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The YAP–TEAD4 complex promotes tumor lymphangiogenesis by transcriptionally upregulating CCBE1 in colorectal cancer

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The secreted protein collagen and calcium-binding EGF domain 1 (CCBE1) is critical for embryonic lymphatic development through its role in the proteolytic activation of mature vascular endothelial growth factor C (VEGFC). We previously reported that CCBE1 is overexpressed in colorectal cancer (CRC) and that its transcription is negatively regulated by the TGFβ-SMAD pathway, but the transcriptional activation mechanism of *CCBE1* in CRC remains unknown. Recent studies have revealed the vital role of the hippo effectors YAP/TAZ in lymphatic development; however, the role of YAP/TAZ in tumor lymphangiogenesis has not been clarified. In this study, we found that high nuclear expression of transcription factor TEAD4 is associated with lymph node metastasis and high lymphatic vessel density in patients with CRC. YAP/TAZ–TEAD4 complexes transcriptionally upregulated the expression of CCBE1 by directly binding to the enhancer region of *CCBE1* in both CRC cells and cancer-associated fibroblasts, which resulted in enhanced VEGFC proteolysis and induced tube formation and migration of human lymphatic endothelial cells *in vitro* and lymphangiogenesis in a CRC cell-derived xenograft model *in vivo*. In addition, the bromodomain and extraterminal domain (BET) inhibitor JQ1 significantly inhibited the transcription of *CCBE1*, suppressed VEGFC proteolysis, and inhibited tumor lymphangiogenesis *in vitro* and *in vivo*. Collectively, our study reveals a new positive transcriptional regulatory mechanism of *CCBE1 via* YAP/TAZ–TEAD4–BRD4 complexes in CRC, which exposes the protumor lymphangiogenic role of YAP/TAZ and the potential inhibitory effect of BET inhibitors on tumor lymphangiogenesis.

Metastasis is the leading cause of death in patients with colorectal cancer (CRC) (1). Regional lymph node metastases can partially seed distant metastases (2, 3) and are indicators of reduced survival in patients with CRC (4). Lymphangiogenesis in the tumor microenvironment is crucial for the entry of cancer cells into the lymphatic circulatory system (5). However, the regulatory mechanism of tumor lymphangiogenesis in CRC remains to be investigated. Collagen and calcium-binding EGF domain 1 (CCBE1) is a secreted protein that is vital for lymphatic development in embryos by promoting the proteolysis and activation of mature VEGFC (6–8). Although CCBE1 has long been known as a vital regulator of embryonic lymphatic development, its role in tumor lymphangiogenesis and lymphatic metastasis remained unknown until recently, when we and others revealed that the overexpression of CCBE1 is a marker of poor prognosis in CRC and lung cancer (9, 10). Indeed, overexpression of CCBE1 promotes lymphangiogenesis and lymphatic metastasis in CRC (10). Transcriptional activation of *CCBE1* in CRC is the key mechanism underlying the enhanced expression of CCBE1 in CRC. *CCBE1* gene transcription is negatively regulated by the TGFβ-SMAD pathway in both colonic epithelial cells and fibroblasts (10). Inactivation of the TGFβ-SMAD pathway in CRC abolishes the inhibitory effect of TGFβ on *CCBE1* gene transcription (10). However, the transcriptional activation mechanism of the *CCBE1* gene in CRC has not been elucidated.

Emerging evidence indicates that a variety of cancers exhibit dysregulation of the Hippo pathway, which is evolutionarily conserved and plays a vital role in organ size control, development, and tissue regeneration (11). The transcriptional coactivators YAP/TAZ are the downstream effectors of the Hippo pathway, whose activation promotes tumorigenesis and tumor progression by regulating the transcription of downstream target genes, mainly through TEAD transcription factor family members (TEAD1/2/3/4) (11, 12). As transcriptional coactivators, YAP/TAZ can recruit the histone acetyltransferase p300 and histone acetylation reader BRD4 to establish a histone code for the transcription of target genes (13). Most YAP/TAZ-regulated target genes display exquisite sensitivity to BRD4-inhibitory drugs, such as the bromodomain and extraterminal domain (BET) inhibitor JQ1 (13). The list of YAP/TAZ target genes continues to increase and includes *CCND1*, *BIRC5*, *TERT*, *FAO*, *CCL2*, *PDL1*, and *ANGPT2*, which endow tumor cells with every hallmark of

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cancer, such as sustained proliferation, deregulated cellular metabolism, activated invasion, and induced angiogenesis (12, 14–16). In melanoma, YAP-dependent metabolic adaptation is required for the survival of metastatic tumor cells in the lymph nodes through activating the expression of genes in the fatty acid oxidation pathway (17). However, the potential role of YAP/TAZ in tumor lymphangiogenesis remains poorly understood. In mouse embryos, Yap/Taz promote the remodeling of lymphatic plexus patterning and postnatal lymphatic valve maintenance by negatively regulating Prox1 expression (18). In addition, Yap is essential in lymphatic vascular budding and initial structure formation and is indispensable for vascular endothelial growth factor C (Vegfc)-induced proliferation in zebrafish (19). The results of these studies demonstrate the crucial role of YAP/TAZ in lymphatic endothelial cells during lymphatic development, which also implies the potential role of YAP/TAZ in tumor lymphangiogenesis.

In this study, we found that *CCBE1* is a new downstream direct target gene of YAP/TAZ in CRC. YAP/TAZ promote human lymphatic endothelial cell (HLEC) tube formation and migration *in vitro* and lymphangiogenesis *in vivo* by upregulating the gene expression of *CCBE1* in CRC cells and cancer-associated fibroblasts (CAFs), which in turn results in the enhancement of VEGFC proteolytic activation. The BRD4 inhibitor JQ1 significantly decreases the mRNA level of CCBE1 and inhibits tumor lymphangiogenesis *in vitro* and *in vivo*. In CRC, high expression of YAP/TAZ and TEAD4 is positively correlated with lymphatic vessel density. Thus, our study reveals the transcriptional activation mechanism of *CCBE1* by YAP/TAZ in CRC, which exposes the vital role of YAP/TAZ in tumor lymphangiogenesis and provides evidence for the clinical therapeutic potential of targeting the YAP/TAZ/BRD4–CCBE1–VEGFC axis to inhibit tumor lymphangiogenesis and lymphatic metastasis.

Results

Increased nuclear TEAD4 expression is associated with lymph node metastasis and high lymphatic vessel density in CRC

In our previous study, we found that TEAD4 is the only TEAD family member that is overexpressed in CRC (20). Analysis of clinical characteristics showed that patients with advanced CRC (stages III and IV) expressed higher levels of nuclear TEAD4 than those with stage I and II tumors, which indicates that increased TEAD4 expression is associated with distal metastasis in CRC (20). Interestingly, in reanalysis of the clinical data, subgroup analysis revealed that patients whose CRC samples exhibited high nuclear TEAD4 expression showed a higher lymph node metastasis rate than those whose CRC samples exhibited low nuclear TEAD4 expression (Fig. 1*A*). Induction of tumor lymphangiogenesis is the first step in lymphatic metastasis; thus, we investigated the correlation between TEAD4 expression and tumor lymphangiogenesis in CRC. Another 30 CRC samples were stained with an anti-podoplanin (PDPN) antibody to evaluate the density of lymphatic vessels infiltrated into tumor areas. As shown in Fig. S1*A*, more PDPN-positive lymphatic vessels were found in CRC tissues with higher TEAD4 expression in the nucleus. These results indicate that overexpression of TEAD4 not only promotes tumor cell migration and metastasis but also plays a potential vital role in tumor lymphangiogenesis in CRC.

YAP/TAZ and TEAD4 in CRC cells and CAFs promote tumor lymphangiogenesis in vitro

To validate the potential lymphangiogenic role of TEAD4 in CRC, we performed a tube formation assay with HLECs. Conditioned medium from TEAD4 knockdown HCT116 cells significantly decreased HLEC tube formation (Fig. 1*B*). Given that the transcriptional output of TEADs is mainly regulated by YAP/TAZ, we further examined whether YAP/TAZ also promote tumor lymphangiogenesis *in vitro*. Since we previously discovered the vital role of CAFs in tumor lymphangiogenesis in CRC, and YAP/TAZ are required for the generation and maintenance of CAFs (10, 21), we established stable YAP/TAZ double knockdown in two CRC cell lines (HCT116 and SW837) and CAFs from two patients with CRC (Fig. S1, *B* and *C*). HLEC tube formation and wound healing assays were performed with cell culture supernatants from the CRC cell line SW837 and the CAFs. The results showed that the tube formation and migration abilities of HLECs treated with conditioned medium from YAP/TAZ knockdown SW837 cells and CAFs were significantly impaired (Fig. 1, *C* and *D*). Similarly, the proliferation ability of HLECs was also attenuated by the conditioned medium from YAP/TAZ knockdown SW837 cells (Fig. S1*D*). These data demonstrate that tumor expression and tumor stromal expression of YAP/TAZ can promote tumor lymphangiogenesis in CRC, probably through activating the transcription factor TEAD4.

YAP/TAZ transcriptionally upregulate CCBE1 expression and sequentially enhance VEGFC proteolysis

To further investigate the regulatory mechanism of tumor lymphangiogenesis by the YAP–TEAD4 complex in CRC, we performed RNA-Seq analysis of *TEAD4* knockdown HCT116 cells (Table S1, GSE176062). Although a recent study showed a decreased mRNA level of *Vegfc* in *Yap/Taz* KO intestinal stromal cells (22), we did not observe a decrease in the *VEGFC* mRNA level in TEAD4 knockdown HCT116 cells (Table S1). However, we noted that *CCBE1*, a VEGFC activation factor, was the only lymphangiogenesis-related gene whose mRNA expression was reduced upon the knockdown of *TEAD4* in HCT116 cells (FC>2, $p < 0.05$) (Table S1). The decreased *CCBE1* mRNA level was confirmed in *TEAD4* knockdown HCT116 and SW837 cells by quantitative PCR (qPCR) (Figs. 2*A* and S2*A*). Similar results were observed when the *TEAD1/2/3/4* were individually silenced by specific siRNA in HCT116 cells (Fig. S2*B*). In addition, *YAP/TAZ* knockdown attenuated *CCBE1* expression in two CRC cell lines HCT116,

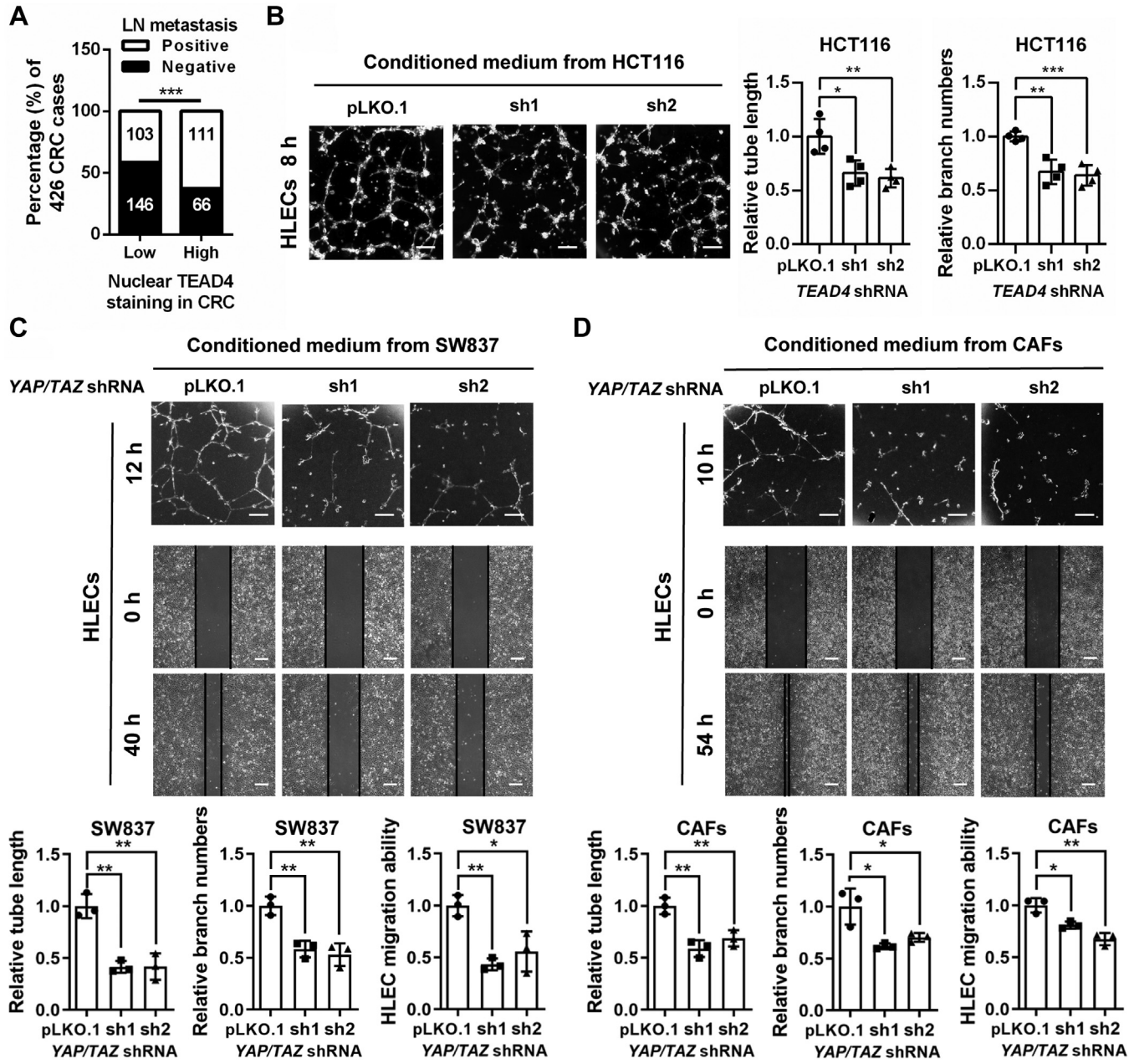

Figure 1. YAP/TAZ and TEAD4 promote tumor lymphangiogenesis in CRC *in vitro*. *A*, in CRC, high nuclear TEAD4 expression is correlated with an increased lymph node metastasis rate compared with that of CRC tumors with low TEAD4 expression. The original immunoreactive score and clinical data were obtained from our previous study (20). ***$p < 0.001$ by the chi-squared test. *B*, HLEC tube formation assay with conditioned medium from *TEAD4* knockdown HCT116 cells. HLECs were cultured with conditioned medium from the indicated HCT116 cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student's *t* test. *C* and *D*, tube formation and wound healing assays of HLECs cultured with conditioned medium from the indicated SW837 cells (*C*) and CAFs (*D*). The scale bars for the tube formation assay represent 100 μm. The scale bars for the wound healing assay represent 200 μm. *$p < 0.05$, **$p < 0.01$ by Student's *t* test. CAF, cancer-associated fibroblast; CRC, colorectal cancer; HLEC, human lymphatic endothelial cell.

SW837 and two primary CAF lines (Fig. 2*B*). Knockdown of either *YAP* or *TAZ* alone impaired the mRNA expression of *CCBE1* in SW837 cells, which implied the redundant function of YAP and TAZ in modulating *CCBE1*'s transcription (Fig. S2*C*). Then, we reexpressed the constitutively active YAP-5SA in shYAP-2 SW837 cells, in which the *YAP* shRNA targets the 3′UTR of *YAP* mRNA (Fig. S2*D*). As expected, both mRNA and protein expression of *CCBE1* were restored by reexpression of YAP-5SA (Fig. S2*E*). By analyzing published chromatin immunoprecipitation (ChIP)-seq datasets (23), we found direct binding of YAP at the *CCBE1* gene locus in the glioma cell line NCI-60 and breast cancer cell line MDA-MB-231 (Fig. 2*C*). Interestingly, these YAP binding peaks overlapped with strong H3K27Ac signals, indicating active gene transcription (Fig. 2*C*). To further explore whether YAP binds to the *CCBE1* gene locus, we performed ChIP-qPCR analysis using a ChIP-grade anti-FLAG antibody in HCT116 cells stably expressing FLAG-YAP. The ChIP-qPCR results showed strong signals of FLAG-YAP binding at the predicted PEAK1 (−46.7 kb) and PEAK2 (−20.8 kb) of the *CCBE1* gene locus in

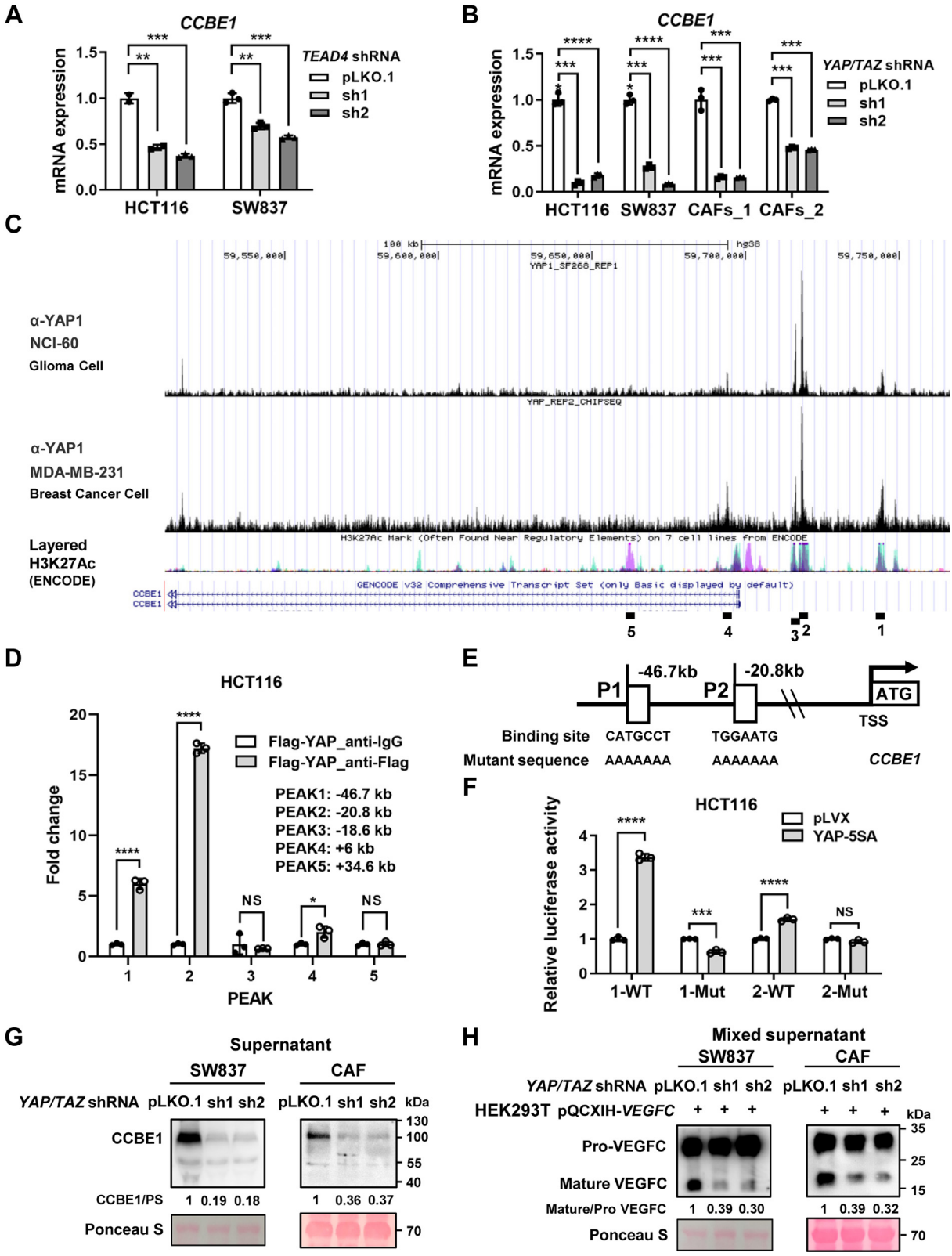

Figure 2. YAP/TAZ transcriptionally upregulate the expression of CCBE1 in colorectal cancer cells and cancer-associated fibroblasts (CAFs). *A*, quantitative PCR (qPCR) analysis of CCBE1 mRNA levels in control and TEAD4 knockdown HCT116 and SW837 cells. $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's *t* test. *B*, qPCR analysis of CCBE1 mRNA levels in control and YAP/TAZ knockdown HCT116 and SW837 cells and in primary CAFs derived from two different colorectal cancer tissues. $^{***}p < 0.001$, $^{****}p < 0.0001$ by Student's *t* test. *C*, YAP binds to the *CCBE1* gene locus in NCI-60 glioma cells and MDA-MB-231 breast cancer cells. Chromatin immunoprecipitation (ChIP)-seq data for YAP and H3K27Ac in seven cell lines from ENCODE were extracted from the Cistrome database (23). The regions targeted by the ChIP-qPCR primers are indicated. *D*, ChIP-qPCR analysis of FLAG-YAP binding to the *CCBE1* gene locus in HCT116 cells. Rabbit IgG was used as the negative control. $^{*}p < 0.05$, $^{****}p < 0.0001$ by Student's *t* test. *E*, schematic depiction of the *CCBE1* enhancers with potential TEAD-binding sites (P1 and P2). The mutation of the TEAD-binding sites for luciferase assay was indicated. *F*, luciferase assay of the WT and mutant

HCT116 cells, while the TEAD-binding defective S94A mutant lost the ability of binding the CCBE1 enhancers (Figs. 2*D* and S2*F*). Consistently, the luciferase assay showed that the relative luciferase activities of these two enhancer regions were both increased by the overexpression of YAP-5SA, while mutation of the potential TEAD-binding sites matching the M-CAT motif abolished the activating effect of YAP-5SA on the luciferase activity (Fig. 2, *E* and *F*). In all, these data indicated that YAP was recruited to the enhancer region of the *CCBE1* gene to regulate *CCBE1* transcription.

CCBE1 cooperates with A disintegrin and metalloproteinase with thrombospondin motifs 3 (ADAMTS3) to regulate VEGFC proteolysis (6, 7). The fully processed 21-kDa mature form of VEGFC has the highest activity toward VEGFR3, which is generated by proteolysis of the 29/31 kDa pro-VEGFC form (8). Next, we verified the decrease in secreted CCBE1 protein in the supernatant of *YAP/TAZ* knockdown SW837 cells and CAFs by Western blot (WB) analysis (Fig. 2*G*). Consistent with the above findings, the amount of the 21-kDa mature VEGFC form was dramatically decreased in the mixed supernatant from VEGFC full length- overexpressed HEK293T cells and *YAP/TAZ* knockdown SW837 cells or CAFs, which indicated that the ability for VEGFC proteolytic processing was reduced by knockdown of *YAP/TAZ* in SW837 cells and CAFs (Fig. 2*H*). Similar results were observed that the protein expression of secreted CCBE1 and the VEGFC proteolysis were also attenuated in the *TEAD4* knockdown SW837 cells (Fig. S2*G*). Taken together, these data implied that YAP/TAZ could transcriptionally activate *CCBE1* and enhance proteolysis-dependent maturation of VEGFC in both CRC cells and CAFs.

CCBE1 mediates the protumor lymphangiogenic function of YAP/TAZ in CRC in vitro *and* in vivo

To further confirm that enhanced expression of CCBE1 mediates the protumor lymphangiogenic function of YAP/TAZ in CRC, we rescued CCBE1 expression in *YAP/TAZ* knockdown CRC SW837 cells by stably expressing CCBE1 (Fig. 3*A*). Rescue of CCBE1 expression reversed the attenuation of VEGFC proteolytic processing and the suppression of HLEC tube formation and migration abilities induced by knockdown of YAP/TAZ in SW837 cells (Fig. 3, *B* and *C*). Furthermore, adding mature human VEGFC (hVEGFC) protein to conditioned medium from *YAP/TAZ* knockdown SW837 cells also rescued the impaired tube formation and migration ability of HLECs (Fig. S3), which supported that the diminished prolymphangiogenic ability of the conditioned medium from *YAP/TAZ* knockdown SW837 cells could be due to impaired VEGFC proteolytic processing by downregulation of CCBE1 expression and subsequently decreased level of mature VEGFC protein. Next, we established a cell-derived xenograft (CDX) model to further confirm the function of YAP/TAZ in tumor lymphangiogenesis and the functional correlation between YAP/TAZ and CCBE1 in CRC *in vivo* by inoculating HCT116 cells into the footpad of a nude mouse, in which lymphatic vessels infiltrating the xenograft tumors were evident. As shown in Figure 3, *D* and *E*, fewer mLyve-1-positive lymphatic vessels were found in the xenografts with *YAP/TAZ* knockdown. As expected, CCBE1 overexpression partially rescued the decrease in lymphatic vessel infiltration induced by *YAP/TAZ* knockdown in HCT116 cells (Fig. 3*E*). Statistical analysis of mLyve-1-positive lymphatic vessel numbers further confirmed the above observation (Fig. 3*D*). Taken together, these data indicated that the promotive effect of YAP/TAZ on tumor lymphangiogenesis in CRC cells was dependent on upregulation of CCBE1 expression.

JQ1 inhibits lymphangiogenesis by decreasing the expression of CCBE1 in CRC cells

Previously, we performed RNA-Seq analysis of HCT116 cells and two lines of primary CAFs and normal fibroblasts treated with the BET inhibitor JQ1 and found that *CCBE1* was one of the differentially expressed genes after JQ1 treatment in all these cell lines (24, 25). Since YAP/TAZ-regulated genes exhibit sensitivity to JQ1, which has also shown promising efficacy in preclinical CRC models (13, 24), we assessed the therapeutic potential of JQ1 in inhibiting tumor lymphangiogenesis induced *via* the YAP–CCBE1 axis. First, we confirmed that JQ1 treatment significantly decreased the mRNA levels of *CCBE1* in HCT116 and SW837 CRC cells and two CAF lines derived from different CRC tissues (Fig. 4*A*). Furthermore, simultaneous knockdown of BRD2/3/4 dramatically suppressed the mRNA expression of CCBE1 in HCT116 and SW837 cells (Fig. 4*B*). Intriguingly, individual knockdown of BRD2, BRD3, and BRD4 all suppressed the mRNA expression of *CCBE1* in HCT116 cells (Fig. S4*A*). Since it is reported that BRD4 is a cofactor for YAP/TAZ transcriptional activity (13), we performed ChIP assay to verify the involvement of BRD4 in the transcriptional regulation of *CCBE1* by YAP. As shown in Fig. S4*B*, the binding of BRD4 to *CCBE1* enhancers was largely abrogated in YAP knockdown HCT116 cells, especially for PEAK1 and PEAK2, which were also occupied by YAP (Fig. 2*D*).

Second, consistent with these findings, the CCBE1 protein secretion (Fig. 4*C*) and VEGFC proteolytic processing (Fig. 4*D*) were also inhibited by JQ1 treatment and were rescued by overexpression of CCBE1 in SW837 cells (Fig. 4, *E* and *F*). Similarly, overexpression of CCBE1 rescued the downregulation of CCBE1 protein secretion and VEGFC proteolytic processing caused by simultaneous knockdown of BRD2/3/4 (Fig. S4, *C* and *D*). In addition, we observed that overexpression of YAP-5SA enhanced both mRNA and protein level of CCBE1 (Fig. S4, *E* and *F*) and VEGFC proteolytic

CCBE1 enhancer reporters in control pLVX and YAP-5SA-expressing HCT116 cells. *G*, Western blot analysis of CCBE1 protein levels in supernatants from the indicated stable SW837 cells and CAFs. *H*, Western blot analysis of pro-VEGFC and mature VEGFC protein levels in the indicated mixed conditioned medium. Conditioned medium from the indicated stable SW837 cells and CAFs was mixed with conditioned medium from full-length VEGFC-expressing 293T cells (1:1), incubated overnight and analyzed by Western blotting. Ponceau S staining was used to control for equal loading of supernatant samples.

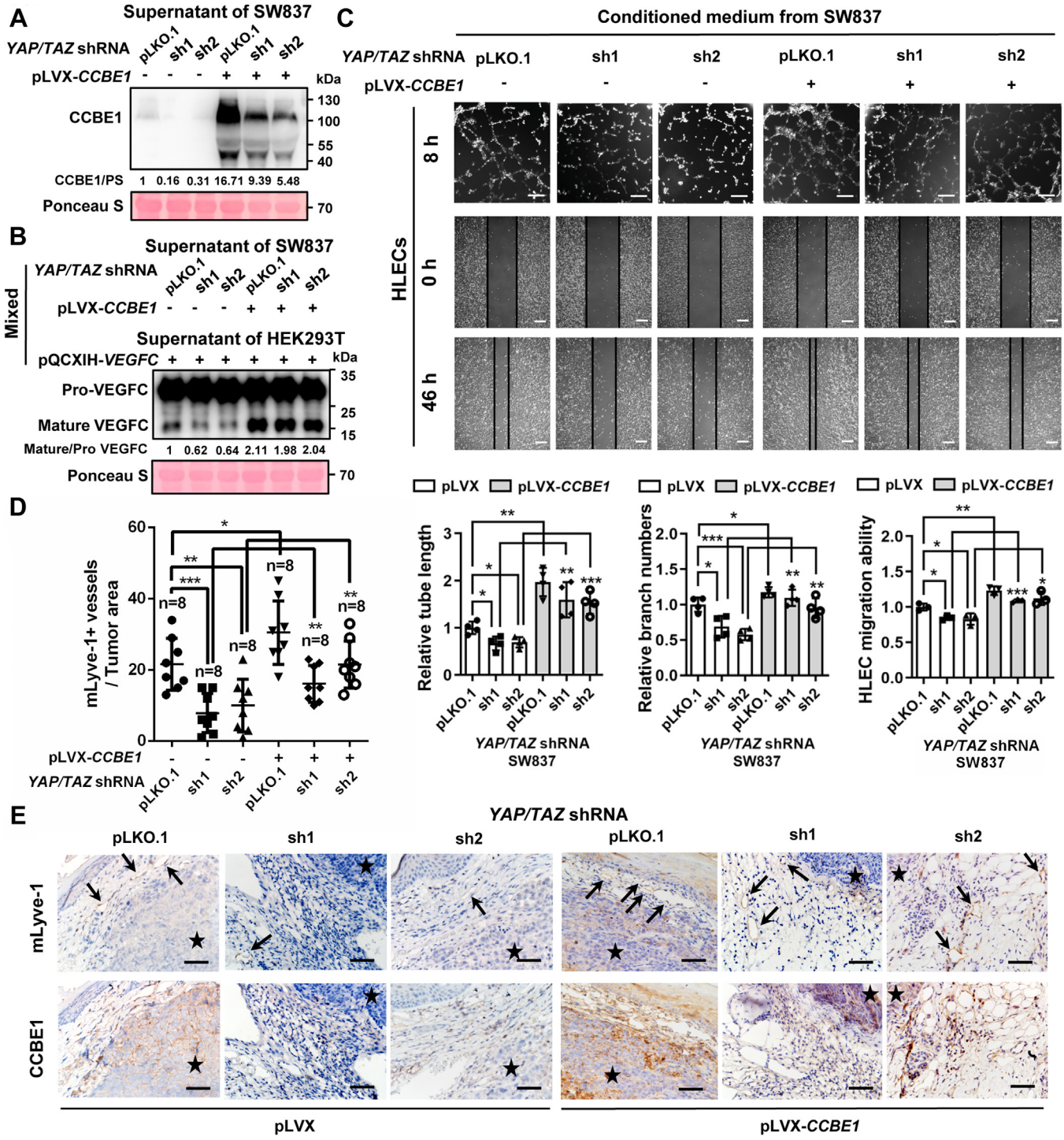

Figure 3. CCBE1 mediates the protumor lymphangiogenic function of YAP/TAZ *in vitro* and *in vivo*. *A*, Western blot analysis of CCBE1 protein levels in supernatants from the indicated stable SW837 cells. *B*, Western blot analysis of pro-VEGFC and mature VEGFC protein levels in the indicated mixed conditioned medium. Conditioned medium from the indicated treated SW837 cells was mixed with conditioned medium from VEGFC-expressing 293T cells (1:1), incubated overnight, and analyzed by Western blotting. Ponceau S staining was used to control for equal loading. *C*, tube formation and wound healing assays of human lymphatic endothelial cells (HLECs) cultured with conditioned medium from the indicated SW837 cells. The scale bars for the tube formation assay represent 100 μm. The scale bars for the wound healing assay represent 200 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student's *t* test. *D*, immunohistochemical analysis of mLyve-1 (lymphatic vessel number) in the indicated HCT116 cell line–derived xenografts in mouse footpads. *E*, representative images of mLyve-1(+) lymphatic vessels and CCBE1 protein expression in the same field are shown. Black arrows: mLyve-1(+) lymphatic vessels. Stars: colorectal cancer cells. The scale bars represent 20 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student's *t* test.

processing (Fig. S4*G*) in HCT116 cells, which were dramatically suppressed by JQ1 treatment.

Third, we examined the effect of JQ1-treated CRC cells on tumor lymphangiogenesis *in vitro* and *in vivo*. As shown in Figure 4*G*, JQ1 markedly inhibited SW837-conditioned medium-induced tube formation and migration of HLECs, both of which were also partially rescued by CCBE1 overexpression. Despite constitutive activation of YAP, JQ1 still

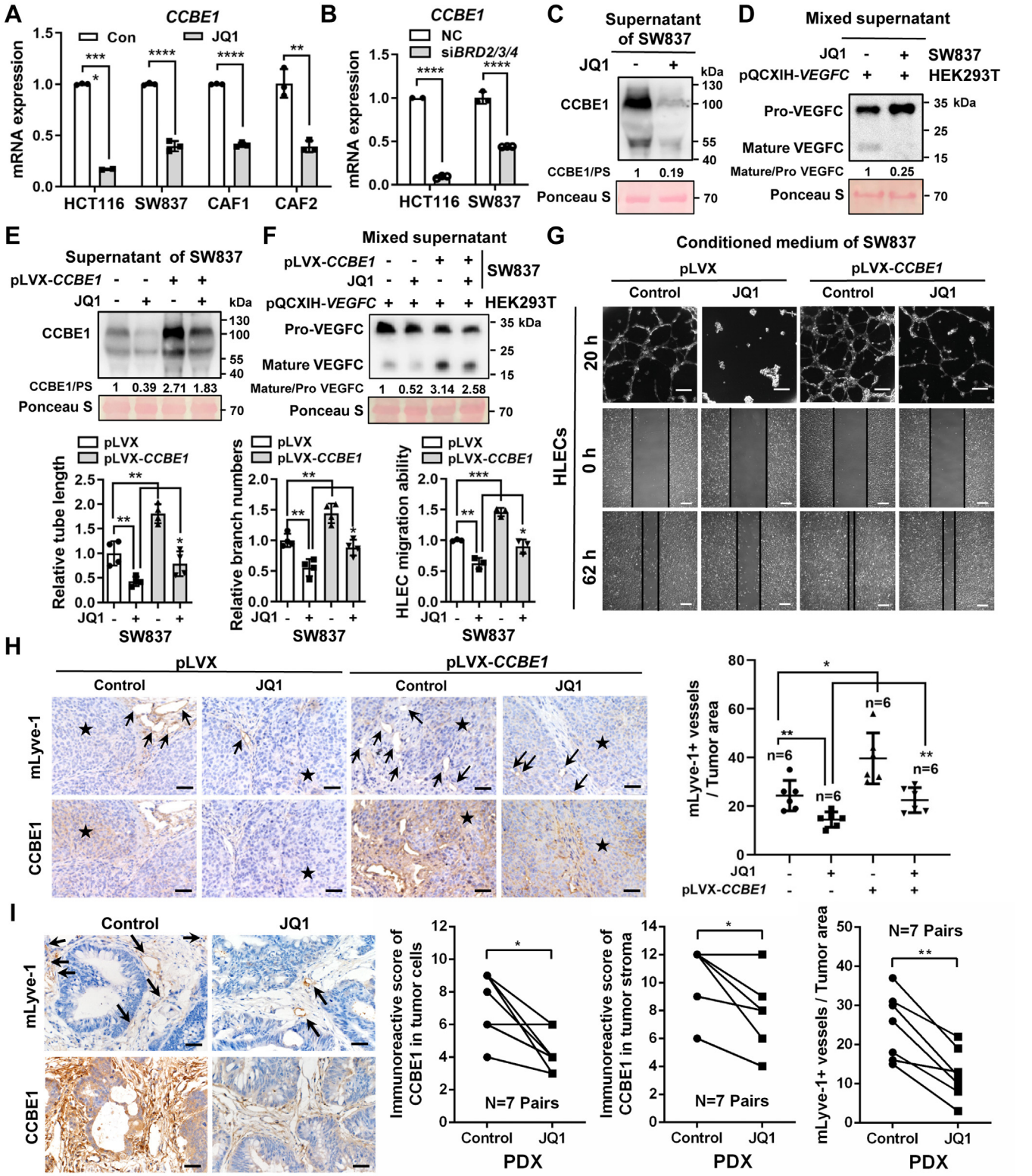

Figure 4. JQ1 inhibits lymphangiogenesis by suppressing the expression of CCBE1 in CRC. *A*, JQ1 decreased the mRNA level of *CCBE1* in HCT116 and SW837 cells and primary cancer-associated fibroblasts derived from two different CRC tissues. Cells were treated with JQ1 (1 μM) or DMSO for 24 h, and the mRNA level of CCBE1 was then determined by quantitative. $^{**}p < 0.01$, $^{****}p < 0.0001$ by Student's *t* test. *B*, knockdown of *BRD2/3/4* by siRNAs decreased the mRNA level of *CCBE1* in HCT116 and SW837 cells. Cells were transfected with the indicated siRNA for 72 h, and the mRNA level of *CCBE1* was then determined by quantitative PCR. $^{****}p < 0.0001$ by Student's *t* test. *C*, Western blot analysis of CCBE1 protein levels in supernatants from the indicated SW837 cells treated with JQ1 (1 μM) or DMSO for 24 h. *D*, Western blot analysis of pro-VEGFC and mature VEGFC protein levels in the indicated mixed conditioned medium. Conditioned medium from the indicated treated SW837 cells was mixed with conditioned medium from full-length VEGFC-expressing 293T cells (1:1), incubated overnight and analyzed by Western blotting. *E*, Western blot analysis of CCBE1 protein levels in supernatants from the indicated treated SW837 cells overexpressing CCBE1 and treated with JQ1 (1 μM) or DMSO for 24 h. *F*, Western blot analysis of pro-VEGFC and mature VEGFC protein levels in the indicated mixed conditioned medium. Conditioned medium from the indicated treated stable SW837 cells was mixed with conditioned

significantly impeded the tube formation and migration of HLECs induced by YAP-5SA HCT116-conditioned medium (Fig. S5*A*). Nevertheless, CCBE1 overexpression partially rescued the impaired tube formation and migration ability of HLECs induced by conditioned medium from SW837 cells with simultaneous knockdown of BRD2/3/4 (Fig. S5*B*). Given that CCBE1 overexpression partially reversed the inhibitory effect of JQ1 (Fig. 4, *E*–*G*), but almost completely reversed the inhibitory effect of YAP/TAZ knockdown on prolymphangiogenesis, these results are consistent with a broader effect of JQ1 on the lymphangiogenic program. Indeed, we observed that the mRNA level of *VEGFC* was dramatically decreased by JQ1 but not YAP/TAZ knockdown in SW837 cells (Fig. S5, *C* and *D*). Since JQ1 unexpectedly suppressed VEGFC expression in SW837 cells, which may lead to partial rescue of the inhibitory effect of JQ1 by CCBE1 overexpression, we performed the tube formation assay with the JQ1-treated conditioned medium mixed with supernatant from VEGFC overexpressed HEK293T cells (Fig. S5*E*). With the presence of exogenous pro-VEGFC protein, the inhibitory effect of JQ1 on tube formation was almost fully rescued by CCBE1 overexpression (Fig. S5*E*).

To next assess the inhibitory effects of JQ1 on CCBE1 expression and tumor lymphangiogenesis *in vivo*, the CDX models were established, in which JQ1 treatment resulted in fewer mLyve-1-positive lymphatic vessels in HCT116 xenograft tumors and overexpression of CCBE1 reversed the inhibitory effect of JQ1 on the infiltration of mLyve-1-positive lymphatic vessels (Fig. 4*H*). Finally, to explore the translational potential of JQ1 in CRC treatment, patient-derived xenograft (PDX) tumors treated with JQ1 were analyzed by immunohistochemical staining of mLyve-1 and CCBE1. In PDX models, the pathological structure of the xenograft tissues mimics that of the primary cancer tissues and the tumor stroma is evident and easily identified. Immunohistochemical staining of CCBE1 showed that CCBE1 was expressed in both CRC epithelial cells and the tumor stroma, consistent with our observation in primary CRC tissues (Fig. 4*I*). In the JQ1-treated group, the protein levels of CCBE1 were significantly decreased in both CRC tumor cells and the tumor stroma, and this decrease was correlated with a decreased number of mLyve-1-positive lymphatic vessels in the tumor stroma (Fig. 4*I*). Collectively, these data suggested that JQ1 inhibits tumor lymphangiogenesis probably by suppressing the expression of CCBE1 through interfering the YAP/BRD4 transcriptional complex in both CRC tumor cells and the tumor stroma, which might be a potential therapeutic strategy for inhibiting tumor lymphangiogenesis and lymphatic metastasis in CRC.

The expression of YAP is positively correlated with the expression of CCBE1 and lymphatic vessel density in CRC

Finally, we assessed the correlation between the expression levels of YAP and CCBE1 and the clinical implication of YAP expression in tumor lymphangiogenesis in CRC. We observed that a high YAP expression level in both tumor cells and the tumor stroma were associated with a higher number of PDPN-positive tumor lymphatic vessels in 30 CRC biopsies (Fig. 5, *A* and *B*). In these 30 CRC biopsies, the expression of TEAD4 in tumor cells is also positively correlated with the expression of CCBE1 (Fig. S6*A*). Furthermore, by immunohistochemical staining of YAP in the CRC tissue array of samples from patients with immunohistochemical data for CCBE1 expression, we found that the protein level of YAP was positively correlated with the CCBE1 protein level in both tumor cells and the tumor stroma (Fig. 5*C*). The mRNA levels of the genes in the YAP/TAZ downstream target gene signature (*CTGF*, *CYR61*, *ANKRD1*, and *AXL*) can reflect the transcriptional activity of YAP/TAZ, and the mRNA levels of *LYVE1* and *PDPN* can indicate the infiltration of lymphatic vessels in the tumor. Consistent with the results of immunohistochemistry (IHC), analysis of mRNA expression in the TCGA CRC dataset further revealed that the YAP/TAZ target gene signature was positively correlated with the mRNA level of *CCBE1* (Fig. 5*D*) and the genes in the lymphatic vessel gene signature (*LYVE1* and *PDPN*) (Fig. 5*E*). Intriguingly, the TCGA CRC dataset also showed the positive correlation between the mRNA levels of *BRD4* and *CCBE1* (Fig. 5*F*) or YAP/TAZ target gene signature (Fig. S6*B*), and between the mRNA levels of *BRD4* and *LYVE1*/*PDPN* (Fig. 5*G*). These data support the positive regulation of CCBE1 by YAP/TAZ/BRD4 and their protumor lymphangiogenesis in CRC. In summary, we concluded that YAP/TAZ can promote tumor lymphangiogenesis by transcriptionally upregulating the expression of CCBE1 and then enhancing proteolytic activation of VEGFC in CRC, which could be effectively blocked by JQ1 (Fig. 5*H*).

Discussion

Tumor lymphangiogenesis facilitates lymphatic dissemination of malignant tumor cells and correlates with cancer progression (26). The generation of new lymphatic vessels and remodeling of existing lymphatics are thought to be definitive processes in lymphangiogenesis and to be regulated by various

medium from full-length VEGFC-expressing 293T cells (1:1), incubated overnight, and analyzed by Western blotting. Ponceau S staining was used to control for equal loading. *G*, tube formation and wound healing assays of HLECs cultured with conditioned medium from the indicated SW837 cells. To exclude the effect of JQ1 on HLECs, SW837 cells were treated with JQ1 (1 μM) or DMSO for 24 h and were then washed and cultured with fresh culture medium without JQ1 for another 24 h. This conditioned medium without JQ1 was collected for the assay with HLECs. The scale bars for the tube formation assay represent 100 μm. The scale bars for the wound healing assay represent 200 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's *t* test. *H*, immunohistochemical analysis of CCBE1 and mLyve-1 (lymphatic vessel number) in the indicated treated stable HCT116 cell line–derived xenografts in mouse footpads. Representative images of mLyve-1(+) lymphatic vessels and CCBE1 protein expression in the same field are shown. Black arrows: mLyve-1(+) lymphatic vessels. Stars: CRC cells. The scale bars represent 20 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, by Student's *t* test. *I*, immunohistochemical analysis of CCBE1 and mLyve-1 in seven CRC patient-derived xenografts treated with JQ1 (50 mg/kg). Representative images are shown. The scale bars represents 20 μm. *Black arrows*: mLyve-1(+) lymphatic vessels. PDX samples treated with JQ1 had lower CCBE1 scores in tumor cells/stroma and *lower* lymphatic vessel densities than those treated with DMSO. $^{*}p < 0.05$ by the Mann–Whitney U test for CCBE1 scores; $^{**}p < 0.01$ by paired Student's *t* test for mLyve-1(+) lymphatic vessel counts. CRC, colorectal cancer; DMSO, dimethyl sulfoxide; HLEC, human lymphatic endothelial cell. PDX, patient-derived xenograft.

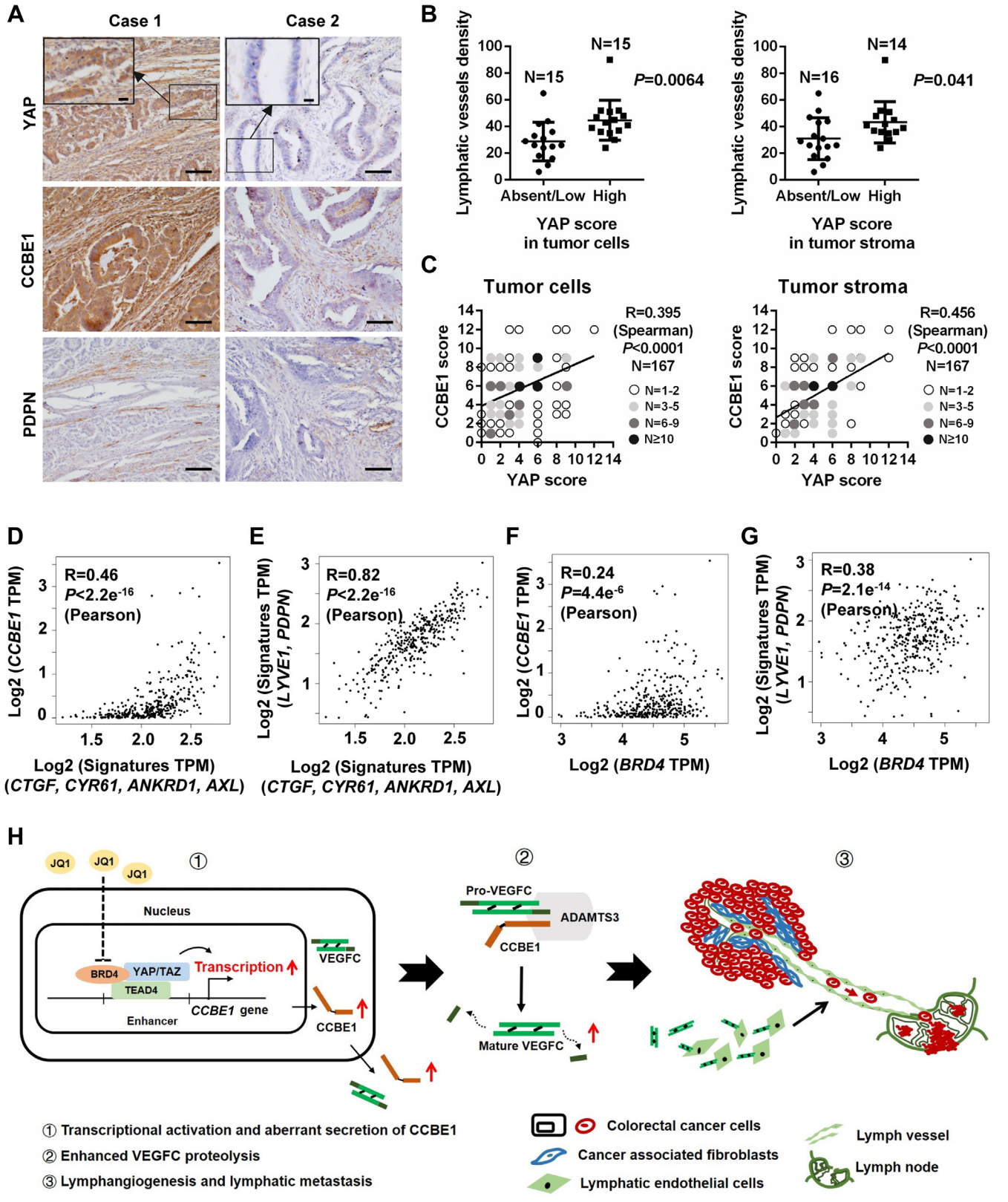

Figure 5. Clinical correlation between YAP and CCBE1 expression and lymphatic vessel density in colorectal cancer (CRC). *A*, representative images of samples with high YAP/high CCBE1/high PDPN expression (case 1) and samples with low YAP/low CCBE1/low PDPN expression (case 2) are shown. The scale bars represent 50 μm for the original image and 10 μm for the zoom magnification image. *B*, CRC samples with a high YAP score in CRC cells and the tumor stroma showed a higher lymphatic vessels density than those with a low YAP score. *C*, positive correlation between the protein levels of YAP and CCBE1 in CRC cells and stroma. A color scale was used to indicate different sample size of each *dot*. Spearman correlation analysis was used to evaluate the associations. *D* and *E*, correlations between the mRNA levels of the YAP target gene signature (*CTGF/CYR61/ANKRD1/AXL*) and *CCBE1* (*D*) and the lymphatic vessel marker signature (*LYVE1/PDPN*) (*E*) in the TCGA CRC dataset. Pearson correlation analysis was used to evaluate the associations. *F* and *G*, correlations

lymphangiogenic factors (such as VEGFC and VEGFD) produced by cancer cells and cells in the tumor microenvironment (26). Increased mRNA levels of *VEGFC/D* are common in multiple cancers, and numerous studies have shown how oncogenic transcription factors enhance the gene expression of *VEGFC/D* to promote tumor lymphangiogenesis and metastasis (27, 28). The VEGFC activating factor CCBE1 is a lymphangiogenic factor, and we recently revealed its dysregulation and protumor lymphangiogenic function in CRC (10). CCBE1 is expressed at very low levels in normal colonic cells, and transcription of *CCBE1* is upregulated in both CRC epithelial cells and CAFs (10). Thus, similar to dysregulation of VEGFC/D in cancer, transcriptional activation of *CCBE1* is the main mechanism underlying CCBE1 overexpression in CRC. It has been reported that atypical E2Fs (E2F7 and E2F8) can regulate lymphangiogenesis through transcriptional activation of the lymphangiogenic genes *CCBE1* and *FLIT4* (29). However, since E2F7/8 are E2F1 repressors and normally act as tumor suppressors (30), and there is a lack of a positive correlation between *E2F7/8* and *CCBE1* in the TCGA mRNA expression dataset, E2F7/8 may not be the transcription factors driving CCBE1 overexpression in CRC. The hippo pathway effectors YAP/TAZ are usually activated in both tumor cells and CAFs during tumor progression (11, 12, 21). In this study, we revealed that YAP/TAZ are indispensable for *CCBE1* gene transcription in CRC cells and CAFs. The clinical correlation between *CCBE1* and the YAP/TAZ gene signature further indicates that increased YAP/TAZ oncogenic activity could be one of the main contributors to the enhancement of *CCBE1* gene expression in CRC. Interestingly, we previously identified an enhancer regulatory region (–20.8 kb) in *CCBE1* that is bound and inhibited by SMAD3/4 in response to TGFβ; this region is also the binding site of the YAP–TEAD complex (10). Although YAP and SMADs are well known to cooperatively regulate the expression of genes such as *CTGF* and *CYR61* (31), YAP may compete with SMADs for binding to this enhancer regulatory region to abolish the inhibitory effect on *CCBE1* and activate *CCBE1* gene expression, and vice versa.

Dysregulation of the Hippo pathway and activation of YAP/TAZ have been related to each cancer hallmark and are associated with the induction of cancer cell hyperproliferation, invasion, and metastasis, as well as cancer cell survival and chemotherapeutic resistance (32). Recently, YAP was found to be required for the survival of metastatic cancer cells in lymph nodes, which promotes a metabolic shift toward fatty acid oxidation and enables tumor cells to adapt to the microenvironment in lymph nodes (17). Our finding that YAP/TAZ promotes tumor lymphangiogenesis by activating the CCBE1–VEGFC axis in CRC further complements the evidence for the vital protumorigenic role of YAP/TAZ in lymphatic metastasis. VEGFC has been shown to promote the proliferation of VEGFR3-positive CRC tumor cells (33). In addition, VEGFC can disrupt the endothelial lymphatic barrier to promote CRC invasion and induce tumor immune escape in CRC (34, 35). Thus, activation of VEGFC by YAP/TAZ through the activation of *CCBE1* expression could endow tumor cells with other progressive characteristics in addition to promoting tumor lymphangiogenesis. YAP/TAZ has recently been revealed to induce gene expression and secretion of VEGFC in intestinal stromal cells to regulate lacteal integrity (22). In contrast, we did not observe transcriptional regulation of *VEGFC* by YAP/TAZ in CRC cells. Although both CCBE1 and YAP/TAZ are involved in lymphatic development, the YAP/TAZ-CCBE1 regulatory axis was not observed in lymphatic endothelial cells or intestinal stromal cells in previous studies (6, 7, 18, 19, 22). This phenomenon may indicate the cell context-dependent transcriptional regulation of *CCBE1* by YAP/TAZ.

Signaling pathways in tumor lymphangiogenesis contain potential diagnostic and therapeutic targets to suppress lymph node metastasis (26). The discovery of the oncogenic role of YAP/TAZ in tumor lymphangiogenesis and cell survival in lymph nodes further demonstrates the promising efficacy of targeting YAP/TAZ in cancer treatment. Recently, BET inhibitors have emerged as inhibitors of YAP/TAZ (13). Zanconato *et al.* (13) revealed that the BET protein BRD4 is a cofactor for YAP/TAZ transcriptional activity and that the BET inhibitor JQ1 can selectively reduce the transcript abundance of most YAP/TAZ-dependent genes in breast cancer cells. We noted that *CCBE1* was one of the differentially expressed genes after knockdown of YAP/TAZ and treatment with JQ1 in breast MDA-MB-231 cells in Zanconato's study (13). These results suggest that *CCBE1* might be a general target gene of YAP/TAZ–BRD4 complexes. Meanwhile, YAP, TAZ, and TEAD4 were reported to be direct targets of BRD4 in lung cancer cells, and we also observed similar results in CRC cells (data not shown) (36). These data suggest that JQ1 could also decrease CCBE1's transcription through indirect suppression of the YAP/TAZ–TEAD4 complex. In addition, our finding that BRD4 can bind to YAP/TAZ-independent CCBE1 enhancers further implicates that BRD4 can also modulate CCBE1's transcription through YAP/TAZ–TEAD4-independent mechanism. Besides BRD4, our study showed that knockdown of BRD2 and BRD3 also decreased the mRNA level of CCBE1 in CRC cells. It is worth exploring the underlying mechanism of BRD2/3's function in regulating CCBE1's transcription in future study. Since BET inhibitors have shown good efficacy in preclinical models of various cancers, including CRC (37). Given that BET inhibitors show proven safety with manageable reversible toxicity in clinical trials, our study provides a rationale for targeting CCBE1–VEGFC by BET inhibitors through inhibiting the YAP/TAZ–TEAD complex–mediated transcriptional activity in CRC.

between the mRNA levels of the *BRD4* and *CCBE1* (*F*) and the lymphatic vessel markers (*LYVE1/PDPN*) (*G*) in the TCGA CRC dataset. Pearson correlation analysis was used to evaluate the associations. *H*, the schematic diagram shows that the YAP/TAZ–TEAD4–BRD4 complex promotes tumor lymphangiogenesis and tumor lymphatic metastasis by transcriptionally upregulating CCBE1 expression and subsequently enhancing proteolytic activation of VEGFC in CRC, which could be effectively blocked by BRD4 inhibitor JQ1.

Experimental procedures

Clinical sample collection and immunohistochemistry

All human CRC samples were collected between January 2008 and December 2016 in the Department of Colorectal and Anal Surgery, XinHua Hospital, Shanghai Jiao Tong University School of Medicine. The Ethics Committee of Xinhua Hospital approved all aspects of clinical sample collection. Informed consent was obtained for all collection. A total of 167 CRC tissues were used to prepare tissue arrays to analyze the correlation between the expression of YAP and CCBE1 by immunohistochemistry. Another 30 CRC tissues were sectioned for immunohistochemical analysis of lymphatic vessel density and expression of YAP and TEAD4. Seven fresh CRC tissues were collected to establish the PDX mouse models. Immunohistochemical experiments were performed as described (10). In this study, the tumor stroma was defined as the population of stromal cells surrounding cancer cells but did not include identifiable blood/lymphatic vessels or muscular tissue.

Cell culture, transfection, Western blotting, qPCR, ChIP, luciferase assay, cell counting kit (CCK-8) assay, and reagents

All experiments were performed as described (10). Experiments were repeated three times, and the results of representative experiments are shown. The following antibodies and reagents were obtained commercially: anti-CCBE1 antibody (Atlas Antibodies, HPA041374, for IHC and WB), anti-VEGFC antibody (Santa Cruz Biotechnology, sc-374628), anti-YAP antibody (Santa Cruz Biotechnology, sc-101199, for IHC and WB), anti-YAP/TAZ antibody (Cell Signaling Technology, D24E4, for WB), anti-BRD4 antibody (Cell Signaling Technology, E2A7X), anti-FLAG antibody (DYKDDDDK Tag, Cell Signaling Technology, D6W5B), anti-human D2-40 (PDPN) antibody (Dako), anti-mouse Lyve-1 antibody (eBioscience, ALY7), Human VEGFC (Pepro Tech, 100-20C), and JQ1 (Selleckchem, S7110). The primer, siRNA, and shRNA sequences are provided in Table S2.

HLEC tube formation and wound healing assays

HLEC tube formation and wound healing assays were performed as described (10). HLEC (#2500, lot no.19415) were purchased from ScienCell and cultured in Endothelial Cell Medium (#1001, ScienCell). Primary human CAFs were isolated from CRC tissues during our previous study and cultured in fibroblast growth medium-2 (FGF-2, Lonza) (25). The indicated cancer cells or CAFs were cultured in fetal bovine serum (FBS)-free culture medium for 24 to 48 h and the FBS-free culture medium was collected. Since fibroblasts and some tumor cells were reported to show similar tube formation activity atop Matrigel (38, 39), the collected culture medium was centrifuged and the supernatants were used as conditioned medium for subsequent experiments. The mixed supernatants were produced by mixing the supernatants from CRC cells/CAFs with FBS-free supernatants from HEK293T cells overexpressing full-length VEGFC protein. The mixed supernatants were then incubated for proteolysis of VEGFC protein at 37 °C for another 24 h before subsequent experiments. For the tube formation assay, 50 μl Growth Factor–Reduced Matrigel was pipetted into each well of a precooled (4 °C) 96-well plate and allowed to polymerize for 30 min at 37 °C. Then, 1×10^4 HLECs in 100 μl conditioned medium were added into each well and incubated at 37 °C and 5% CO_2 for indicated time. The tube formation ability was quantified by measuring the total length and counting the branch numbers of the cord and tubule structures formed on the Matrigel in each well. For the wound healing assay, HLECs in complete medium were seeded into a six-well plate (1×10^6 cells/well). After 8 h, the medium was replaced with serum-free medium, and the HLECs were cultured overnight. A scratch in the cell monolayer was made with a 10-μl pipette tip, and then, the medium was replaced with serum-free conditioned medium from the indicated cells. The migration ability of HLECs was assessed by measuring the unhealed area at 0 h and indicated time to calculate the migration area. Experiments were repeated three times, and the results of representative experiments are shown.

In vivo *xenograft models*

All mouse protocols were approved by the Xinhua Hospital Ethics Committee, Shanghai Jiao Tong University School of Medicine. Patient-derived xenograft (PDX) models were established as described in our previous study (24). For CDX models, 1×10^6 HCT116 cells in 40 μl of PBS were injected into the unilateral footpads of nude mice (4–5 weeks old, male). For JQ1 treatment, mice bearing palpable hindfoot tumors (30–50 mm3) were randomized into the control (vehicle) or JQ1-treated (50 mg/kg, intraperitoneal administration, q.d.) group. Nude mice were sacrificed when the xenograft volume was 0.5 cm^3. The xenograft tumors were sectioned and stained. The mLyve-1-positive vessels were counted in each section. The lymphangiogenic ability *in vivo* was quantified by counting the mLyve-1-positive vessels on each slide.

Statistical analysis

A chi-square test was performed to evaluate the correlation between the lymph node metastasis rate and TEAD4 nuclear expression in clinical samples. Student's *t* test was performed to assess the statistical significance of lymphatic vessel counts and the tube formation and migration abilities of HLECs. The Mann–Whitney U test was performed to assess the statistical significance of the immunoreactive score of CCBE1 in the PDX models. Spearman correlation analysis was performed to evaluate the correlation between the immunoreactive scores of CCBE1 and YAP in CRC samples. Pearson correlation coefficients were used to evaluate the correlations between the mRNA expression of YAP/TAZ target genes (*CTGF, CYR61, ANKRD1, and AXL*) and that of *CCBE1* or the lymphatic vessel signature genes (*LYVE1 and PDPN*). For each analysis, a Bonferroni-adjusted alpha level was used to determine statistical significance. All *p* values were based on two-sided tests and considered to be statistically significant when <0.05. The error bars in the statistical figures indicated the standard deviation.

Data availability

RNA-Seq raw data generated in this study have been deposited in the GEO database under accession code GSE176062.

Supporting information—This article contains supporting information (10, 20, 25, 40, 41).

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: BET, bromodomain and extraterminal domain; CAF, cancer-associated fibroblast; CCBE1, collagen and calcium-binding EGF domain 1; ChIP, chromatin immunoprecipitation; CRC, colorectal cancer; FBS, fetal bovine serum; HLEC, human lymphatic endothelial cell; PDPN, podoplanin; PDX, patient-derived xenograft; qPCR, quantitative PCR; VEGFC, vascular endothelial growth factor C; WB, Western blot.

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