3-control or pcDNA3-CRNDE plasmids with shRNA-silenced p300. Cell numbers were assessed with the proliferation assay. C, D Huh7 and J7 cells from pcDNA3-control and CRNDE overexpression groups were incubated with or without C646 and evaluated via the MTT assay. E Nude mice were subcutaneously injected with pcDNA3-control or CRNDE overexpressing J7 cells followed by vehicle control (DMSO) or C646 treatment and tumor growth monitored. Tumor sizes (cm) are indicated at the bottom. F, G Chart showing tumor sizes and weights from E. H Kinetic changes in tumor sizes of CDX following C646 treatment. I Survival rates of nude mice from E. Kaplan–Meier plots using standard tumor sizes (150 mm³) as a criterion for euthanasia. J qRT-PCR analysis of EGFR mRNA in tumors of mice. K Determination of EGFR protein expression in mouse tumors via immunohistochemistry. Positive staining (brown) in tumor tissues. Size reference (10 μm) is indicated. Statistical significance (p value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean±SD (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001)
J7 cells (transfected with pcDNA3-control or CRNDE overexpressing constructs). CRNDE overexpressing J7 cells induced a significant increase in tumor sizes examined on day 5 following injection. A representative tumor sample is depicted in Fig. 6E. Final tumor sizes were considerably greater in the CRNDE overexpressing than pcDNA3-control cell-injected groups (Fig. 6F). C646 treatment for 21 days induced the strongest inhibition of tumor growth, resulting in markedly smaller tumor sizes and weights in CRNDE overexpressing cancer cell line-derived xenograft (CDX) mice (Fig. 6G, H). As expected, the survival curve for the CRNDE overexpression group treated with C646 at the sub-pharmacologic dose was longer than that for untreated mice (Fig. 6I). Additionally, EGFR mRNA levels were significantly increased upon CRNDE overexpression and conversely, decreased following treatment with C646 (Fig. 6I). Consistently, IHC analysis revealed marked inhibition of EGFR protein expression in cells treated with C646 for 21 days compared to untreated cells overexpressing CRNDE (Fig. 6K). The collective results support the utility of the p300 inhibitor in suppressing tumor growth under conditions of CRNDE expression.

**CRNDE enhances sorafenib resistance through upregulation of EGFR with p300 in HCC**

The influence of p300 inhibitors on sorafenib resistance in HCC has not been extensively investigated to date. Based on the finding that C646 is effective against HCC in vitro and in vivo (Fig. 6), we further examined whether C646 could reduce cell viability in vitro upon co-treatment with sorafenib in CRNDE overexpressing Huh7 and J7 cells. Notably, sorafenib resistance was reduced in C646-treated CRNDE overexpressing Huh7 and J7 cells in a dose-dependent manner (Fig. 7A, Additional file 9: Fig. S8A). To assess the effects of C646 on sorafenib resistance of CRNDE overexpressing HCC cells in vivo, a mouse xenograft model was used. CRNDE overexpressing Huh7 xenografts displayed elevated tumor sizes compared to those expressing pcDNA3-control (Fig. 7B, C). Simultaneously, sorafenib resistance was enhanced under CRNDE overexpression, with no significant decrease in tumor size following sorafenib treatment relative to the pcDNA3-control (Fig. 7B, C). Only the pcDNA3-control group showed a marked reduction in tumor size following treatment with sorafenib (Fig. 7B, C). Under conditions of CRNDE overexpression, combined treatment with C646 and sorafenib for 21 days dramatically reduced resistance compared to treatment with sorafenib only (Fig. 7B–D). However, in the presence of C646 only, HCC tumor size was equivalent to that recorded following treatment with a combination of C646 and sorafenib under conditions of CRNDE overexpression (Fig. 7B, C). In terms of survival curves, pcDNA3-control mice died within 43 days, with a median survival of 27 days, while mice in the CRNDE overexpression group died within 10 days, with a median survival of 10 days (Fig. 7D, E). Treatment of pcDNA3-control mice with either sorafenib or C646 led to increased survival times (Fig. 7D). Sorafenib treatment under CRNDE overexpression conditions increased survival, with a median survival time of 30 days (Fig. 7E). Notably, C646-treated or sorafenib and C646-treated mice displayed significantly longer survival times compared to non-treated or sorafenib only groups (Fig. 7E). EGFR mRNA and protein expression were significantly induced upon CRNDE overexpression and reduced by both C646 alone and in combination with sorafenib (Fig. 7F, G). These results support a critical role of CRNDE in sorafenib resistance and suggest that the p300 inhibitor C646 exerts inhibitory and additive effects on sorafenib resistance to prolong overall survival through downregulation of EGFR expression.

**Discussion**

Currently, sorafenib is the most effective clinical drug for treatment of advanced HCC, but progressive therapy with this drug is often associated with emergence of resistance. Therefore, only 30% of HCC patients effectively benefit from sorafenib treatment. The mechanisms underlying sorafenib resistance remain to be established. Here, we have demonstrated an oncogenic role of CRNDE in sorafenib resistance of HCC, consistent with previous reports [13]. In our experiments, CRNDE was upregulated in HCC tumor specimens and induced elevation of EGFR expression, thereby increasing proliferation and sorafenib resistance. Mechanistically, CRNDE enhanced p300/YY1 binding to the EGFR promoter and simultaneously increased H3K9Ac mark expression at

(See figure on next page.)

**Fig. 7** CRNDE enhances sorafenib resistance through upregulation of EGFR with p300 in HCC. A Huh7 cells from pcDNA3-control and CRNDE overexpression groups were incubated with or without sorafenib and assessed via the MTT assay. B Nude mice were subcutaneously injected with pcDNA3-control or CRNDE overexpression Huh7 cells, followed by DMSO (vehicle control), C646 or sorafenib treatment, and tumor growth monitored. Tumor sizes (cm) are indicated at the bottom. C Chart showing tumor sizes and weights from B. D, E Survival rates of nude mice from B. Kaplan–Meier plots using the standard tumor size (200 mm³) as a criterion for euthanasia from pcDNA3-control and CRNDE overexpression groups. F, G qRT-PCR and western blot analyses of EGFR mRNA and protein expression in tumors of mice. H Working model of the function of the CRNDE/p300/H3K9Ac/YY1 complex in regulation of EGFR expression. Statistical significance (p value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001)
Fig. 7 (See legend on previous page.)
the promoter region, resulting in marked transcriptional activation of EGFR via the mediation of chromatin relaxation. Treatment with the p300 inhibitor C646 induced repression of EGFR transcription activity under CRNDE overexpression, with consequent suppression of HCC proliferation and enhancement of sensitivity to sorafenib, leading to additive effects in prolonging overall survival (Fig. 7H). Based on these findings, we propose that EGFR is positively upregulated by the epigenetic p300/YY1 complex in a manner dependent on CRNDE expression, thereby leading to sorafenib resistance. The regulatory role of CRNDE in the therapeutic resistance of HCC uncovered in this study offers a novel therapeutic avenue to combat tumor resistance to sorafenib.

Earlier studies have shown that EGFR signaling promotes sorafenib resistance in HCC, which could be effectively reversed by EGFR signaling inhibitors or siRNA knockdown of EGFR [3]. Additionally, CRNDE promotes glioma cell growth through the enhancement of the activation status of the EGFR pathway [24]. We observed that CRNDE promotes EGFR transcription activity to affect signaling. However, knockdown of epigenetic regulatory factors in HCC cells with low CRNDE expression reduced EGFR mRNA but not protein expression and further induced cell proliferation. EGFR protein expression may therefore be mediated to maintain normal biological function by other feedback mechanisms under conditions of low expression of CRNDE in HCC cells. In a previous study, the long non-coding RNA Inc-EGFR was shown to promote human tongue cancer proliferation via upregulation of EGFR mRNA and protein, potentially through direct binding [39]. Furthermore, EGFR-AS1 and EGFR expression are reported to be positively correlated, with EGFR-AS1 promoting the expression of EGFR by inhibiting degradation of its mRNA via direct interactions [40]. Long intergenic non-protein coding RNA 52 (LINC00052) promotes head and neck squamous cell carcinoma (HNSCC) progression through the regulation of EGFR by sponging miR-608 [41]. Our results indicate that CRNDE could modulate EGFR transcription through epigenetic regulators, further influencing tumorigenesis and growth in HCC. Both the GSEA and Oncomine datasets suggested that the EGFR expression was no difference between normal (N) and tumor (T). However, EGFR expression in our 240 paired HCC tumor (T) tissue specimens was markedly higher relative to normal (N), and it was increased at the advanced pathological stage, larger tumors, and virus infection. These outcomes are consistent with CRNDE. Thus, CRNDE possibly induces EGFR transcription under specific risk factors such as the hepatitis C virus (HCV) or hepatitis B virus (HBV)/HCV infection. Multiple lncRNAs regulate gene transcription through the activity as a scaffold that recruits p300 and enhances H3K9Ac [42–44]. p300 also acts as a coactivator that can interact directly or indirectly with multiple transcription factors to regulate downstream target genes [27, 33]. YY1 is a common multifunctional transcription factor that interacts with p300. In glioblastoma, YY1 acts as an oncogene that stimulates EGFR expression via miRNA effects, in turn, promoting cellular proliferation [45]. In this study, we showed that CRNDE regulates p300/YY1 interactions and promotes the binding of the complex to the EGFR promoter in association with H3K9Ac, in turn, influencing EGFR transcriptional activity.

In contrast to genetic mutations, epigenetic alterations are reversible. Given the importance of epigenetic marks in tumorigenesis, the identification of corresponding inhibitors has attracted extensive attention [36]. Considerable evidence has emerged showing that C646 influences tumor proliferation, metastasis, and drug resistance through suppression of lncRNA transcription owing to its function as a p300 inhibitor [46, 47]. C646 is under investigation as a promising candidate anti-tumor drug [48], but the specific mechanisms underlying its inhibitory effect on sorafenib resistance in HCC remain largely unclear. Data from the current study confirmed that tumor growth and sorafenib resistance in HCC were improved by the epigenetic inhibitor. Under conditions of high CRNDE expression, C646 treatment reduced chromatin relaxation, in turn, decreasing YY1 binding to the EGFR promoter and suppressing transcriptional activity, resulting in a significant decrease in sorafenib resistance and prolonged overall survival. Additionally, we also found no significant regulation of EGFR mRNA and protein levels by sorafenib treatment. These results are consistent with The phase 3 STORM trial, which compared sorafenib with placebo and showed that EGFR is not significantly different between the placebo group and the sorafenib-treated group (GSE109211 ([49]). Thus, EGFR played an important mediator in regulating sorafenib resistant phenotype, but its expression level is only repressed by C646 treatment.

In summary, we have uncovered a novel molecular mechanism involving CRNDE that underlies HCC proliferation and sorafenib resistance through chromatin relaxation and induction of epigenetic regulatory complex binding to the EGFR promoter to enhance its transcriptional activity. Our findings support the development of effective therapeutic strategies based on p300 inhibitors that significantly inhibit HCC growth and decrease sorafenib resistance to extend total median survival. CRNDE overexpression clearly contributes to the development and progression of HCC, supporting its utility as a target for blocking or reversing tumor progression and sorafenib resistance. Overall, p300 inhibitors, such as C646, may present an efficient alternative
therapeutic option to combat sorafenib resistance in patients with advanced HCC.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-022-01326-3.

Additional file 1: Fig. S1. (A and B) Data are presented as relative expression levels in tumor tissues. CRNDE expression was significantly increased in HCC patients with HCV, HBV+HCV and cirrhosis. Statistical significance (P-value) was calculated with the two-tailed Student's t-test for a single comparison between two groups. Data are presented as mean ± SD (*P<0.05; **P<0.01; ***P<0.001).

Additional file 2: Fig. S2. (A and B) Hep3B and SKHep1 cells were infected with shRNAs of CRNDE (shCRNDE1 and shCRNDE2) and cell numbers assessed relative to control cell groups via the proliferation assay. (C and D) Huh7 and J7 cells were transfected with pcDNA3 control or pcDNA3-CRNL. Cell numbers were assessed after 48 h using the proliferation assay and expressed relative to control cells. (E) Nude mice were subcutaneously injected with the two groups (pcDNA3-control and CRNDE overexpression, N=11 per group) of J7 cells. Representative tumors were excised after 21 days. Sizes (cm) are indicated at the bottom. (F) Examination of cell line-derived xenograft tumor sizes of 17 cells expressing pcDNA3-control or CRNDE overexpression plasmids in nude mice. (G) Survival rates of nude mice injected with J7 cells containing pcDNA3-control or CRNDE overexpression plasmid. Kaplan–Meier plots were generated, using standard tumor sizes (200 mm3) as a criterion for expressing pcDNA3-control or pcDNA3-CRNL. Cell numbers were assessed after 48 h using the proliferation assay and expressed relative to control cells. (H) Nude mice were subcutaneously injected with the two groups (pcDNA3-control and CRNDE overexpression, N=11 per group) of J7 cells. Representative tumors were excised after 21 days. Sizes (cm) are indicated at the bottom. (I) Examination of cell line-derived xenograft tumor sizes of 17 cells expressing pcDNA3-control or CRNDE overexpression plasmids in nude mice. (J) Relative luciferase activities of each promoter were determined in J7cells with conditions of CRNDE depletion by reporter assay, taking control (shLuc) EGFR promoter as 100%. (K) Relative luciferase activities of each promoter were determined in J7 cells with conditions of CRNDE overexpression by reporter assay. Statistical significance (P-value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (***P<0.001).

Additional file 3: Fig. S3. (A) IF analysis of EGFR protein expression in Huh7 cells (pcDNA3-control and CRNDE-overexpression) with siRNA-induced silencing of CRNDE. EGFR mRNA levels in SkHep1 cells from control and CRNDE depletion groups were determined by qRT-PCR. (B) EGFR mRNA expression in SkHep1 cells transfected with control and CRNDE-depletion groups was determined by qRT-PCR. (C) EGFR mRNA expression in J7 cells from pcDNA3-control and CRNDE overexpression groups was determined by qRT-PCR. (D) EGFR mRNA expression in J7 cells from pcDNA3-control and CRNDE overexpression groups was determined by qRT-PCR assay. (E–H) qRT-PCR analysis of the efficiency of CRNDE silencing in Hep3B and SKHep1 cells and CRNDE overexpression in Huh7 and J7 cells. (I) IF analysis of EGFR protein expression in Huh7 cells (pcDNA3-control and CRNDE overexpression).

Additional file 4: Fig. S3. (J) The EGFR mRNA expression level in human HCC. (T) (n=369) compared to adjacent normal tissue (N) (n=160) were no different, as calculated from The Cancer Genome Atlas datasets. (K) There are no different expressions of EGFR in HCC (n=35) compared with liver (n=10) specimens from the Wurmbach Liver dataset in the Oncomine database. (L–N) Data are presented as relative expression levels in different tumor tissues. EGFR expression was significantly increased in a paired in-house HCC patients with higher pathological stages, larger tumors, and HCC, HCV, HBV+HCV infection. (O) J7 cells were transfected with pcDNA3-control or pcDNA3-CRNL plasmids with siRNA-induced silencing of EGFR. Cell numbers were assessed after 48 h using the proliferation assay and expressed relative to control cells. (P) J7 cells from pcDNA3-control and CRNDE-overexpression with siRNA-induced silencing of EGFR were incubated with or without sorafenib and cell viability relative to control cells was assessed after 72 h using the MTT assay. (Q) EGFR protein levels in J7 cells from pcDNA3-control and CRNDE-overexpression with siRNA-induced silencing of EGFR were determined by western blot. Statistical significance (P-value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (***P<0.001).

Additional file 5: Fig. S4. (A) Relative luciferase activities of each promoter were determined in SkHep1 with conditions of CRNDE depletion by reporter assay, taking control (shLuc) EGFR promoter as 100%. (B) Relative luciferase activities of each promoter were determined in J7 cells with conditions of CRNDE overexpression by reporter assay, taking control (pcDNA3) EGFR promoter as 100%. (C and D) Subcellular fractionation of CRNDE in Hep3B and SkHep1 cells. qRT-PCR analysis using GAPDH and U1 as cytoplasmic and nuclear localization markers revealed that CRNDE is primarily located in the nucleus. (E) H3K9Ac, H3K27Ac, and Histone H3 protein levels in SkHep1 cells from control and CRNDE depletion groups were determined by western blot. (G) H3K9Ac, H3K27Ac, and Histone H3 protein levels in J7 cells from pcDNA3-control and CRNDE-overexpression groups were determined by western blot. (H) Relative luciferase activity of EGFR promoter in J7 cells transfected with CRNDE-overexpression plasmid and using shRNA for silencing of p300 were determined by reporter assay. (J) J7 in pcDNA3-control and CRNDE-overexpression groups were incubated with DMSO or 20 μM C646 and EGFR, H3K9Ac, H3K27Ac, and histone H3 protein levels were assessed after 48 h by western blot. (I) EGFR mRNA level was assessed by qRT-PCR. (J) J7 in pcDNA3-control and CRNDE-overexpression groups were incubated with DMSO or 20 μM C646 and relative luciferase activities of the promoter were assessed after 48 h by reporter assay. Statistical significance (P-value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (***P<0.001).

Additional file 6: Fig. S5 (A and B) YY1 and EGFR protein and EGFR mRNA expression in J7 cells under conditions of CRNDE overexpression and silencing of YY1 using shRNA were determined by western blot and qRT-PCR analysis, respectively. (C) Relative luciferase activity of EGFR promoter in J7 cells transfected with CRNDE overexpression plasmid and using shRNA for silencing of YY1 were determined by reporter assay. (D) Reporter assay of relative luciferase activity of each promoter in J7 cells transfected with pcDNA3-control and CRNDE overexpression constructs. Luciferase activities of EGFR promoter with mutant YY1 binding site 1 (M1), YY1 binding site 2 (M2), and YY1 binding site 1 and 2 (M3), taking wild-type (WT) EGFR promoter activity as 100% (E and F) ChIP assay of YY1 on J7 cells on the EGFR promoter in Hep3B transfected with control and CRNDE-depletion constructs and in Huh7 cells transfected with pcDNA3-control and CRNDE overexpression plasmids. Co-IP DNA for negative control region (GAPDH) was detected via q-PCR. (G and H) YY1 binding to the EGFR promoter in SkHep1 cells transfected with control or CRNDE-depletion constructs and in J7 cells transfected with pcDNA3-control and CRNDE overexpression plasmids were determined by ChIP assay. Images of representative gels of ChIP PCR products. Chromatin of cells subjected to the indicated treatments was immunoprecipitated with YY1 antibody or IgG control. Co-IP DNA was detected with PCR for the YY1 binding region (EGFR) or negative control region (GAPDH). Statistical significance (P-value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (***P<0.001).

Additional file 7: Fig. S6. (A and B) Hep3B and J7 extracts were immunoprecipitated using mouse or p300 antibody. Immunoprecipitated of p300-associated RNA was reverse transcription and quantitative PCR were performed and presented as relative fold of RNA enrichment. GAPDH
was used as a negative control. (C and F) p300/YY1 complex binding to the EGFR promoter in Hep3B transfected with control and CRNDE-depletion constructs and in HuH7 cells transfected with pcDNA3-control and CRNDE overexpression plasmids were determined by re-ChIP assay. (D and E) re-ChIP assay of p300/YY1 complex binding to the EGFR promoter in Hep3B and SkHeP1 transfected with control and CRNDE-depletion constructs. (G and H) re-ChIP assay of p300/YY1 complex binding to the EGFR promoter in HuH7 and J7 transfected with control and CRNDE overexpression constructs. (J and L) H3K9Ac on EGFR promoter in SkHeP1 transfected with control and CRNDE-depletion constructs and in J7 cells transfected with pcDNA3-control and CRNDE overexpression plasmids were determined by ChIP assay. (J and K) ChIP assay of H3K9Ac on the EGFR promoter in Hep3B and SkHeP1 transfected with control and CRNDE-depletion constructs. (M and N) ChIP assay of H3K9Ac on the EGFR promoter in HuH7 and J7 transfected with control and CRNDE overexpression constructs. (O and R) H3K9Ac/YY1 complex binding to EGFR promoter in SkHeP1 transfected with control and CRNDE-depletion constructs and in J7 cells transfected with pcDNA3-control and CRNDE overexpression plasmids were determined by re-ChIP assay. (P and Q) re-ChIP assay of H3K9Ac/YY1 complex binding to the EGFR promoter in Hep3B and SkHeP1 transfected with control and CRNDE-depletion constructs. (S and T) re-ChIP assay of H3K9Ac/YY1 complex binding to the EGFR promoter in HuH7 and J7 transfected with control and CRNDE overexpression constructs. (U) H3K9Ac binding to EGFR promoter in HuH7 cells with CRNDE overexpression and incubation with or without C646 treatment were determined by ChIP assay. (V) H3K9Ac/YY1 binding to EGFR promoter in HuH7 cells with CRNDE overexpression and incubation with or without C646 treatment were determined by re-ChIP assay. Statistical significance (P value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (P<0.05, **P<0.01, ***P<0.001).

Additional file 8: Fig. S7. (A and B) Matrigel migration and invasion of pcDNA3-control or CRNDE overexpression HuH7 cell groups with shRNA-mediated silencing of p300 (shp300#1 and shp300#2) and YY1 (shYY1#1 and shYY1#2), determined via transwell assay. Scale bar = 200 μm. (C and D) HuH7 cells were quantified based on transwell assay data (A and B). Statistical significance (P value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (P<0.05; **P<0.01; ***P<0.001).

Additional file 9: Fig. S8. (A) J7 cells from pcDNA3-control and CRNDE overexpression groups were incubated with or without sorafenib and cell viability relative to control cells assessed after 72 h using the MTT assay. Statistical significance (P value) was calculated with the two-tailed Student’s t-test for a single comparison between two groups. Data are presented as mean ± SD (P<0.05; **P<0.01; ***P<0.001).

Additional file 10. The shRNA and siRNA oligonucleotides sequences.

Additional file 11. The quantitative reverse transcription-PCR (qRT-PCR) primer sequences.

Additional file 12. The Chromatin immunoprecipitation (ChIP) and re-ChIP primer sequences.

Additional file 13. The primers used for construction of EGFR promoter mutant fragments.

Additional file 14. The western blot and immunohistochemistry primary antibodies against.

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Author contributions

Y-CL and K-HL conceived and designed the study, drafted the manuscript, critically revised the manuscript, and approved the final manuscript. Y-CL acquired the data. Y-CL, Y-HL, H-CC, P-SH, C-JL, Y-SL, C-CL, C-JY, Y-HH, C-CL, and K-HL analyzed and interpreted the data. All authors read and approved the final manuscript.

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Availability of data and materials

All other data are within the main text or its Supplementary Information.

Declarations

Ethics approval and consent to participate

All the paired human HCC specimens were obtained from the Taiwan Liver Cancer Network (TLCN). The protocol was approved by the Medical Ethics and Human Clinical Trial Committee at Chang-Gung Memorial Hospital (IRB: 20200004580). Animal treatments were performed following the Guideline of Experimental Animal Ethics Committee of the Chang-Gung University (IACUC Approval No.: CGU108-015).

Consent for publication

All authors have read and approved the manuscript including content and presentation to the clinical epigenetic.

Competing interests

The authors have no competing interests to disclose.

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