CXCL12/CXCR7/β-arrestin1 Biased Signal Promotes Epithelial-to-Mesenchymal Transition of Colorectal Cancer by Repressing Mirnas Through YAP1 Nuclear Translocation

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Research

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Abstract

Background: CXCR7 is an atypical chemokine receptor that transmits biased signal independent of G-protein activation. However, whether CXCL12/CXCR7 biased signal activation plays an essential role in colorectal cancer (CRC) progression and metastasis remains obscure.

Methods: The functional role of CXCL12/CXCR7 biased signal in CRC was investigated by RNA-sequencing, Transwell assay and in vivo tumor xenografts. YAP1 nuclear translocation and molecular mechanisms were determined by cell transfection, luciferase activity assay, immunofluorescence, coimmunoprecipitation and immunohistochemistry and RT-qPCR analysis.

Results: In this study, CXCR7 CXCL12/overexpression promotes Epithelial-to-mesenchymal transition (EMT) and upregulates the expression of stem marker doublecortin-like kinase 1 (DCLK1) in CRC cells with concurrent repression of miR-124-3p and miR-188-5p. Further luciferase assay prove that these miRNAs could regulate EMT by direct targeting vimentin and DCLK1. More importantly, CXCL12/CXCR7/β-arrestin1-mediated biased signal induces YAP1 nuclear translocation, which functions as a transcriptional repressor by interacting with Yin Yang 1 (YY1) and recruiting YY1 to the promoter of miR-124-3p and miR-188-5p. Pharmacological inhibitor of YAP1 recapitulates the anti-tumorigenesis and anti-metastasis effects of YAP1 depletion upon CXCR7 activation in tumor xenografts. Clinically, the expression of CXCR7 was positively correlated with nuclear YAP1 levels and EMT markers.

Conclusions: Our findings revealed the novel role of YAP1 nuclear translocation in promoting EMT of CRC by repressing miR-124-3p and miR-188-5p through CXCL12/CXCR7/β-arrestin1 biased signal activation. These findings highlight the potential of targeting YAP1 nuclear translocation in hampering CXCL12/CXCR7 biased signal-induced metastasis of CRC.

Background

Colorectal cancer (CRC), known as the third most common cancer worldwide, accounts for about 10% of all new cancer cases globally, remaining the second most frequent cause of cancer-related deaths[1]. Despite the improved treatment strategies, nearly half of patients are challenged with metastasis, showing a poor 5-year survival rate of < 10%[2, 3].

CXCR4 has long been thought to be the unique receptor for CXCL12 and play important roles in chemotaxis, inflammation, and cancer dissemination and organotropic liver metastasis of CRC. Until recently, CXCR7, an atypical chemokine receptor 3 (ACKR3), was identified to bind to CXCL12 with even higher affinity than CXCR4[4]. CXCR7 has a dramatic effect on the signaling response resulting from CXCR4 activation because CXCR7 and CXCR4 can form heterodimers, whereby CXCR7 changes the conformation of the CXCR4/G-protein complexes and abrogates its signaling[5, 6]. Although the seven trans-membrane protein CXCR7 could bind to CXCL12, it functions in a G-protein-independent manner. It has been proposed that CXCR7 interacts with β-arrestin (β-arr) for a biased signal transduction as it fails

to activate heterotrimeric G proteins. Much less is known about CXCL12 signaling via CXCR7, which was initially considered to be a sink for CXCL12[7]. It has been recently reported that CXCR7 interacts with β-arr2 and recruits MAPK proteins for the phosphorylation of ERK[8]. Notably, the serine/threonine residues present at the C-terminal of CXCR7 are essential for β-arr recruitment after receptor activation [9]. However, it remains unknown how CXCL12/CXCR7 biased signal plays a role in the progression and metastasis of CRC.

Epithelial-to-mesenchymal transition (EMT) is a critical step that initiates metastasis. It refers to epithelial cells acquire the mesenchymal phenotype to facilitate invasion and metastasis. EMT has been linked with stemness, as evidenced by the reactivation of embryonic signaling pathways such as Wnt and Notch [10]. Doublecortin-like kinase 1 (DCLK1), a microtubule associated protein kinase, is overexpressed in colorectal cancer and specifically marks cancer stem cells (CSC) in the intestine[11]. It has been demonstrated that DCLK1$^+$ intestinal cells of *Apc*$^{Min/+}$ mice display higher pluripotency and pro-survival signaling[12]. DCLK1 has been shown to regulate EMT and associate with metastasis and poor prognosis in several cancers[13, 14], suggesting DCLK1 is a potential therapeutic target for cancer invasion and metastasis[15].

The Hippo signaling pathway has emerged as a tumor suppressive pathway that acts to control the transcriptional activity of Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), which plays a central role in regulating EMT plasticity and metastatic potential[16]. YAP/TAZ activity underlies several key hallmarks of cancer through promoting tumor invasion, metastasis and acquisition of CSC characteristics[17]. Activation of the pathway can be triggered by environmental cues such as cell contact, cytoskeletal remodeling, nutrient status and activation of G-protein-coupled receptors[18–20]. Sequential phosphorylation and activation of mammalian STE20-like kinase 1 and 2 (MST1/2), and then large tumor suppressor kinases 1/2 (LATS1/2), culminates in the phosphorylation, and then degradation of the YAP and TAZ. Conversely, when the pathway is deactivated, YAP/TAZ accumulates in the nucleus, associate with DNA-binding proteins, most notably transcriptional enhanced associate domain (TEAD), driving a pro-oncogenic transcriptional program[21].

CXCR7 is markedly overexpressed in tumors compared with normal tissues and growing studies have demonstrated an association of CXCR7 upregulation with tumor growth, neovascularization, invasion and metastasis[22, 23]. Activation of CXCL12/CXCR7 biased signal pathway may be critical for tumor progression by promoting cancer cell invasion and stem cell phenotype. It has been shown that nuclear YAP1 levels positively correlate with tumor grade, metastasis, and induction of CSC-like activity[24]. However, whether CXCL12/CXCR7 biased signal contributes to CRC invasion and metastasis through YAP1 nuclear translocation and subsequent target gene regulation remains elusive. In this study, we unraveled the intricate roles of CXCL12/CXCR7 biased signal on EMT and stemlike phenotype. More important, we revealed that YAP1 nuclear translocation activated by the biased signal plays a pivotal role in the regulation of EMT by repressing miRNAs. All this raises the exciting possibility that blocking CXCL12/CXCR7 biased signal pathway may be a valid strategy to inhibit metastasis of CRC.

Materials And Methods

Cell culture and transfection

Human colorectal adenocarcinoma cell lines HCT116, HT29 and SW620 were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium (Corning, USA) containing 10% fetal bovine serum (FBS) (Biological Industries, Israel) and 1% penicillin-streptomycin (Gibco, USA) at 37 °C in a humid atmosphere (5% CO_2).

To determine the role of miR-124-3p and miR-188-3p, cells were transfected with 100 nM of these miRNA mimics or inhibitors (GenePharma, China) using Lipofectamine 2000 (Invitrogen, USA) and Opti-MEM medium (Gibco, USA) for 48 h according to manufacturer's instructions. Silencing of YAP1 and YY1 were performed by using YAP1 and YY1 siRNAs (GenePharma, China). The YAP1 siRNA sequence-1 and -2 were 5'-CACCAAGCUAGAUAAAGAATT-3' and 5'-AGCUUCUCUGCAGUUGGGAGCUGTT-3' respectively. The YY1 siRNA sequence-1 and -2 were 5'-GACGACUACAUUGAACAAATT-3' and 5'-CCCAAACAACUGGCAGAAUTT-3' respectively. To overexpress YAP1 and YY1 in cancer cells, the cells were transfected with plasmids of Flag-YAP5SA (addgene #27371) and HA-YY1 (addgene #104395) by using lipofectamine 2000 (Invitrogen, USA). Cancer cells with overexpression and knockdown of CXCR7 were constructed by infecting lentivirus expressing CXCR7 or shRNA targeting CXCR7 (Genechem, China). CXCL12 (SDF-1α) was used at 100ng/ml and was purchased from PeproTech (PeproTech, USA).

Transcriptome sequencing of mRNA and miRNA

Total RNA was extracted from HCT116 cells infected with vector control and lenti-CXCR7 (HanBio, Shanghai) using Trizol reagent (Invitrogen). RNA integrity and concentration was evaluated by Bioanalyzer 2100 system (Agilent, CA). Library construction and transcriptome sequencing was performed on Hiseq 4000 sequencer and miRNA sequencing was performed on BGISEQ-500 platform by Beijing Genomics Institute (Wuhan, China). Raw reads were filtered using SOAPnuke software and mapped to the human reference genome hg38 using HISAT and Bowtie2 software. Differentially expressed genes (fold change $\geq$ 1.5 with $p < 0.05$) were analyzed using DESeq2.

Immunoblotting and Co-Immunoprecipitation

Colon tissue and cell pellets were lysed in RIPA buffer (Beyotime, China) supplemented with complete protease inhibitor mixture (Roche Pharmaceuticals, China) and 1mM PMSF (Sigma, USA). Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) was used to separate cytoplasmic and nuclear fractions. Protein content of the extracts was determined using BCA Protein Assay Kit (Beyotime, China). Whole cell lysates or separated fractions were resolved by SDS/PAGE and transferred to PVDF membranes (Millipore, USA). The membranes were blocked and incubated overnight at 4 °C with primary antibodies against CXCR7 (ab72100) (Abcam, UK), E-cadherin (3195), N-cadherin (13116), Vimentin (5741), Phospho-YAP (Ser127) (13008), Notch1 (3608), Cleaved Notch1 (4147) (Cell Signaling Technology, USA), YAP1 (13584-1-AP), YY1 (22156-1-AP), β-arr1 (15361-1-AP), β-arr2 (10171-1-AP),

DCLK1 (21699-1-AP), FLAG tag FLAG (20543-1-AP) and HA tag (51064-2-AP) (Proteintech, USA) followed by HRP-conjugated secondary antibodies (7074 and 7076) (Cell Signaling Technology, USA). Densitometric analyses of the bands were normalized with β-actin (66009-1-Ig), GAPDH (60004-1-Ig) (Proteintech, USA) and Histone H3 (4499) and Lamin B1 (13435) (Cell Signaling Technology, USA) functioning as a loading control.

For immunoprecipitation, precleared whole cell lysates were incubated with indicated antibodies or with normal anti-rabbit IgG (2729) (Cell Signaling Technology, USA), and protein A/G magnetic beads (BEAVER, China) at 4 °C overnight. Immunoreactive products were visualized using Fluorchem FC3 system (ProteinSimple, USA) by chemiluminescence (Millipore, USA) and quantified by using NIH image program (Image J).

Immunofluorescence staining

Cells stimulated with 100ng/ml CXCL12 (PeproTech, USA) with or without AMD3100 (Sigma, USA) were fixed in 4% formaldehyde (Beyotime, China) for 20 min at room temperature. Cells were then washed with PBS, permeabilized in 0.5% Triton X-100 in PBS for 10 min and blocked in 5% BSA for 60 min at room temperature. After cells were incubated at 4 °C overnight with anti-YAP (1:250, 13584-1-AP) or (1:250, CL594-66900), anti-YY1 (1:200, 22156-1-AP), anti-β-arr1 (1:150, 15361-1-AP) or anti-β-arr2 (1:150, 10171-1-AP). Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:1000, CA11008S, Invitrogen, USA) was incubated for 1 h at room temperature. DAPI (Beyotime, China) was used for nuclear counterstain for 5 min. Images of representative cells for each labeling condition were captured at ×64 magnitudes under confocal laser imaging system (TCS SP8, Leica, Germany).

RT-qPCR analysis

Total RNA was reverse-transcribed by the miScript reverse transcription kit (Qiagen, USA) according to the manufacturer's protocol. miScript SYBR Green PCR kit (Qiagen, USA) and miR-124-3p and miR-188-5p specific primers were used to determine the expression of mature miRNAs. RUN6B was used as an internal control. The expression of CXCR7, Vimentin, DCLK1, E-cadherin and Notch1 mRNA was converted into cDNA using ReverTra Ace qPCR RT Kit (TOYOBO, Japan). RT-qPCR was carried out using SYBR Green Realtime PCR Master Mix (TOYOBO, Japan) in the 7500 Fast Real-Time PCR System (Applied Biosystems, USA) as described previously[25] . The sequences of primers for PCR are listed in Supplementary Table 1.

Immunohistochemistry

Colorectal tissues were fixed in 4% polyformaldehyde followed by paraffin embedding and then the sections were deparaffinized, rehydrated and immersed in 10 mM citrate buffer for heat-induced antigen retrieval. The tissues were stained with antibodies against CXCR7 (ab72100) (Abcam, UK), Vimentin (5741) (Cell Signaling Technology, USA) and anti-YAP1 (13584-1-AP) (Proteintech, USA) in accordance with manufacturer's suggestions. The staining results were scanned using a digital slide scanning

system (Pannoramic Scan, 3DHISTECH Ltd.) and semi-quantified of mean density (IOD/area) by Image-Pro Plus 6.0 software (IPP, USA).

Luciferase reporter gene assay

To confirm that DCLK1 and Vimentin were the target genes of miR-188-5p and miR-124-3p, the 3'UTR of DCLK1 and Vimentin containing miRNA binding sites were synthesized and digested by *Xba* I and cloned into GV272 vector (GeneChem, Shanghai) to make the luciferase constructs. Wild type and mutant inserts were confirmed by sequencing. GV272 empty vector was used as control (Con081). To investigate the effect of transcriptional factor YY1 on the promoter activity of miR-188-5p and miR-124-3p, the promoter sequences of these miRNAs containing YY1 binding sites were digested by Nhe I and Hind Ⅲ and cloned into pGL3-basic plasmid (Promega).

The luciferase reporter assay was performed as previously described[25]. Briefly, HCT116 cells were co-transfected with wild type or mutant DCLK1-3'UTR-Luc/Vimentin-3'UTR-Luc firefly luciferase constructs (100 ng) and 40 nM miR-188-5p/miR-124-3p mimics using lipofectamine 2000 reagent. 2 ng of pRL-SV40 plasmid was transfected to monitor transfection efficiency. Luciferase activity was determined by a dual-luciferase reporter assay system (Promega). All assays were performed in triplicates for twice.

Animal models

Villin-CXCR7 transgenic mice (*Villin-CXCR7*) overexpressing CXCR7 in intestinal epithelial cells (IEC) were generated by Cyagen Biosciences Inc. (Guangzhou, China). To establish AOM/DSS-induced inflammatory colonic adenocarcinoma, wild type C57BL/6 mice and *Villin-CXCR7* mice (aged at 8 weeks, n = 5) were treated by a single intraperitoneal injection of AOM (10 mg/kg, Sigma-Aldrich) and subsequent oral administration of 1% DSS (MP Biomedicals) in drinking water ad libitum for 7 consecutive days and then returned to normal drinking for 14 days. *Villin-CXCR7*mice were injected intraperitoneally daily with YAP1 inhibitor verteporfin (10mg/kg, MedChemExpress LLC) in the vehicle (40% PEG300, 5% Tween 80 and 45% normal saline) or vehicle control for 8 consecutive weeks. After 3 cycles of DSS treatment, these mice were euthanized and the colons were excised and opened longitudinally to evaluate the number and size of tumors with a caliper.

Six-week-old female athymic *Balb/c-nu/nu* mice were purchased from Charles River Laboratories (Beijing, China) and maintained in a specific pathogen-free environment. 2×10^6 HCT116 cells infected with Lenti-CXCR7 and vector control were injected into the tail veins of nude mice. The mice injected with $HCT116^{LV\text{-}CXCR7}$ cells were administered with YAP1 inhibitor verteporfin (10mg/kg, MedChemExpress LLC) or solvent control. After 3 weeks, the mice were sacrificed and livers and lungs were removed for histologic examination. All animal experiments were approved by the Institutional Animal Care and Use Committee of Capital Medical University.

Clinical specimens

Human CRC tissue specimens and adjacent normal mucosa were obtained from XuanWu Hospital of Capital Medical University (Beijing, China) and were confirmed by pathological analysis. The experiment was approved by the Institutional Review Board of XuanWu Hospital of Capital Medical University. The informed consents were obtained from all patients.

Bioinformatics

Gene Expression Profiling Interactive Analysis (GEPIA) (http://GEPIA.cancer-pku.cn/index.html) was a web server to analyze gene sequencing data from the cancer genome atlas (TCGA) and Genotype-Tissue Expression (GTEx). GEPIA was used to perform pair-wise gene expression correlation analysis of CXCR7 and Vimentin or DCLK1 in CRC patients. The association of the expression of Vimentin and DCLK1 with overall survival was analyzed by Kaplan-Meier (KM) plotter (https://kmplot.com/analysis) in CRC. The patients were divided with high and low gene expression levels using the auto-select best cutoff and log-rank *P* value was shown.

Statistical analysis

All data were presented as mean ± SD and statistical data were analyzed using SPSS 20.0 or graphpad prism 8.0. Statistical differences among multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett (multiple comparisons to the same control) post hoc tests. Student's *t* test was used to compare differences between two groups. The Pearson correlation was used to evaluate potential correlations between miR-124-3p and Vimentin expression in paired CRC tissues. A $P < 0.05$ was considered to indicate statistical significance.

Results

CXCR7 overexpression promotes EMT and upregulates the expression of stem marker DCLK1

CXCR7 is highly expressed in many cancers and has predominantly pro-metastatic roles in cancer[26]. EMT is the initial step of metastasis which is characterized by decreasing of E-cadherin in epithelial cells as well as increasing of vimentin in mesenchymal cells. To explore whether CXCR7 contribute to EMT and stemness, we performed RNA-sequencing in CXCR7-overexpressing HCT116 cells and control cells. Among the differentially expressed genes, vimentin and ZEB1 were significantly increased and the intestinal stem cell marker DCLK1 was also markedly enhanced when CXCR7 was overexpressed (Fig. 1A). The most significantly upregulated genes were listed in Supplementary Table 2. To further confirm the association of CXCR7 on the regulation of EMT and DCLK1, colorectal cells of HCT116, HT29 and SW620 cells were infected by lentivirus expressing CXCR7 (LV-CXCR7) and siRNA targeting CXCR7 (CXCR7-KD). The results showed that Vimentin were prominently upregulated with concurrent downregulation of E-cadherin in CXCR7-overexpressing cells compared with control (Fig. 1B, C). Transwell assay indicated the enhanced invasive capacity in CXCR7-overexpressing CRC cells than that of control cells (Supplementary Fig. 1). In contrast, CXCR7 knockdown produced opposite effects (Fig.1C), indicating CXCR7 overexpression triggered EMT in CRC. In parallel, as a stem marker, DCLK1 has similar

changes in line with EMT progression, suggesting overexpression of CXCR7 enhanced multipotency with the progression of CRC.

To further determine the clinical relevance of CXCR7 expression with EMT and cell stemness, we analyzed the correlation of expression of CXCR7 with vimentin and DCLK1 in human CRC tissues by Gene Expression Profiling Interactive Analysis (GEPIA) (http://GEPIA.cancer-pku.cn/index.html) using TCGA datasets. Notably, we found a robust and statistically significant association between vimentin, DCLK1 and CXCR7 (R = 0.58 and 0.49 respectively, $p = 0$) (Fig.1D). Furthermore, we evaluated the expression of vimentin and DCLK1 as prognostic gene signature by using CRC datasets, we found that high vimentin and DCLK1 mRNA expression significantly correlated with a worse prognosis (Fig. 1E). Collectively, these findings suggest CXCR7 overexpression promotes EMT and upregulates the expression of stem marker DCLK1, which is associated with poor clinical outcome.

CXCR7 overexpression contributes to EMT by repressing miR-124-3p and miR-188-5p

In order to investigate the mechanism that CXCR7 signal activation contributes to CRC progression and EMT, miRNA sequencing was performed in above HCT116 cells ($HCT116^{Control}$ and $HCT116^{LV\text{-}CXCR7}$), and the significantly upregulated and downregulated miRNAs are listed in Supplementary Table 3. Among the differentially expressed miRNAs, miR-124-3p and miR-188-5p were significantly downregulated in $HCT116^{LV\text{-}CXCR7}$ comparing with $HCT116^{Control}$, indicating that these miRNAs were downregulated by CXCR7 activation (Fig. 2A). To verify the results, we determine the expression of these miRNAs in CRC cells in response to activation of the CXCL12/CXCR7 axis. As a result, RT-QPCR analysis showed that miR-124-3p and miR-188-5p were significantly downregulated upon activation of the CXCL12/CXCR7 axis. In contrast, knockdown of CXCR7 markedly elevated these miRNAs in HCT116 and HT29 cells (Fig. 2B). CXCL12 is the common ligand for activation of CXCR4 and CXCR7. As the atypical chemokine receptor, CXCR7 could mediate biased signal activation independent of activation of heterotrimeric G proteins. Importantly, to elucidate whether activation of CXCR7 repressed the expression of miRNAs through biased signaling, we used AMD3100, the specific inhibitor of CXCR4, to exclude the effect of activation of CXCL12/CXCR4. The results showed that CXCL12/CXCR7 produced a similar effect on the downregulation of miR-124-3p and miR-188-5p with or without the treatment of AMD3100. Specifically, the biased signal of CXCL12/CXCR7 can profoundly suppressed miR-124-3p and miR-188-5p. (Fig. 2B).

To gain an insight into the molecular mechanism of these miRNAs on CRC progression and EMT, two mRNA target-predicting algorithms (MiRDB and Targetscan) were utilized to identify the potential downstream targets of miR-124-3p and miR-188-5p. Vimentin and DCLK1 were predicted to be the potential target gene of miR-124-3p and miR-188-5p respectively. To verify this, HCT116, HT29 and SW620 cells were transfected with miR-124-3p and miR-188-5p mimics and inhibitors respectively, and the results showed that the expression of vimentin and DCLK1 was significantly suppressed by miR-124-3p and miR-188-5p mimics, while substantially enhanced by miR-124-3p and miR-188-5p inhibitors (Fig. 2C, 2D). Of note, we also observe the regulation of DCLK1 by miR-124-3p, possibly due to indirect impact on gene expression imposed by multiple targets of miRNAs. Furthermore, luciferase reporter assay was

performed to confirm the direct binding of miR-124-3p and miR-188-5p with Vimentin 3'-UTR and DCLK1 3'-UTR. The predicted binding sites of the miRNAs with wild type and mutant 3'-UTR luciferase reporter constructs are shown in Fig. 2E. In line with these results, the binding was abolished by mutation of the binding site of these miRNAs on Vimentin 3'-UTR and DCLK1 3'-UTR, suggesting that these miRNAs could directly bind to Vimentin 3'-UTR and DCLK1 3'-UTR and regulate its expression at the post-transcriptional level (Fig.2F). Taken together, these results establish that CXCR7 biased signal activation contributes to CRC progression and EMT by repressing miR-124-3p and miR-188-5p which targeting Vimentin and DCLK1.

YAP1 manipulates CXCR7 biased activation-induced EMT by repressing miR-124-3p and miR-188-5p

EMT is a highly dynamic and reversible process, conferring cancer cells with the plasticity for distant dissemination and stemness properties. YAP1/TEAD transcriptional activation has emerged as the core regulator in EMT by cooperation with ZEB1 and AP-1[27]. In this case, we hypothesize that YAP1 promotes EMT and stemness via the regulation of miRNAs in CRC cells.

As shown in Fig. 3A, YAP1 silencing strongly enhanced the expression of miR-124-3p and miR-188-5p. Of relevance, the mRNA levels of stemness marker DCLK1 and mesenchymal marker vimentin were remarkably reduced with YAP1 depletion (Fig. 3B). Consistently, knockdown of YAP1 with two different siRNAs led to a pronounced decrease of DCLK1 and vimentin proteins in CRC cells (Fig. 3C).

As a transcriptional coactivator, YAP1 translocated into nucleus to exert the regulatory effects with TEAD that has DNA-binding domains. To further interrogate whether nuclear YAP1 orchestrates the expression of vimentin and DCLK1 by regulating miRNAs, we transfected CRC cells with a construct expressing the constitutive active form of YAP1 (YAP5SA), resistant to LATS-mediated phosphorylation, which directly leads to YAP1 nuclear translocation. Intriguingly, enforced expression of YAP5SA rescued, to a large extent, the marked downregulation of DCLK1 and Vimentin caused by YAP1 knockdown (Fig. 3D). More importantly, enforced expression of YAP5SA resulted in a drastic reduction of miR-124-3p and miR-188-5p and it could also attenuate the prominent elevation of these miRNAs in YAP1 knockdown cells (Fig. 3E). The results suggest that YAP5SA could rescue the suppression of EMT marker caused by silencing of YAP1 through repression of miR-124-3p and miR-188-5p. Altogether, these data reveal that nuclear YAP1 is critically involved in promoting EMT and stemness by repressing miR-124-3p and miR-188-5p in CRC cells.

CXCR7/β-arr1-mediated biased signal induces YAP1 nuclear translocation in CRC cells

Since nuclear YAP1 plays a crucial role in regulation of EMT by repression of miR-124-3p and miR-188-5p, next, we explored whether CXCL12/CXCR7 biased activation promoted YAP1 nuclear translocation. Notably, CXCL12 induced the reduction of YAP1 in the cytoplasm paralleled with YAP1 nuclear accumulation, which was potentiated by overexpression of CXCR7 (Fig. 4A). The CXCL12-induced YAP1 nuclear accumulation was also confirmed by immunofluorescence analysis in HCT116 and HT29 cells,

showing the time-dependent nuclear translocation of YAP1 and reached plateau after 60 min stimulation by CXCL12 (Fig. 4B, 4C and Supplementary Fig. 2).

β-arr1, previously known as a cytosolic regulator and scaffold of GPCR signaling, has recently been revealed to translocate to the nucleus mediating receptor endocytosis and signal transduction. Therefore, it is likely that β-arr1 shuttles between the cytoplasm and the nucleus mediating CXCL12/CXCR7 biased signal activation. To assess whether β-arr1 could functionally contribute to YAP1 activity regulation consequently to CXCL12/CXCR7 axis activation, we performed co-immunoprecipitation analysis in whole cell lysates derived from HCT116 cells, and found that endogenous YAP1 physically interacted with β-arr1 (Fig. 4D). Here we ask whether the interaction between β-arr1 and YAP1 facilitate the nuclear translocation of YAP1 with the concurrent nuclear shuttling of β-arr1 or not? Immunofluorescence analysis indicated that although CXCL12 stimulation at early stage (for 90 min) led to substantial YAP1 nuclear translocation, β-arr1 and β-arr2 were predominantly located in cytoplasm and not involved in the YAP1 nuclear co-translocation (Fig. 4E). However, we do observe the nuclear translocation of β-arr1 at later stage (> 6 h) of CXCL12 stimulation (Supplementary Fig. 3). Furthermore, nuclear-cytoplasmic fractions indicated that CXCL12 induced dramatic reduction of YAP1 in the cytoplasm accompanied by the significant increase of nuclear YAP1. Concurrently, AMD3100, as a CXCR4 antagonist, was used to rule out any effects of CXCL12/CXCR4 signaling activation (Fig. 4F). These results indicated that CXCL12/CXCR7 biased signal activation substantially promoted nuclear translocation of YAP1. Noticeably, β-arr1 does not exhibit a simultaneous nuclear translocation at early stage of CXCL12 stimulation in CRC cells (Fig. 4F). Then we ask whether β-arr1 is required for YAP1 nuclear translocation upon CXCL12/CXCR7 biased signal activation? As shown in Fig. 4G and 4H, we demonstrated that knockdown of β-arr1 but not β-arr2 interfered with the nuclear translocation of YAP1 in response to CXCL12 stimulation.

These results revealed that CXCL12/CXCR7 biased signal activation promoted YAP1 nuclear translocation by recruiting β-arr1 in the cytoplasm, which could not be abrogated by AMD3100 pretreatment. Overall, these findings establish that CXCR7 activation by CXCL12 induces YAP nuclear enrichment in CRC cells and prove the critical role of β-arr1 in transducing CXCL12/CXCR7-dependent YAP1 cytoplasmic-nuclear shuttling.

YAP1 inhibits miR-124-3p and miR-188-5p expression by recruiting YY1 to the promoter

Nuclear YAP1 functions as a potent transcriptional cofactor by binding with TEAD1. YAP1/TEAD1 interacts with other transcriptional factors to regulate the expression of target genes[28]. Generally, YAP1/TEAD complex activates the oncogenic downstream genes to trigger carcinogenesis. To further explore the inhibitory effects of nuclear YAP1 on the expression of miR-124-3p and miR-188-5p, TransmiR v2.0 database (http://www.cuilab.cn/transmir) and interface of mirTrans (http://mcube.nju.edu.cn/jwang/lab/soft/mirtrans/) were used to predict the transcriptional factor binding sites at the promoter of miR-124-3p and miR-188-5p. As a result, Yin Yang 1 (YY1) is predicted to be the potentially common transcriptional factor that could suppress the expression of these miRNAs.

We hypothesized that YAP1 functions as a transcriptional repressor by interacting with YY1, transcriptionally repressing the expression of miR-124-3p and miR-188-5p, thereby promoting EMT and metastasis. To prove this, HCT116 and SW620 cells are used to confirm whether YY1 was involved in the regulation of miR-124-3p and miR-188-5p expression. As shown in Fig. 5A, miR-124-3p and miR-188-5p were robustly upregulated by YY1 silencing. Expectedly, as shown in Fig. 5B-5C, knockdown of YY1 led to remarkably downregulation of mesenchymal marker vimentin in SW620 cells, consistently, the expression of stem cell marker DCLK1 was also profoundly impaired with YY1 depletion. These results indicated that YY1 was implicated with the regulation of EMT and stemness. To further assess the specificity of YY1 in promoting stemness and EMT, we transfected CRC cells with a construct expressing YY1 with HA-tag which led to YY1 overexpression. Remarkably, cells with YY1 silencing, characterized by DCLK1 reduction was rescued by re-expression of YY1 at protein levels (Fig. 5D). Moreover, RT-PCR was used to determine the expression of miR-124-3p and miR-188-5p. As results, the level of miR-124-3p and miR-188-5p were significantly increased by YY1 silencing which can be strongly reduced by enforced expression of HA-YY1 (Fig. 5E).

In order to address our hypothesis that YAP1 inhibit miR-124-3p and miR-188-5p expression by recruiting YY1, co-immunoprecipitation analysis was performed in HCT116 cells, and we found that endogenous YAP1 could physically interact with endogenous YY1 in HCT116 cells. Moreover, when HCT116 cells were transfected with flag- YAP5SA and HA-YY1, YAP1 and YY1 was found to interact in the nucleus by exogenously overexpression using Co-IP assay (Fig. 5F). Consistently, immunofluorescence analysis further showed the nuclear co-localization of YAP1 and YY1 in CRC cells (Fig. 5G). Moreover, luciferase reporter assay showed that the promoter activities of miR-124-3p and miR-188-5p were robustly enhanced compared with control vector of pGL3-baisc, which was significantly hampered upon YY1 overexpression. (Fig. 5H). Taken together, these results unveiled that YAP1 inhibit miR-124-3p and miR-188-5p expression by recruiting YY1 to the promoter, therefore, YAP1/YY1 cooperate to regulate EMT plasticity, stemness and metastasis in CRC cells.

YAP1 inhibitor suppresses CXCL12/CXCR7-induced EMT and tumor metastasis *in vitro and in vivo*

In light of the crucial role of YAP1 in regulating EMT by repressing miR-124-3p and miR-188-5p via interacting with YY1, we wonder if YAP1 inhibitor verteporfin could blunt CXCL12/CXCR7-induced EMT and distant metastasis. As shown in Fig.6A, CXCL12/CXCR7 biased activation strongly upregulated the expression of DCLK1, which was attenuated by verteporfin. We further detect the effects of verteporfin on distant metastasis when injected with HCT116 cells overexpressing CXCR7 via tail veins. The results indicated that CXCR7 overexpression facilitated more distant metastasis such as lung metastasis, which was greatly impaired by verteporfin. The representative Hematoxylin and Eosin (HE) staining of metastatic nodules in lungs were illustrated in Fig. 6B.

To investigate the role of YAP1 in colitis-associated carcinogenesis and progression upon CXCL12/CXCR7 biased activation in vivo, wild type (WT) and *villin-CXCR7* transgenic mice (*villin-CXCR7*) were treated with AOM and DSS for 3 cycles as described in methods. We found that AOM/DSS exposure

seriously aggravated colonic inflammation and tumor burden in *villin-CXCR7*mice compared with WT mice, as indicated by the larger size of colonic adenocarcinoma. Importantly, Verteporfin, a YAP1 inhibitor which disrupts YAP-TEAD interactions, led to a significant reduction of the colonic adenocarcinomas (Fig. 6C). To investigate whether YAP1 inhibitor hindered EMT process in CRC by regulation of miR-124-3p and miR-188-5p in vivo, RT-qPCR analysis indicated that pharmacological inactivation of YAP1 with verteporfin reversed the repression of miRNAs in AOM/DSS induced *villin-CXCR7* mice (Fig. 6D). Further IHC and Western blot analysis showed that vimentin and DCLK1 were highly expressed in colonic adenocarcinoma of *villin-CXCR7* mice, which was abrogated by Verteporfin (Fig. 6E, 6F). These results suggest that YAP1 inhibitor verteporfin recapitulates the anti-tumorigenesis and anti-metastasis of YAP1 depletion upon the activation of CXCL12/CXCR7, highlighting the therapeutic potential of targeting YAP1 in the control of CRC progression and metastasis.

CXCL12/CXCR7/β-arr1-induced YAP1 nuclear translocation is associated with EMT and metastasis in human CRC tissues

To extend current knowledge to colorectal cancer patients, we collected 22 pairs of human CRC specimens and adjacent normal colon tissues for immunohistochemistry, Western Blot and RT-PCR analysis. As shown in Fig. 7A, CXCR7 was highly expressed in human CRC tissues compared with normal colon tissues, particularly with higher expression in metastatic CRC than non-metastatic counterpart. YAP1 was remarkably overexpressed in nuclei in CRC tissues whereas predominantly expressed in cytoplasm in adjacent normal colon tissues. More importantly, there was also a higher expression of nuclear YAP1 in metastatic CRC than non-metastatic counterpart. Similarly, the expression of vimentin was higher in CRC particularly metastatic CRC tissues compared with adjacent normal colon tissue (Fig. 7A). Further protein analysis exhibited prominently high expression of YAP1 and DCLK1 in CRC tissues compared with adjacent normal tissues (Fig. 7B). To explore the potential link between YAP1, vimentin, DCLK1 and upstream CXCR7, we used public accessible online tool GEPIA (http://GEPIA.cancer-pku.cn/index.html). It revealed a significant positive correlation of YAP1 and vimentin ($R = 0.24$, $p < 0.001$). The expression of YAP1 and DCLK1 also displays a potent correlation ($R = 0.27$, $p < 0.001$) (Fig.7C). Notably, the expression levels of CXCR7 strongly correlated with those of YAP1 ($R = 0.33$, $p < 0.001$) in CRC tissues. miR-124-3p expression were significantly reduced in human CRC tissues and was statistically significant negatively correlated with the expression of Vimentin at mRNA levels (Pearson $R = -0.3386$, $p = 0.0326$) (Fig. 7D, 7E), highlighting that miR-124-3p functions as a tumor suppressive miRNA in CRC tissues. Taken together, these data suggest that CXCL12/CXCR7/β-arr1 biased activation triggered YAP1 nuclear translocation, which contributes to EMT and CRC metastasis by repressing miR-124-3p and miR-188-5p in clinical CRC specimens (Fig.8).

Discussion

Increasing evidence has shown that CXCR7 is highly expressed in aggressive colon carcinoma, playing an important role in CRC progression and metastasis[22, 29]. However, the underlying mechanism remains elusive. After CXCR7 was identified as another novel CXCL12 receptor with higher affinity[4], considerable

efforts have been made to investigate the functional role and mechanism of CXCR7. CXCR7 has been initially reported to form heterodimers with CXCR4 to modulate CXCR4-mediated cellular responses[5], but some contradicting results also exist[30, 31]. Recently, CXCR7 has been proposed to transmit a biased signal by recruiting β-arrestins to activate MAPK by binding to chemokine CXCL12 without activating typical G-protein signaling pathways[32]. Ligand binding to CXCR7 results in activation of G Protein-Coupled Receptor Kinase 2 (GRK2) and receptor phosphorylation with β-arr2 recruitment. Moreover, the homodimers of CXCR7 but not heterodimers were verified. Notably, CXCR7 functions as a β-arr1-biased receptor and play an essential role but not auxiliary role in potentiating cell migration[31]. In the present study, we first demonstrated that CXCL12/CXCR7/β-arr1 biased signal promoted EMT and CRC metastasis by inducing YAP1 nuclear translocation and consequent repressing the miRNAs through recruiting YY1, highlighting the important role of CXCR7 in cancer metastasis and could serve as a potential therapeutic target.

β-arrestins (β-arr1 and β-arr2) serve as scaffolds and adapters in receptor trafficking and signal transduction. They are cytosolic proteins connecting the receptors to various cytoplasmic effector pathways such as MAPK cascades [33]. Recent studies uncovered a novel role of β-arrestins in guiding receptor-mediated extracellular signals from cell membrane and transmiting through the cytoplasm to the nucleus by a complicated signaling network. In the present study, we revealed that CXCL12/CXCR7 biased signal transmit predominantly through β-arr1 to facilitate YAP1 nuclear translocation. We further confirmed that interaction of YAP1 with β-arr1 by Co-IP analysis. These results suggest that β-arr1 could bind to YAP1 and promote the accumulation of YAP1 in the nucleus.

Although β-arr1 and β-arr2 share roughly 70% sequence identity and function similarly in GPCR signaling, β-arr1 tends to shuttle between the cytoplasm and the nucleus at steady state, while β-arr2 is excluded from the nucleus due to the presence of a Nuclear Export Signal (NES)[34]. Importantly, recent findings shed light on the role of scaffold protein β-arr1 in mediating the interplay between endothelin-1 receptor (ET-1R) and the hippo pathway[35]. Other findings also display the function of β-arr1 as a cytoplasm-nucleus messenger in GPCR signaling and elucidate an epigenetic mechanism for transcriptional regulation stimulated by delta-opioid receptor[36]. In this study, we found that β-arr1, interacts with YAP1 in the cytoplasm at early stage of CXCL12 stimulation, triggering YAP1 nuclear translocation and recruiting YY1 to the YAP-TEAD transcriptional complex. In the canonical Hippo pathway, LATS1/2 kinases phosphorylate YAP1 and thereby suppress its nuclear translocation and transcriptional activity. It is possible that CXCL12/CXCR7/β-arr1 biased signal affects the regulation of phosphorylation of YAP1, further studies on the mechanism are warranted.

EMT, a crucial process in which cells lose their epithelial features and acquire mesenchymal characteristics, is associated with migration, invasion and metastasis of cancer cells[37]. During EMT, epithelial cells gradually lose the expression of E-cadherin, while concomitantly increasing the expression of mesenchymal markers Vimentin. EMT can be induced through the upregulation of transcriptional regulators such as SNAIL, ZEB1 and TWIST[38]. In this study, we found that activation of CXCL12/CXCR7

axis upregulates the expression of EMT marker as shown by increased expression of vimentin and ZEB1. Furthermore, we characterized the regulation of vimentin expression by miR-124-3p at the post-transcriptional level, luciferase activity assay confirmed the direct binding of miR-124-3p to 3'UTR of vimentin and repressed its expression. Interestingly, it has been reported that ZEB1 is also a target gene of miR-124-3p and the upregulation of ZEB1 could promote EMT and stemness by acting as the most important EMT-activating transcription factor[39].

DCLK1, a member of the protein kinase superfamily and the double cortin family, is highly expressed in several types of cancers and has been recently identified as a tumor stem cell marker in CRC[40, 41]. Increasing studies have demonstrated that DCLK1 can promote EMT through downregulating several key tumor suppressor microRNAs and activating NF-κBp65 through the PI3K/Akt pathway[13, 42], indicating targeting DCLK1 may be a therapeutic option for hampering CRC metastasis. In this study, we found that CXCR7 upregulated DCLK1 by repressing miR-188-5p expression, which represents the potential of stemness and EMT.

Among the oncogenic drivers activated by GPCR, YAP1 plays a pivotal role in the progression and metastasis of CRC, linking with worse prognosis of cancer patients[43, 44]. YAP1 is a dual function transcription factor and could function as either a transcriptional activator or repressor which depends on the proteins it interacts. Recently YAP1 has been shown as a potent transcriptional coactivator to form a complex with ZEB1 to promote EMT plasticity[28]. Additionally, YAP1 could also form a transactivation complex with AP-1 and ZEB1 to activate predominantly tumor-promoting genes[27]. YAP1 also functions as a transcriptional repressor to repress transcription of key cell cycle kinase inhibitor p27[45]. In this study, we identified YAP1 could interact with YY1 to repress the transcription of miR-124-3p and miR-188-5p. YY1, a member of the GLI-Kruppel family of zinc finger DNA binding proteins, has been identified as a transcriptional repressor and employs multiple mechanisms to achieve the specific repression[46]. YY1 was predicted to bind to the promoter of these miRNAs by TransmiR 2.0 program. Furthermore, luciferase activity assay proved that YY1 could indeed bind to the promoter and repress the transcription of these miRNAs.

Increasing findings have unveiled the correlation between miR-124-3p and miR-188-5p and CRC progression, indicating that these miRNAs could serve as potential targets for CRC therapy[47]. Intriguingly, we elucidated the regulatory role of these miRNAs on EMT and stemness. More importantly, miR-124-3p was also significantly downregulated in CRC compared to adjacent normal colon tissues, suggesting miR-124-3p could serve as a potential diagnostic biomarker and therapeutic target in CRC. These findings were consistent with previous studies[48]. However, we did not find the significant difference of miR-188-5p between CRC and normal tissues, indicating the function of miR-188-5p depend on the role of target genes and cell context.

In summary, our studies revealed a novel mechanism and clinical significance of CXCL12/CXCR7 biased signal in promoting EMT and invasion in CRC progression. Although GPCR represents the highly

druggable targets, some signaling downstream of a GPCR may be responsible for drug adverse effects. Biased signals hold great promise to become next-generation GPCR drugs with less side effects due to their potential to preferentially activate desired signaling pathways[49]. Targeting CXCL12/CXCR7 biased signal would open a new avenue for improving drug designs to achieve higher efficacy and selectivity. We elucidate that biased activation of CXCL12/CXCR7/β-arr1 promotes EMT by repressing miRNAs (miR-124-3p, miR-188-5p) through YAP1 nuclear translocation and recruitment of YY1. More importantly, YAP1 inhibitors attenuate AOM/DSS-induced inflammatory colonic adenocarcinoma in *villin-CXCR7* transgenic mice as well as impairing distant metastasis of $HCT116^{CXCR7}$ tumor xenografts in nude mice. These findings provide new insights into the nuclear YAP1 in mediating EMT and invasion, highlighting the potential of targeting YAP1 nuclear translocation in hampering CXCL12/CXCR7 biased signal-induced metastasis of CRC.

Conclusions

The present work unveiled that activation of CXCL12/CXCR7/β-arrestin1 biased signal promoted EMT of CRC by repressing miR-124-3p and miR-188-5p through YAP1 nuclear translocation and recruitment of YY1. Targeting CXCL12/CXCR7 biased signal would open a new avenue for improving drug designs. These findings highlight the potential of targeting YAP1 nuclear translocation in hindering CXCL12/CXCR7 biased signal-induced metastasis of CRC.

Abbreviations

EMT: Epithelial-to-mesenchymal transition; CRC: colorectal cancer; DCLK1: Doublecortin-like kinase 1; CSC: cancer stem cells; TAZ: transcriptional coactivator with PDZ-binding motif; MST1/2: mammalian STE20-like kinase 1 and 2; LATS1/2: large tumor suppressor kinases 1/2; YAP: Yes-associated protein; YY1: Yin Yang 1; ACKR3: atypical chemokine receptor 3; β-arr: β-arrestin; GRK2: G Protein-Coupled Receptor Kinase 2; TEAD: transcriptional enhanced associate domain.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Capital Medical University. Informed consent was obtained from all the patients.

Consent for publication

We have obtained consents to publish this paper from all the participants of this study.

Availability of data and materials

Please contact the corresponding author for all data requests.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XY conceived the study, designed the research and drafted the manuscript. MS carried out the Western blot, RT-qPCR analysis and animal experiments, JS participated in the luciferase assay, XW collected the clinical specimens, DW participated in the animal experiments, XL participated in the design and lab managements, XQ helped revise the manuscript.

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Figures

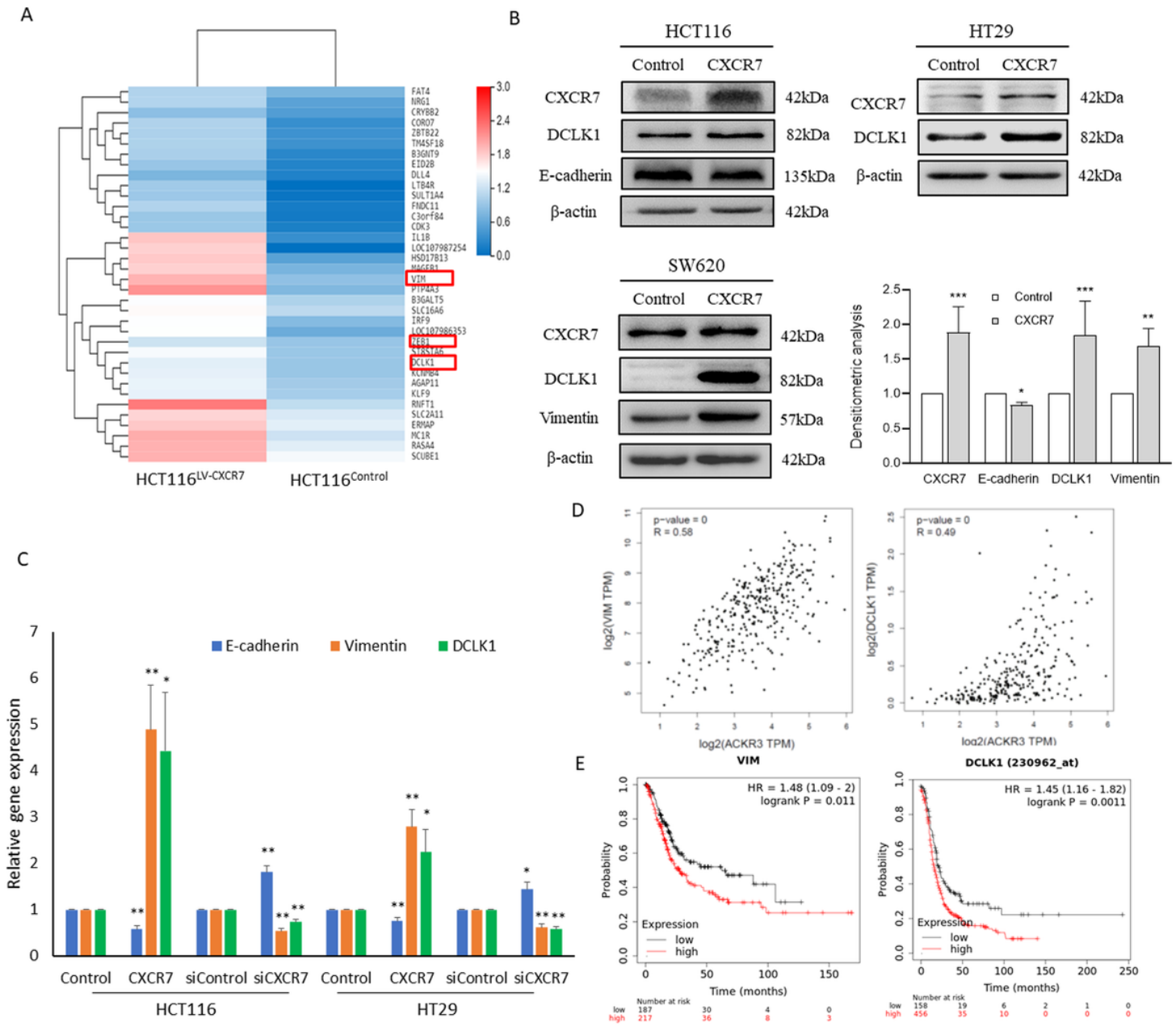

Figure 1

CXCR7 overexpression promotes EMT and upregulates the expression of stem marker DCLK1. (A) RNA-sequencing was performed in HCT116 cells infected with lentiviral expressed CXCR7 (lenti-CXCR7) and control. Hierarchical clustering analysis of differentially expressing mRNAs between HCT116Control and HCT116LV-CXCR7. (B) Western blot analysis of the expression of CXCR7, DCLK1, E-cadherin or Vimentin in HCT116, SW620 and HT29 cells infected with LV-CXCR7 or shRNA-CXCR7 and control lentivirus. β-actin was used as loading control. (C) RT-QPCR was performed to determine the mRNA of E-cadherin, Vimentin and DCLK1 in HCT116 and HT29 cells overexpressing or knockdown of CXCR7. (D) The correlation of CXCR7 with DCLK1 and Vimentin was determined in CRC tissues by Gene Expression Profiling Interactive Analysis (GEPIA) online tools. (E) KM plotter was used to analyze the overall survival

of DCLK1 and Vimentin in CRC. The association of the expression of DCLK1 and Vimentin with overall survival was analyzed by Kaplan-Meier (KM) plotter (https://kmplot.com/analysis) in colonic adenocarcinoma. The patients were divided with high and low gene expression levels using the auto-select best cutoff and log-rank P value was shown. Bars are means ± SD; *p < 0.05, **p < 0.01, ***p < 0.001 (n = 3).

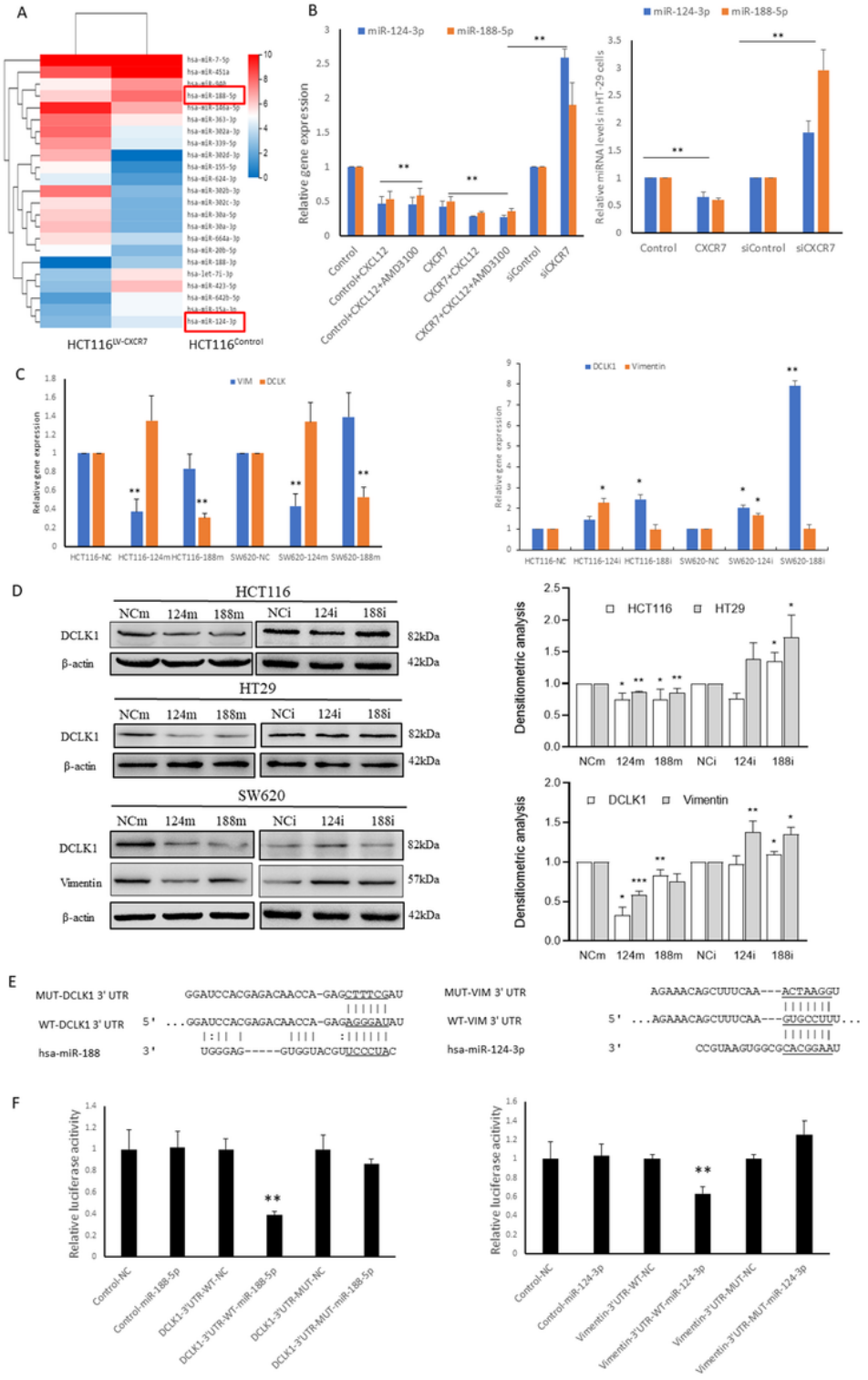

Figure 2

CXCR7 biased signal activation contributes to EMT by repressing miR-124-3p and miR-188-5p. (A) RNA-sequencing was performed in HCT116 cells infected with lenti-CXCR7 and control. Hierarchical clustering analysis of differentially expressing miRNAs between HCT116Control and HCT116LV-CXCR7 cells. (B) RT-qPCR analysis of miR-124-3p and miR-188-5p levels in HCT116 and HT29 cells infected with LV-CXCR7 or shRNA-CXCR7 and control lentivirus with or without CXCL12 (100 ng/ml) stimulation for 48 h. (C, D) RT-qPCR and Western blot analysis of DCLK1, Vimentin mRNA and protein in CRC cells transfected with miR-124-3p and miR-188-5p mimics (124 m, 188 m) or inhibitors (124i, 188i). β-actin was used as loading control. (E, F) HCT116 cells were co-transfected with DCLK1 and Vimentin luciferase constructs and NC, miR-188-5p and miR-124-3p mimics respectively. The comparison of luciferase activity of wild-type (WT) and mutant (MUT) DCLK1-3'UTR or Vimentin-3'UTR constructs was performed 36 h after transfection. Con081 luciferase plasmid was used as the vector control. Data was normalized to Renilla activity. Bars are means ± SD; *p < 0.05, **p < 0.01, ***p < 0.001 (n = 3).

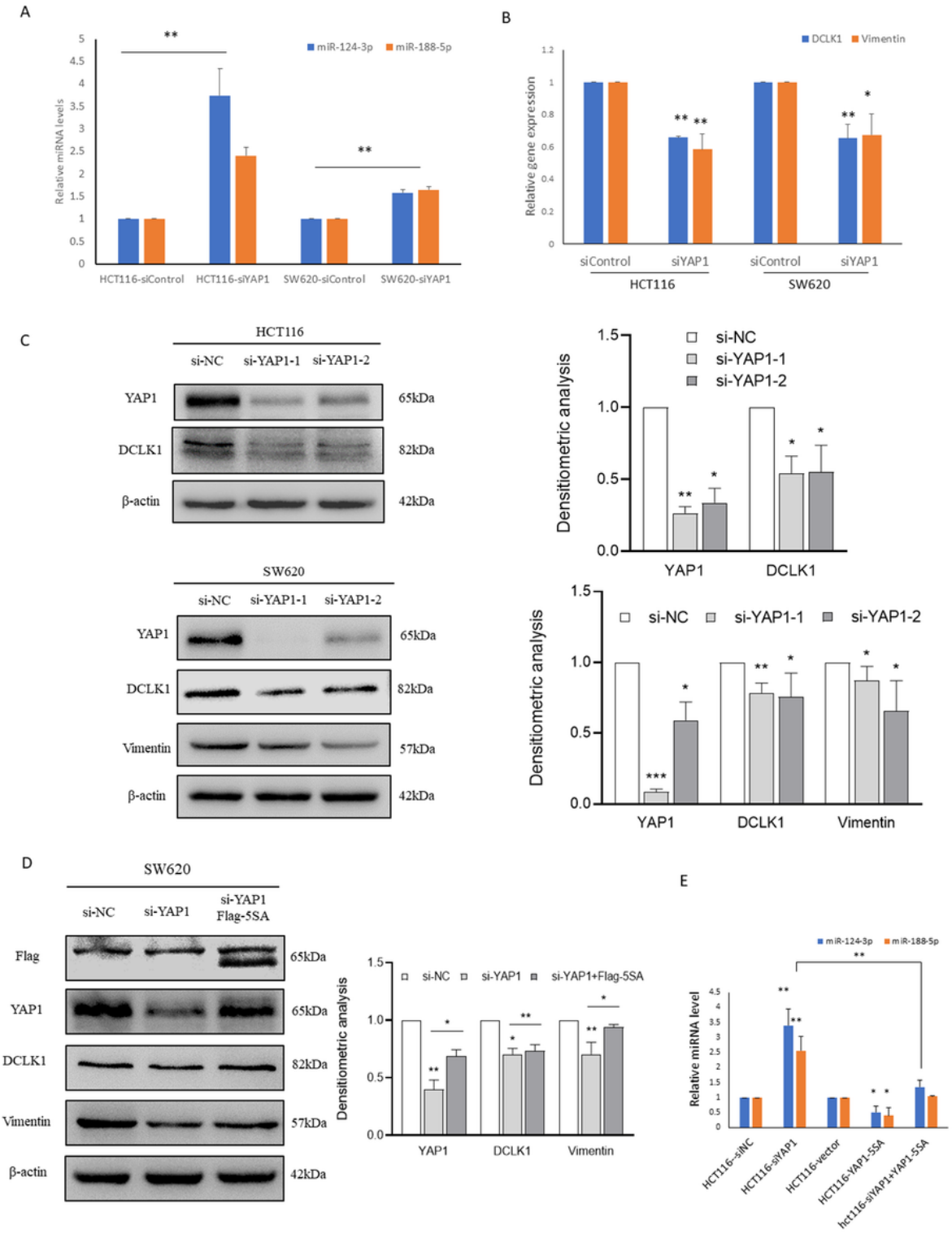

Figure 3

YAP1 promotes EMT and stemness by repressing miR-124-3p and miR-188-5p. (A, B) RT-qPCR analysis of DCLK1, Vimentin and concurrent expression of miR-124-3p and miR-188-5p in HCT116 and SW620 cells transfected with YAP1 siRNAs. (C) Western blot analysis of the expression of YAP1, DCLK1 and Vimentin in HCT116 and SW620 cells transfected with two different YAP1 siRNAs. β-actin was used as loading control. (D) Western blot analysis of YAP1, DCLK1 and Vimentin in SW620 cells transfected with YAP1

siRNAs and then rescued with transfection of Flag-YAP5SA plasmid. β-actin was used as a loading control. (E) RT-qPCR analysis of miR-124-3p and miR-188-5p in HCT116 cells co-transfected with YAP1 siRNAs and Flag-YAP 5SA plasmid. pcDNA3.1 plasmid was used as the vector control. Bars are means ± SD; *p < 0.05, **p < 0.01, ***p < 0.001 (n = 3).

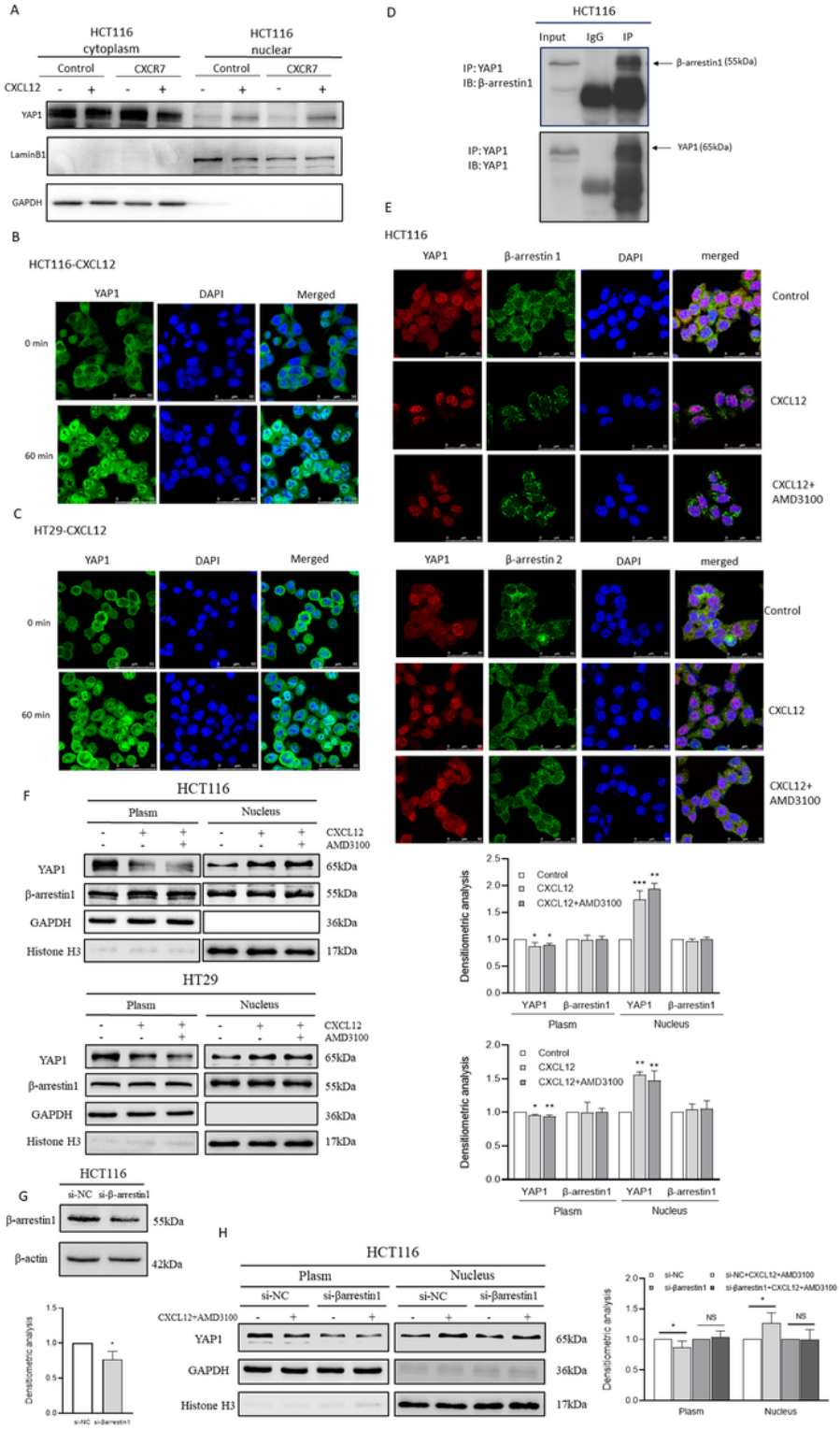

Figure 4

CXCR7/β-arr1-mediated biased signal induces YAP1 nuclear translocation in CRC cells. (A) Western blot analysis of YAP1 expression in cytoplasmic and nuclear extracts of HCT116 control and CXCR7 overexpressing cells treated with or without CXCL12 (100 ng/ml) for 60 min. GAPDH and Lamin B1 was used as cytoplasmic and nuclear loading control, respectively. (B, C) YAP1 localization evaluated by immunofluorescence (IF) in HCT116 and HT29 cells treated with or without CXCL12 (100 ng/ml). YAP1 was labeled with Alexa Fluor® 488 donkey anti-rabbit secondary antibody, Nuclei were visualized with DAPI, shown in blue. Scale bars, 50 μm. (D) Analysis of endogenous YAP1-β-arr1 interaction in HCT116 cells by immunoprecipitation. Normal rabbit IgG antibody was used as control. (E) IF staining was performed to determine the colocalization of YAP1(red) and β-arr1(green) or β-arr2 (green) in HCT116 treated with CXCL12 (100 ng/ml) in the presence of AMD3100 (2 μM). DAPI was used for nuclear staining. Scale bars, 50 μm. (F) Western blot analysis of YAP1 and β-arr1 expression in cytoplasmic and nuclear extracts of HCT116 and HT29 cells treated with or without CXCL12 (100 ng/ml) in the presence of AMD3100 (2 μM). GAPDH and Histone H3 was used as cytoplasmic and nuclear loading control, respectively. (G) Western blot analysis of β-arr1 expression in HCT116 cells transfected with β-arr1 siRNAs. β-actin was used as loading control. (H) Western blot analysis of YAP1 expression in cytoplasmic and nuclear extracts of HCT116 cells transfected with β-arr1 siRNAs and treated with or without CXCL12 (100 ng/ml) in the presence of AMD3100 (2 μM). GAPDH and Histone H3 was used as cytoplasmic and nuclear loading control, respectively. Bars are means ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (n=3).

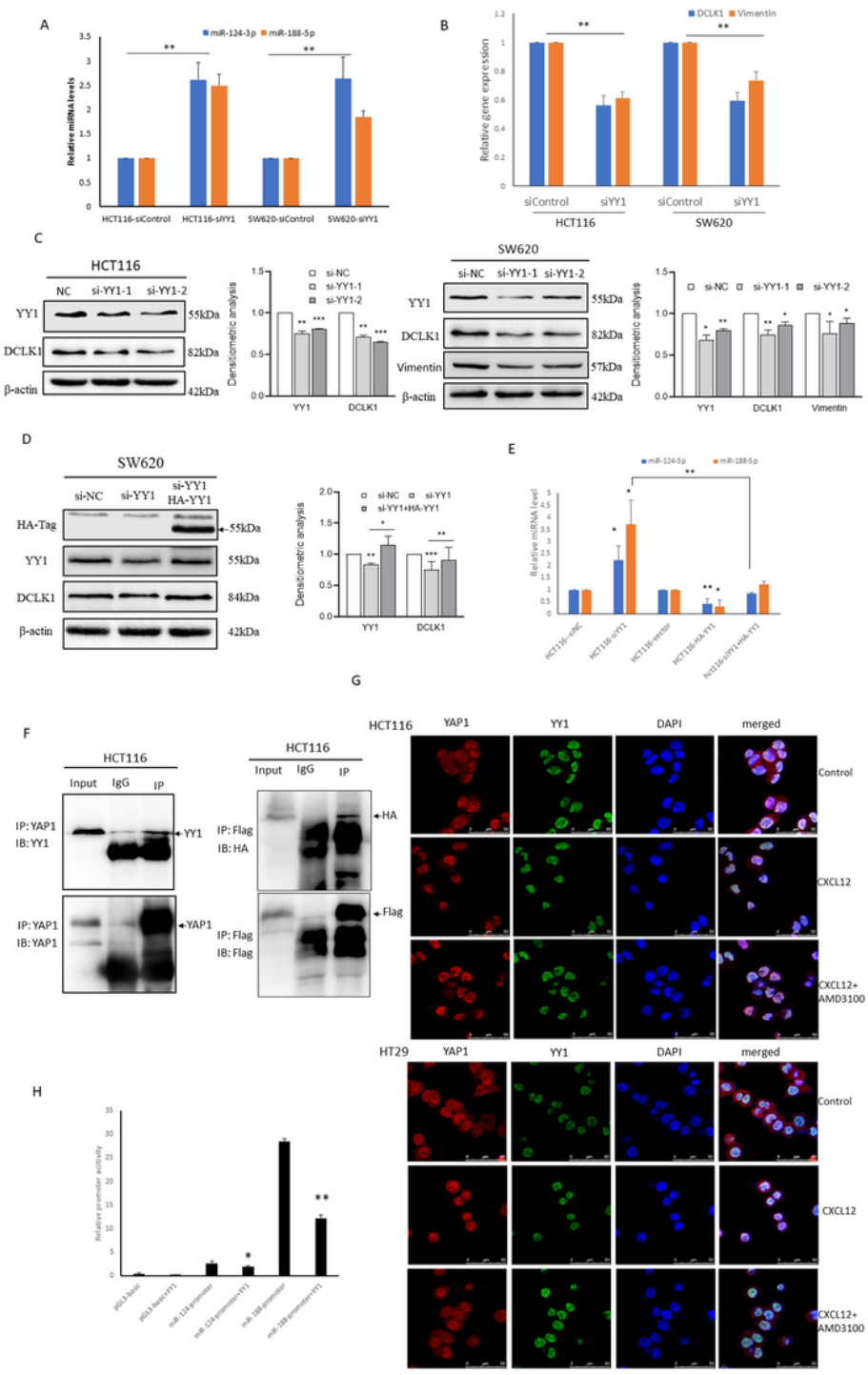

Figure 5

YAP1 inhibits miR-124-3p and miR-188-5p expression by recruiting YY1 to the promoter. (A, B) RT-qPCR analysis of the mRNA level of DCLK1, Vimentin and concurrent expression of miR-124-3p and miR-188-5p in HCT116 and SW620 cells transfected with YY1 siRNAs. (C) Western blot analysis of the expression of YY1, DCLK1 and Vimentin in HCT116 and SW620 cells transfected with YY1 siRNAs. β-actin was used as an internal control. (D) Western blot analysis of YY1 and DCLK1 in SW620 cells transfected with YAP1

siRNAs and then rescued with HA-YY1 plasmid. β-actin was used as loading control. (E) RT-qPCR analysis of miR-124-3p and miR-188-5p in HCT116 cells co-transfected with YY1 siRNAs and HA-YY1 plasmid. pcDNA3.1 plasmid was used as the vector control. (F) Analysis of the interaction of endogenous YAP1 with YY1 and transfected Flag-YAP5SA with HA-YY1 by immunoprecipitation (IP). IP was performed using anti-YAP1 or anti-Flag antibody and normal rabbit IgG antibody. (G) IF staining was performed to determine the colocalization of YAP1(red) and YY1(green) in HCT116 and HT29 cells treated with CXCL12 (100 ng/ml) in the presence of AMD3100 (2 μM). DAPI was used for nuclear staining. Scale bars, 50 μm. (H) HCT116 cells were co-transfected with miR-124-3p and miR-188-5p promoter luciferase constructs (miR-124 and miR-188 promoter) together with HA-YY1 plasmid. The comparison of luciferase activity of promoter constructs normalized to Renilla activity was indicated. pGL3-basic plasmid was used as the vector control. Bars are means ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ($n = 3$).

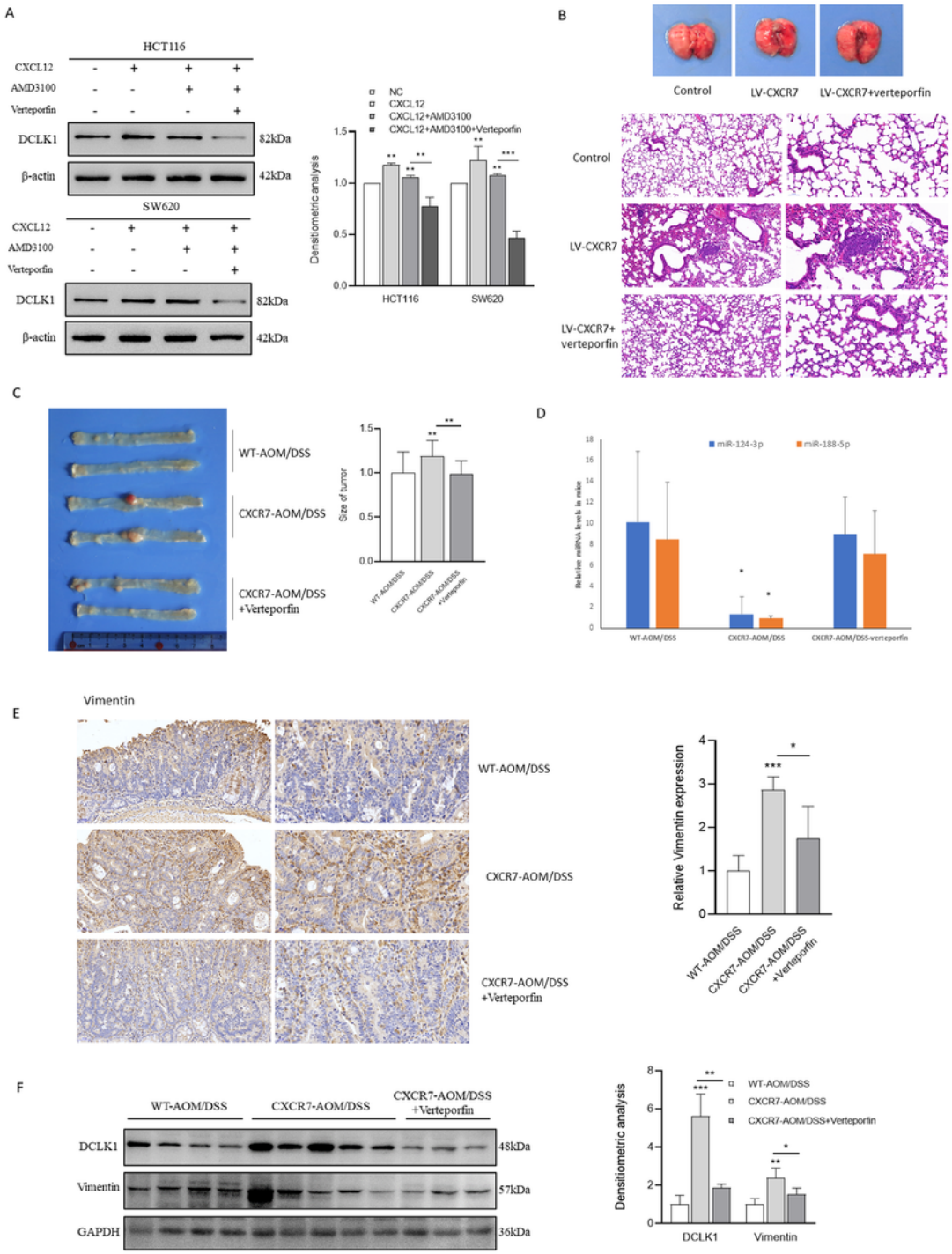

Figure 6

YAP1 inhibitor suppresses CXCR7 induced tumor progression and metastasis. (A) Western blot analysis of the expression of DCLK1 normalized to β-actin in HCT116 and SW620 cells treated with CXCL12 in the presence of AMD3100 and Verteporfin (3 μM) for 48 h. **p < 0.01,***p < 0.001. (B) Representative images of lungs and H&E-stained sections from lung metastatic nodules of nude mice inoculated with HCT116LV-CXCR7 and HCT116Control cells via tail veins with or without the treatment of verteporfin (n =

3). (C) Representative images of colons from AOM/DSS-treated WT, villin-CXCR7 mice and villin-CXCR7 mice treated with verteporfin. Average size of colon polyps was analyzed in different groups. (D) RT-qPCR analysis of expression levels of miR-124-3p and miR-188-5p in colon cancer tissues from mice. (E) Representative IHC staining of Vimentin in AOM/DSS-induced colon adenocarcinoma tissues from wild type C57BL/6 mice and villin-CXCR7 mice administered with verteporfin (10 mg/kg) or vehicle control via intraperitoneal injection daily. (F) Western blot analysis of DCLK1 and Vimentin expression in colon cancer tissues from mice. GAPDH was used as loading control and statistical analysis was performed.

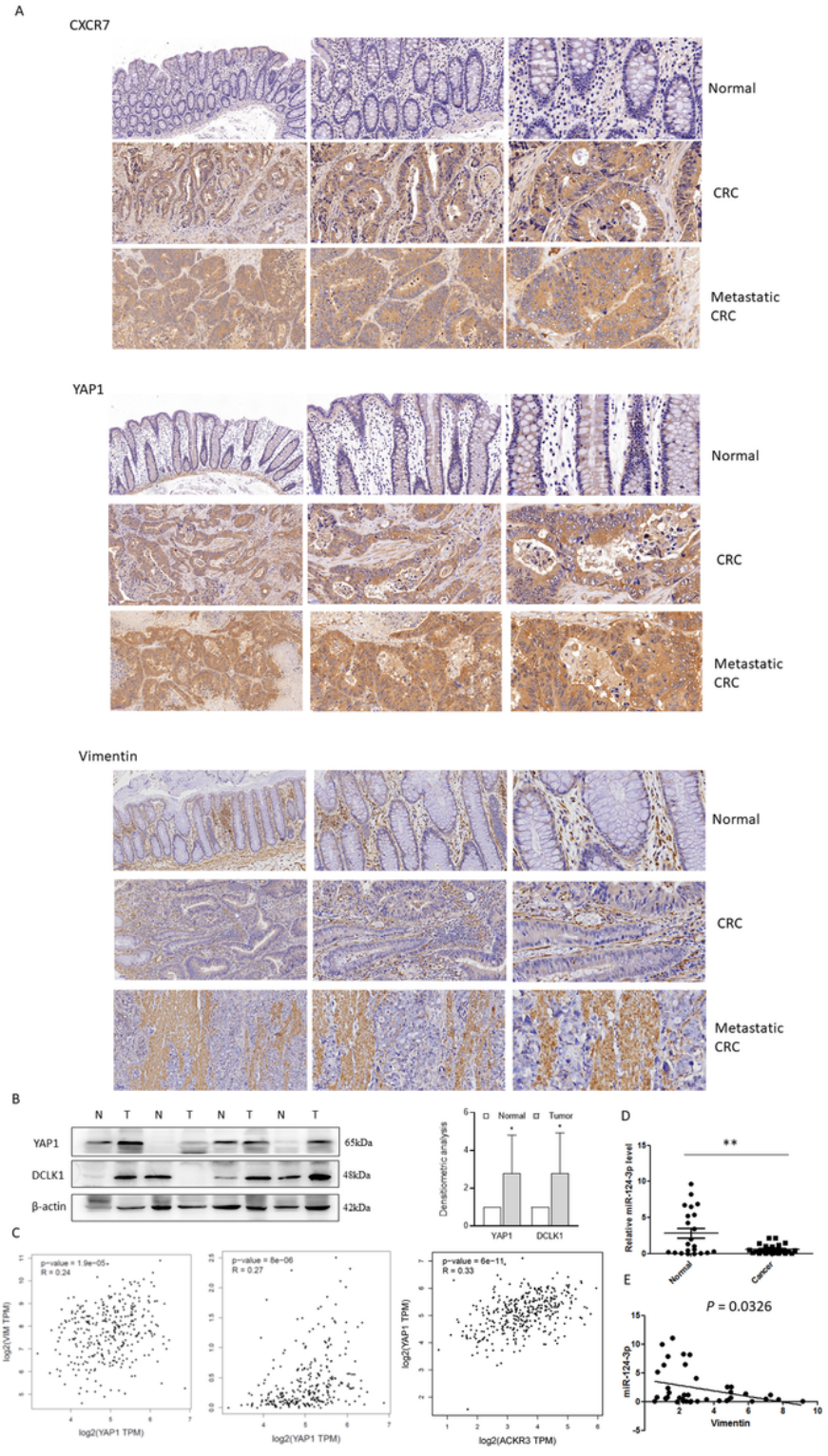

Figure 7

YAP1 nuclear translocation is associated with EMT and metastasis in human CRC tissues. (A) Immunohistochemistry analysis of the expression of CXCR7, YAP1 and Vimentin in human CRC tissues. Scale bar = 100, 50, 20 μm. (B) Western blot analysis of YAP1 and DCLK1 expression in 22 pairs of human CRC tissues and adjacent normal tissues. β-actin was used as an internal control. (C) The correlations of YAP1 with DCLK1 and Vimentin as well as association of CXCR7 with YAP1 were determined in CRC tissues by GEPIA. (D) RT-qPCR analysis of miR-124-3p levels in 22 pairs of human CRC tissues and adjacent normal tissues. (E) The correlation of miR-124-3p and Vimentin mRNA level was performed by Pearson correlation analysis. *p < 0.05, **p < 0.01.

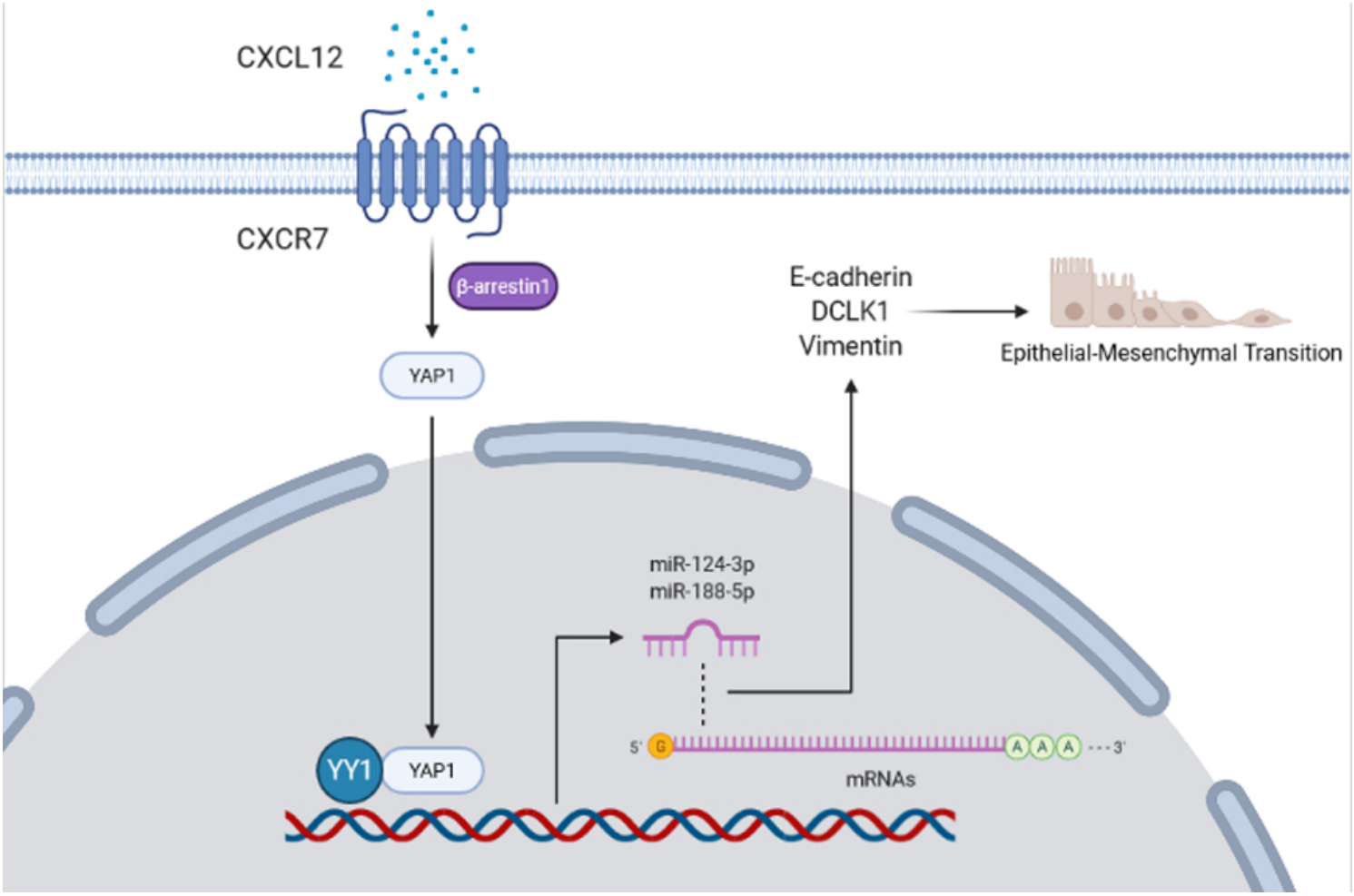

Figure 8

Schematic depiction of the working model for CXCL12/CXCR7/β-arrestin1 biased signal promoting EMT through YAP1 nuclear translocation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable.docx
- SupplementaryFigures.pdf