



CREPT facilitates colorectal cancer growth through inducing Wnt/ β -catenin pathway by enhancing p300-mediated β -catenin acetylation

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Abstract

Using whole genome sequencing, we identified gene amplification of *CREPT* in colorectal cancer (CRC). In this study, we aim to clarify its clinical significance, biological effects, and mechanism in CRC. *CREPT* was upregulated in CRC cell lines and in 47.37% (72/152) of primary CRC tumors. Amplification of *CREPT* was detected in 48.28% (56/116) of primary CRC tumors, which was positively correlated with its overexpression ($P < 0.001$). Multivariate analysis showed that CRC patients with *CREPT* protein overexpression were significantly associated with poor disease-free survival ($P < 0.05$). *CREPT* significantly accelerated CRC cell proliferation and metastasis both in vitro and in vivo. RNA-sequencing (seq) analysis uncovered that the tumor-promoting effect by *CREPT* was attributed to enhancing Wnt/ β -catenin signaling. Using co-immunoprecipitation coupled with mass spectroscopy, we identified p300 protein was a novel *CREPT* interacting partner. *CREPT* greatly increased the interaction between p300 and β -catenin, thus promoting p300-mediated β -catenin acetylation and stabilization. Moreover, *CREPT* cooperated with p300, leading to elevated active histone acetylation markers H3K27ac and H4Ac and decreased repressive histone marker H3K9me3 at the promoters of Wnt downstream targets. In summary, *CREPT* plays a pivotal oncogenic role in colorectal carcinogenesis through promoting Wnt/ β -catenin pathway via cooperating with p300. *CREPT* may serve as a prognostic biomarker of patients with CRC.

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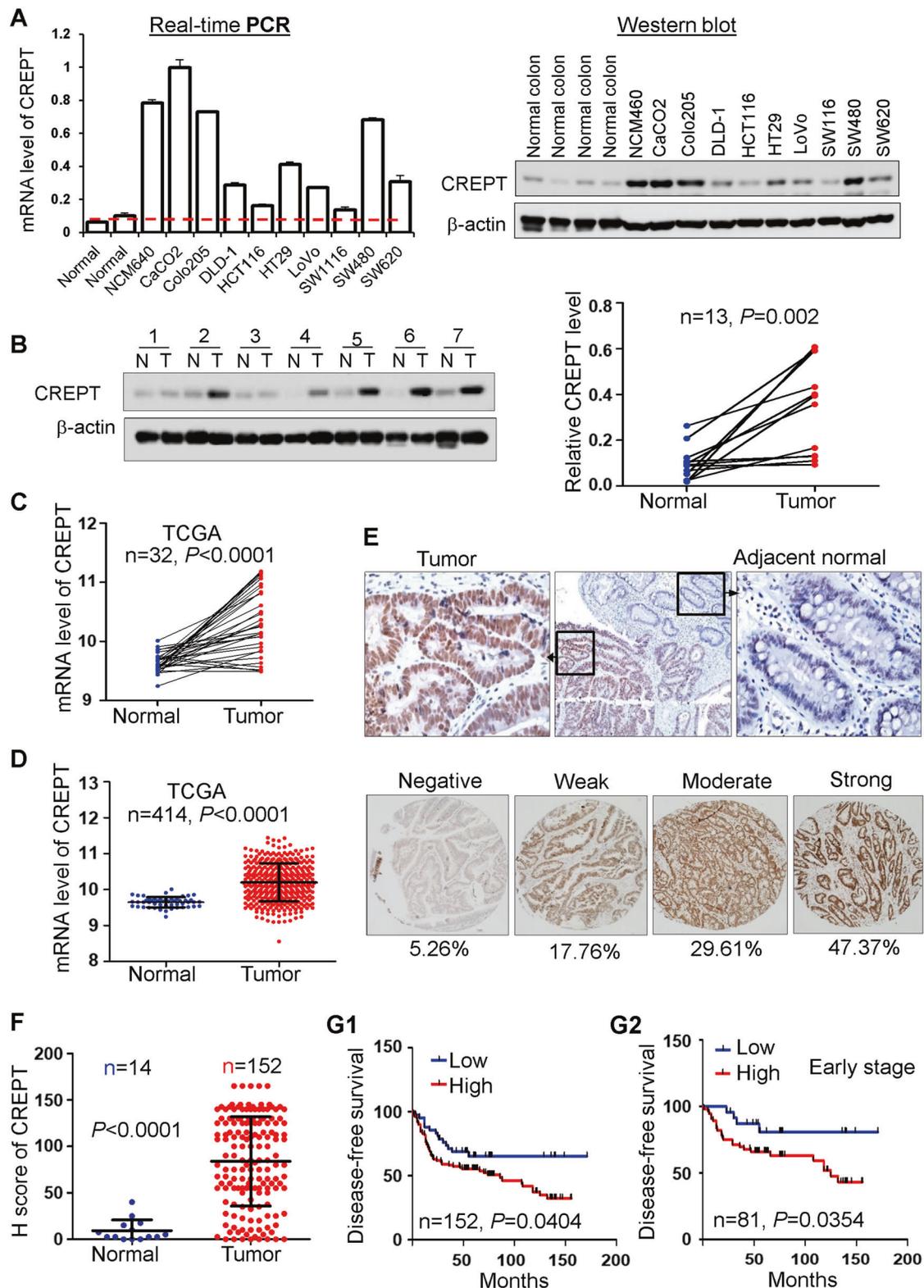
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Introduction

Colorectal cancer (CRC) is the third most common diagnosed cancer in men and the second in women worldwide [1]. More than 1 million individuals are diagnosed with CRC annually, with around 700,000 deaths occurring [2]. CRC is a multistep process with the accumulation of genetic and epigenetic alterations [3, 4]. Previous studies showed that somatic copy number alterations (CNAs) is crucial for the development of CRC [5]. Increased frequency of 7pq, 8q, 13q, and 20q gains, and of 4pq, 5q, 8p, 15q, 17p, and 18q losses were observed in colorectal adenoma and carcinoma progression [6–9]. Therefore, it is important to identify novel genes with CNAs and their biological functions in colorectal tumorigenesis.

Cell-cycle related and expression-elevated protein (CREPT) was firstly identified by our group as a potential oncogene [10]. The expression of *CREPT* was deregulated in many types of tumors [11–15]. *CREPT* is localized at human chromosome 20q11.23, which is a highly amplified region in CRC [16, 17]. *CREPT* functions as a scaffolds of C-terminal domain of human RNA polymerase II (RNAPII)



and cooperates with RPA2 to dephosphorylate of phospho-Ser5, thereby promoting gene transcription [18, 19]. We found that CREPT interacted and coordinated with RNAPII and β -catenin proteins, promoting oncogenic Wnt/

β -catenin signaling and tumorigenesis [10, 20]. However, the role and the molecular mechanism of CREPT in CRC are still unclear. In this study, we investigated the

◀ **Fig. 1** CREPT was overexpressed in colon cancer cell lines and primary tissues. **a** The mRNA and protein level of CREPT was upregulated in most of the colon cancer cell lines detected by real-time PCR or western blot respectively. **b** The protein expression level of CREPT was significantly higher in primary CRC tumors as compared with their adjacent tissues by western blot. **c** The mRNA level of CREPT in tumors was dramatically elevated compared with their paired adjacent normal tissues from TCGA CRC database. **d** CREPT mRNA level was significantly increased in tumor tissues compared with normal tissues from TCGA CRC database ($P < 0.0001$). **e** Representative immunohistochemistry image of CREPT protein expression in CRC tumor tissues and their adjacent tissues. **f** Statistics histogram based on H-score of tissue microarray ($n = 152$) and normal tissues ($n = 14$) ($P < 0.0001$). **g** Disease-free survival in relation to expression was evaluated by the Kaplan–Meier survival curve and the log-rank test ($P < 0.05$) (**g1**). (**g2**) Patients at TNM I and II was defined as early stage

expression regulation, clinical application, biological function, and molecular basis of CREPT in CRC.

Results

CREPT is upregulated in colon cancer cell lines and in primary CRC tissues

We first evaluated the expression of CREPT in CRC cell lines and human tissue samples. CREPT mRNA and protein expression was dramatically enhanced in all nine CRC cell lines compared with normal human colon tissues and two normal human colonic epithelial cells HCEC 1CT and 2CT [21] by real-time PCR and western blot (Fig. 1a, S Fig. 1a). Keeping with the findings, CREPT protein was significantly upregulated in 13 primary CRC tissues compared with their adjacent normal tissues by western blot ($P = 0.002$) (Fig. 1b). To further confirm our findings, the mRNA expression of CREPT in CRC samples from TCGA database (<https://genome-cancer.ucsc.edu>) was analyzed. CREPT was significantly increased in 32 CRC tissues compared with paired adjacent normal tissues ($P < 0.0001$) (Fig. 1c). The upregulation was also observed in unpaired CRC ($n = 364$) compared with normal samples ($n = 50$) from TCGA cohort ($P < 0.0001$) (Fig. 1d). Taken together, CREPT is significantly upregulated in CRC, indicating that it might play an oncogenic role in CRC.

CREPT is an independent predictor of cancer recurrence in CRC patients

To evaluate the association of CREPT with the clinicopathological features and clinical outcomes of patients with CRC, we evaluated the CREPT protein expression in a tissue microarray of 152 CRC tumors. We found that 47.37% (72/152) of CRC tumors showed strong nuclear

Table 1 Cox regression analysis of potential recurrence predictor for patients with CRC

Variables	Univariate analysis		Multivariate analysis	
	RR (95%CI)	P value	RR (95%CI)	P value
<i>Gender</i>				
Female	1			
Male	0.878 (0.549–1.403)	0.587		
<i>Age</i>				
≤60	1			
>60	1.074 (0.678–1.702)	0.761		
<i>Primary tumor location</i>				
Colon	1			
Rectum	0.764 (0.509–1.145)	0.193		
<i>Differentiation</i>				
Well	1			
Moderate	2.801 (0.387–20.310)	0.308		
+Poor				
<i>TNM</i>				
I	1		1	
II	2.054 (0.715–5.901)	0.181	2.109 (0.734–6.056)	0.166
III	3.454 (1.203–9.922)	0.021	3.382 (1.179–9.704)	0.023
IV	11.460 (3.659–35.889)	<0.001	12.320 (3.931–38.614)	<0.001
<i>CREPT</i>				
Low	1		1	
High	1.796 (1.002–3.219)	0.049	1.897 (1.053–3.418)	0.033

The TNM Staging System is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M).

The bold entries highlight the P value which is less than 0.05 and significant

staining of CREPT, 29.61% (45/152) were moderate and 17.76% (27/152) were weak staining, and 5.26% (8/152) cases showed negative staining (Fig. 1e). CREPT was significantly upregulated in 152 tumor tissues compared with 14 normal colon tissues ($P < 0.0001$) (Fig. 1f). There was no significant correlation between CREPT expression and age, gender, tumor localization, differentiation, and the TNM stage of CRC patients (S Table 1). Kaplan–Meier analysis showed that CREPT high expression was significantly associated with poor disease-free survival in patients with CRC ($P < 0.05$, log-rank test) (Fig. 1g1, S Fig. 1b). Upregulated CREPT expression indicated a shorter disease-free survival of patients at early stage (TNM stages I and II, Fig. 1g2), but not at advanced stage (TNM stages III and IV, S Fig. 1b). In univariate Cox regression analysis,

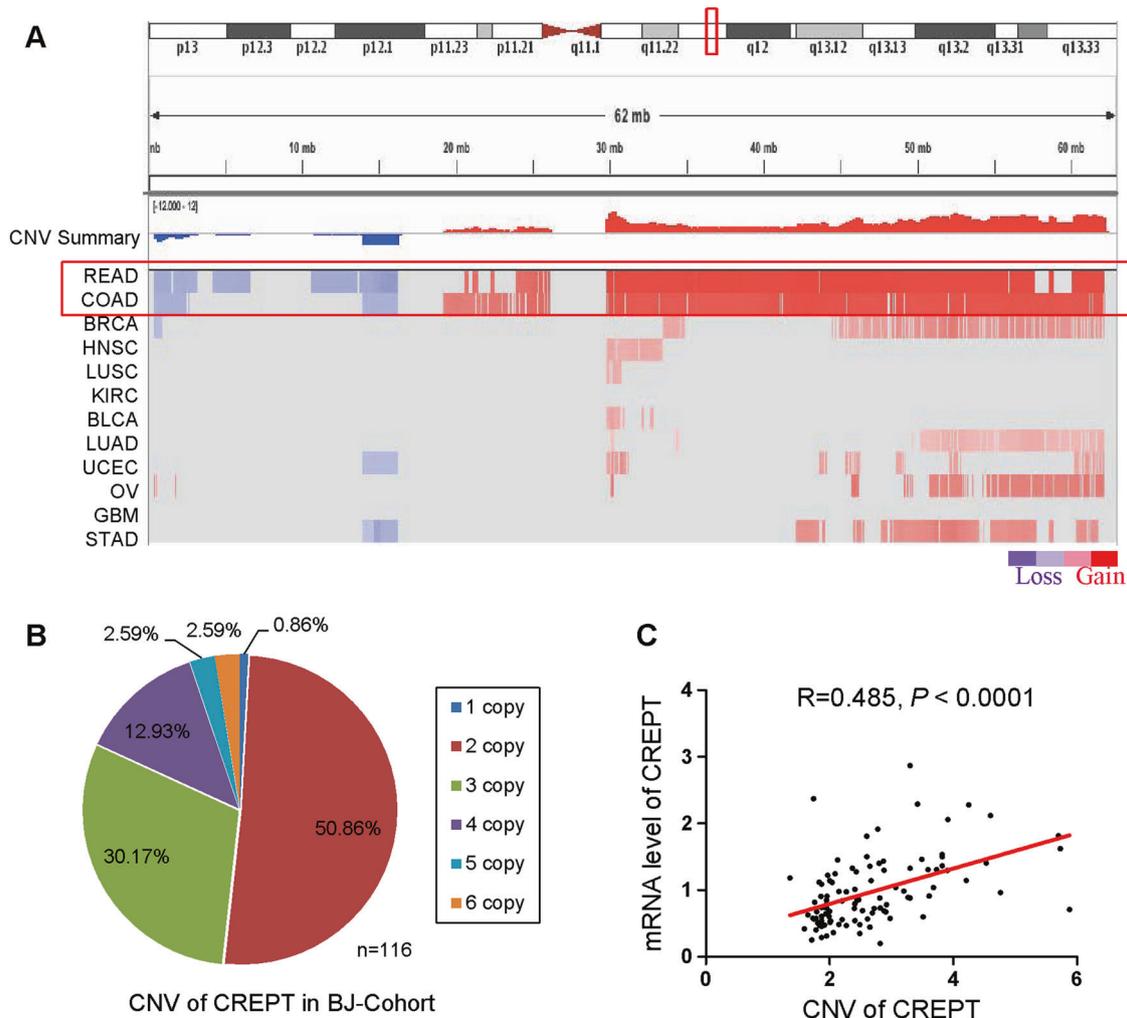


Fig. 2 *CREPT* gene was amplified in CRC. **a** Significant genomic alterations of chromosome 20p13-q13 across 12 while blue color refers to copy number loss. Red box indicates the location of *CREPT* gene. BLCA bladder urothelial carcinoma, BRCA breast invasive carcinoma, KIRC kidney renal clear cell carcinoma, COAD colorectal adenocarcinoma, GBM glioblastoma multiformed, HNSC head and neck squamous cell carcinoma, LAML acute myeloid leukemia,

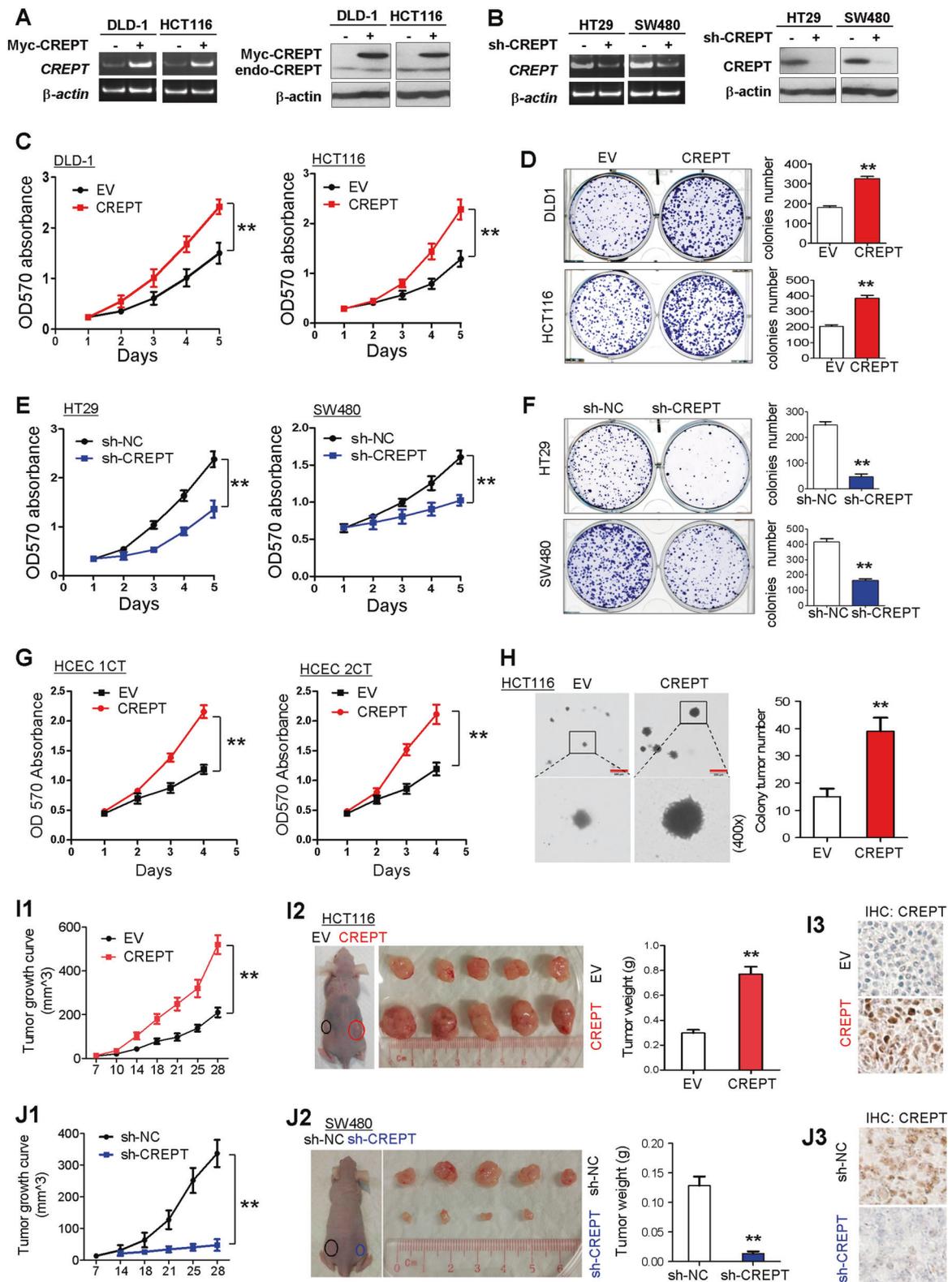
LUAD lung adenocarcinoma, LUSC lung squamous cell carcinoma, READ rectum adenocarcinoma, OV ovarian serous cystadenocarcinoma, UCEC uterine corpus endometrioid carcinoma. **b** DNA copy number variation of *CREPT* was found in 48.28% (56/116) CRC cases by real-time PCR with a Taqman probe targeting *CREPT*. **c** The correlation between copy number changes of *CREPT* and mRNA overexpression ($R = 0.485$, $P < 0.0001$)

CREPT high expression was associated with an increased risk of cancer recurrence (RR = 1.897, 95%CI, 1.053–3.418; $P < 0.05$) (Table 1). After adjustment for potential confounding factors, multivariate Cox regression analysis showed that *CREPT* high expression was an independent predictor of cancer recurrence in patients with CRC (RR 1.897, 95% CI, 1.053–418; $P < 0.05$) (Table 1)

Copy number gain of *CREPT* contributes to its upregulation in CRC

Human *CREPT* gene localizes at the region q11.23 of chromosome 20, which is a highly amplified region in CRC [16, 17]. By whole genome sequencing analyses of the paired CRC tissues from our group, we identified *CREPT*

amplification in CRC [17]. To further confirm our identification, we evaluated the copy number variation from TCGA (<http://www.cbioportal.org>) and found the region that *CREPT* localizes was markedly amplified in both rectum cancer and colon cancer (Fig. 2a). We next examined the DNA copy number variation of *CREPT* in a cohort of 116 primary CRC tumor tissues using a specific Taqman probe against *CREPT*. *CREPT* copy number gain or amplification (defined as more than two copies) was found in 48.28% (56/116) CRC cases (Fig. 2b). Furthermore, a significantly positive correlation between *CREPT* mRNA level and its DNA copy number gain/amplification was observed using linear regression analysis ($R = 0.458$, $P < 0.01$) (Fig. 2c). Therefore, these results suggested that copy



◀ **Fig. 3** CREPT promoted colon cancer cell growth and tumor metastasis. **a** Expression levels of CREPT were increased after transfection with CREPT plasmids in colon cancer cell lines DLD-1 and HCT116 by RT-PCR and western blotting. **b** Knockdown efficiency of CREPT in HT29 and SW480 cell was confirmed by RT-PCR and western blotting. **c** Ectopic expression of CREPT significantly enhanced cell viability in both cell lines by MTT assay. **d** The number of colonies increased when transfected with CREPT plasmids in DLD-1 and HCT116. **e** Knockdown of CREPT significantly inhibited cell viability in HT29 and SW480 cell lines. **f** The number of colonies dramatically reduced after knockdown of CREPT in HT29 and SW480 cell lines. **g** Ectopic expression of CREPT significantly promoted cell viability in HCEC 1CT and 2CT cells. **h** CREPT overexpression accelerated HCT116 cells colony formation in soft agar. **i** Ectopic expression of CREPT in HCT116 cells promoted tumor growth and tumor weight in xenograft model. **j** Knockdown of CREPT in SW480 cells repressed tumor growth and tumor weight. IHC staining showed CREPT expression in xenograft tumor. $n = 5$. * $P < 0.05$, ** $P < 0.01$

number gain of *CREPT* contributes to its upregulation in CRC.

CREPT promotes CRC cell proliferation and tumorigenesis

To elucidate the function of CREPT in CRC, we examined the effects of CREPT on growth characteristics of colon cancer cells by MTT assay and colony formation assay. CREPT was overexpressed in DLD-1 and HCT116 cell lines that showed low CREPT expression and knockdown in HT29 and SW480 that showed high CREPT expression (S Fig. 1a). The efficiency of overexpression (Fig. 3a) and knockdown (Fig. 3b) of CREPT were verified by RT-PCR and western blot. Ectopic expression of CREPT significantly accelerated cell growth (Fig. 3c) and increased clonogenicity (Fig. 3d) in DLD-1 and HCT116 cells compared with empty vector transfected control cells. In contrast, knockdown of CREPT attenuated cell growth (Fig. 3e) and inhibited clonogenicity (Fig. 3f) in HT29 and SW480 cells. Furthermore, CREPT overexpression significantly accelerated the growth of two normal human colonic epithelial cells HCEC 1CT and 2CT (Fig. 3g). Soft agar assay revealed that overexpression of CREPT in transfected HCT116 cells dramatically enhanced their colony-formation ability (Fig. 3h, S Figure 2). To further assess the function of CREPT on tumorigenesis in vivo, we performed a xenograft model assay through subcutaneously injecting CREPT-overexpressing and control HCT116 cells or SW480 cells with CREPT knockdown and control cells, respectively. As shown in Fig. 3i, the growth of tumor volume by CREPT-overexpressing cells was significantly increased compared with control cells ($P < 0.01$). The net weight of tumors formed by CREPT-overexpressing cells was also significantly increased compared with controls at termination of the experiment ($P < 0.01$; Fig. 3i2).

Immunohistochemistry showed that protein expression of CREPT was greatly enhanced in xenograft tumors of the CREPT-overexpressing group compared with control group (Fig. 3i3). The tumor-promoting effect of CREPT in vivo was further confirmed in the knockdown assay of CREPT in nude mice (Fig. 3j).

CREPT enhances invasion and migration abilities of CRC cells

We also investigated the effect of CREPT on metastasis in trans-well migration and invasion assays. Ectopic expression of CREPT significantly increased cell migration (Fig. 4a) and invasion in HCT116 cells (Fig. 4b) compared with control. In contrast, knockdown of CREPT by shRNA dramatically reduced the migratory (Fig. 4c) and invasive abilities (Fig. 4d) of SW480 cells. To further evaluate the ability of CREPT on metastasis in vivo, we intravenously injected HCT116 cells with overexpression of CREPT or empty vector in nude mice. After 6 weeks of injection, mice were killed and the lungs were collected. The number of metastasis nodules in lungs was significantly increased in mice injected with CREPT overexpression cells than in mice injected with control cells ($P < 0.05$) (Fig. 4e). Therefore, CREPT had an important role in colon cancer metastasis.

CREPT promotes Wnt/ β -catenin signaling pathway in CRC

To probe the pathways regulated by CREPT in CRC, we performed RNA-seq on HCT116 cells stably transfected with CREPT or control vector. KEGG Pathway Enrichment Analysis revealed alterations in several pathways, especially WNT signaling pathway ($P < 0.01$, Fig. 5a, detailed gene list in S Table 2). Notably, a considerable number of differential genes were well-established WNT/ β -catenin downstream targets, including *CCND1*, *c-MYC*, and *AXIN2*. Wnt-response luciferase reporter assay further confirmed this finding (Fig. 5b). Ectopic expression of CREPT significantly enhanced the activity of Wnt signaling reporter Top-Flash by 2.67-fold (Fig. 5b1), while knockdown of CREPT attenuated the activity of Top-flash by 50% ($P < 0.01$) (Fig. 5b2). We also validated the expression of these three key Wnt signaling downstream targets by RT-PCR and western blot in CREPT overexpressing and knockdown cells. The mRNA and protein expression of *CCND1*, *c-MYC*, and *AXIN2* were evidently enhanced in CREPT-overexpressing DLD-1, HCT116, HCEC 1CT and 2CT cells compared to the control cells (Fig. 5c, e1, 2); while knockdown CREPT in HT-29 and SW480 cells showed reduced expression (Fig. 5d, e3).

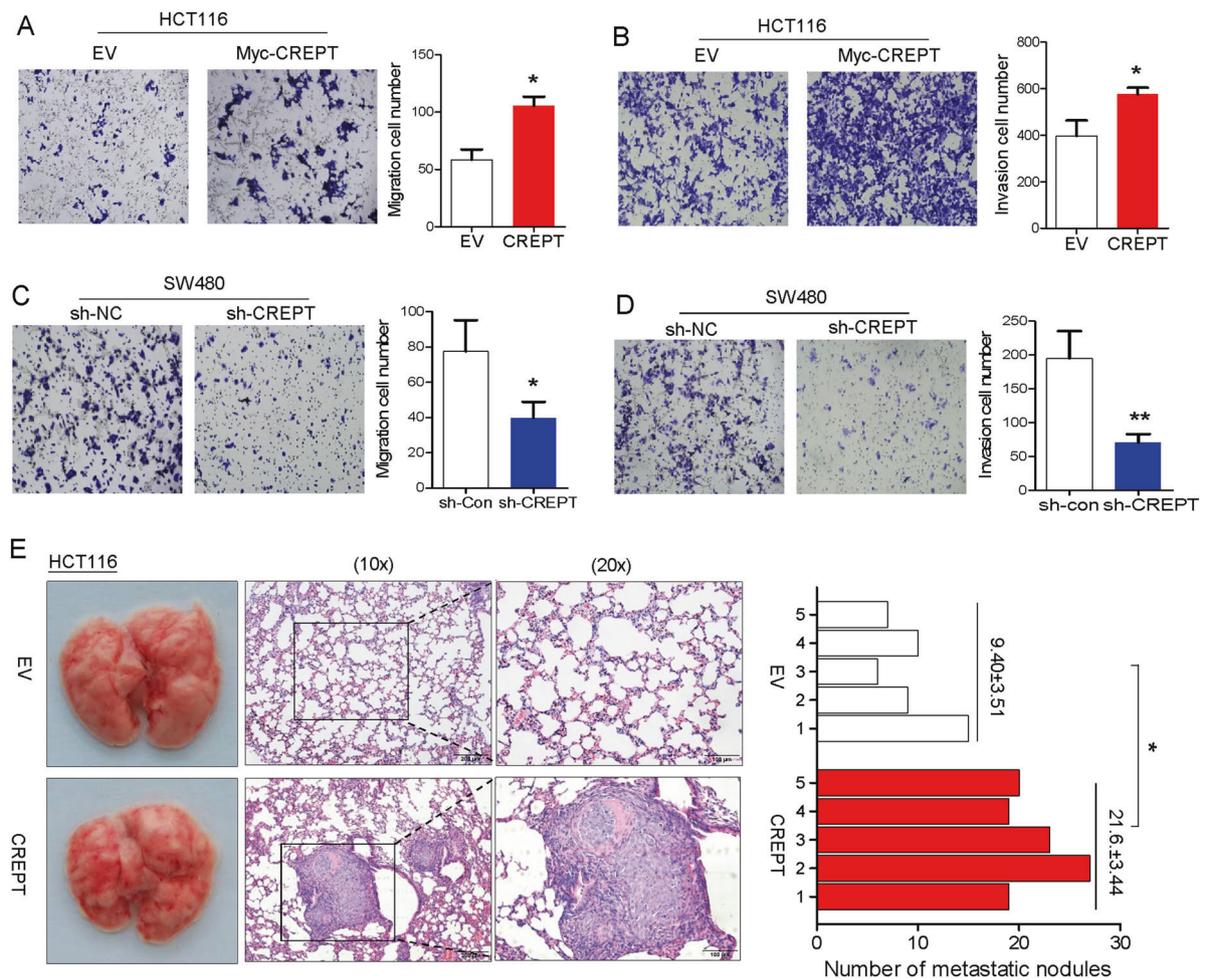


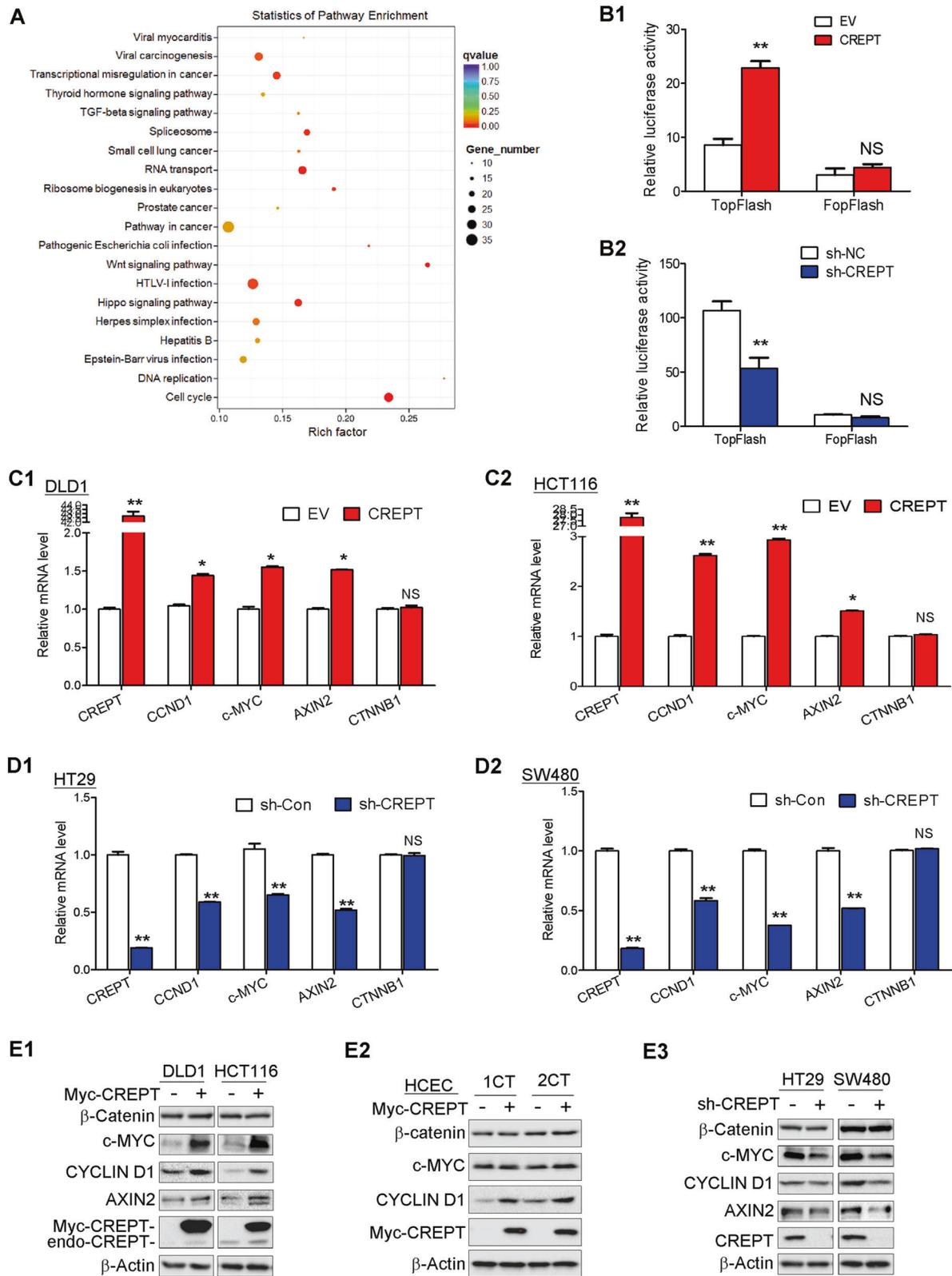
Fig. 4 CREPT enhanced CRC metastasis in vitro and in vivo. Ectopic expression of CREPT promoted cell invasion **a** and migration **b** in HCT116 cells. Knockdown of CREPT suppressed cell invasion **c** and migration **d** in SW480 cells. **e** Ectopic expression of CREPT accelerated lung metastasis of HCT116 cells injected via tail vein. Pictures

on the left showed representative lung morphology. HE staining showed representative lungs section. Histogram showed statistic result of metastasis nodules in nude mice lung tissues. $n = 5$, * $P < 0.05$, ** $P < 0.01$

p300 is identified as a novel crucial co-partner of CREPT

Our group previously performed an immunoprecipitation/mass spectrometry experiment in HEK293T cells over-expressing CREPT and identified RNAPII as an interacting protein of CREPT to stimulate *CCND1* expression and promote oncogenesis [10]. Using this method, p300 was also identified as a potential CREPT interacting partner (S Fig. 3a). The interaction of CREPT with p300 was further confirmed by transient transfection of Myc-tagged CREPT and HA-tagged p300 followed by co-immunoprecipitation with an anti-HA antibody (Fig. 6a). Furthermore, co-immunoprecipitation using endogenous anti-p300 antibody showed that CREPT had a strong binding to endogenous p300 in SW480 cells (Fig. 6b). To elucidate the role of p300 in CREPT-mediated tumorigenesis, we silenced p300 in CREPT-overexpressed cells (Fig. 6c) and examined the

effect on cell growth and colony-formation ability. As shown in Fig. 6d, e, CREPT significantly promoted DLD-1 cell growth, whereas it was significantly dampened after p300 knockdown, suggesting that p300 was an important mediator in CREPT-promoted tumorigenesis. Increasing evidence showed that β -catenin recruits co-activator p300 to form a transcriptionally active complex [22–25]. The binding of p300 has an important effect on Wnt/ β -catenin signaling, leading to differential regulation of target gene expression and different functional outcomes [26, 27]. As a critical partner of CREPT, p300 might be an important co-regulator of CREPT-mediated Wnt/ β -catenin signaling activation. To test this hypothesis, we performed a luciferase reporter assay to evaluate the transcriptional activity of β -catenin/TCF4 complex under different transfection conditions. CREPT overexpression significantly increased the activity of Top-flash, whereas co-transfection of CREPT and p300 further enhanced the transcriptional activity of β -



catenin/TCF4 complex (Fig. 6f). Furthermore, co-immunoprecipitation assay results showed that β -catenin increased its affinity for TCF4 when cells were transfected

with CREPT alone or p300 alone (Fig. 6g, lanes 3 and 4). Furthermore, the interaction between β -catenin and TCF4 was stronger when cells were co-transfected with CREPT

◀ **Fig. 5** CREPT regulated Wnt/ β -catenin signaling pathway in CRC cells. **a** KEGG analysis based on RNA-sequencing data of HCT116 cells stably overexpressing CREPT or control cells. **b** Ectopic expression of CREPT in DLD-1 cell line enhanced TopFlash luciferase reporter activity but not FopFlash reporter (**b1**), while depletion of CREPT reduced dual-luciferase reporter assays in SW480 cells (**b2**). TopFlash, a classic Wnt-response luciferase reporter containing 3xTCF4 binding site. FopFlash, a negative reporter of which TCF4 binding site is mutant. **c** The mRNA expression of Wnt downstream targets *CCND1*, *c-MYC*, and *AXIN2* were enhanced in the presence of CREPT in DLD-1 cells and HCT116 cells. **d** The mRNA expression of *CCND1*, *c-MYC*, and *AXIN2* was diminished under CREPT knockdown in HT29 and SW480 cells. **e** The protein expression of β -catenin, c-MYC, CYCLIN D1, and AXIN2 was increased upon ectopic expression of CREPT in DLD1, HCT116 (**e1**), HCEC 1CT, and 2CT cells (**e2**), while decreased in CREPT knockdown HT29 and SW480 cells (**e3**).

and p300 (Fig. 6g, lane 5). Moreover, CREPT-mediated Wnt/ β -catenin signaling activation was significantly inhibited after p300 knockdown (Fig. 6h, i), further confirming that CREPT cooperated with p300 in promoting Wnt/ β -catenin signaling activation.

CREPT enhances the interaction between p300 and β -catenin and promotes p300-mediated acetylation and stability of β -catenin

To further explore the mechanism how CREPT cooperates with p300 to stimulate Wnt signaling, we examined the effect of CREPT on the formation of β -catenin and p300 transcriptional complex by immunoprecipitation assay. As shown in Fig. 7a, a clear increased associated β -catenin was observed in p300 precipitates from CREPT-overexpressing cells, whereas depletion of CREPT attenuated the interaction between p300 and β -catenin (Fig. 7b), indicating that the interaction between p300 and β -catenin was enhanced in the presence of CREPT. p300 is capable of functioning as acetyltransferase for nonhistone targets [28–31]. p300/CBP coactivator family-mediated acetylation of β -catenin increased its stability and the interaction between β -catenin and TCF4, contributing to β -catenin-mediated neoplastic transformation [22–24, 32–34]. Thus, we proposed that CREPT might regulate β -catenin acetylation by recruiting p300. Our results showed that β -catenin acetylation was significantly enhanced in CREPT-overexpressing cells (Fig. 7c), but decreased in CREPT knockdown cells (Fig. 7d). Especially, acetylation of endogenous β -catenin was increased in DLD-1 cells with CREPT overexpression (Fig. 7e), whereas decreased in SW480 CREPT knockdown cells (Fig. 7f). To further confirm whether CREPT affects β -catenin stability, we examined the degradation rate of β -catenin after cycloheximide treatment. The results showed that the protein level of β -catenin was significantly higher in the presence of CREPT (Fig. 7g). Therefore, our findings

suggested that CREPT-mediated Wnt/ β -catenin signaling activation is through cooperating with p300 and promoting β -catenin acetylation and stability.

CREPT enhances active epigenetic modification of histone in the promoter of Wnt downstream targets

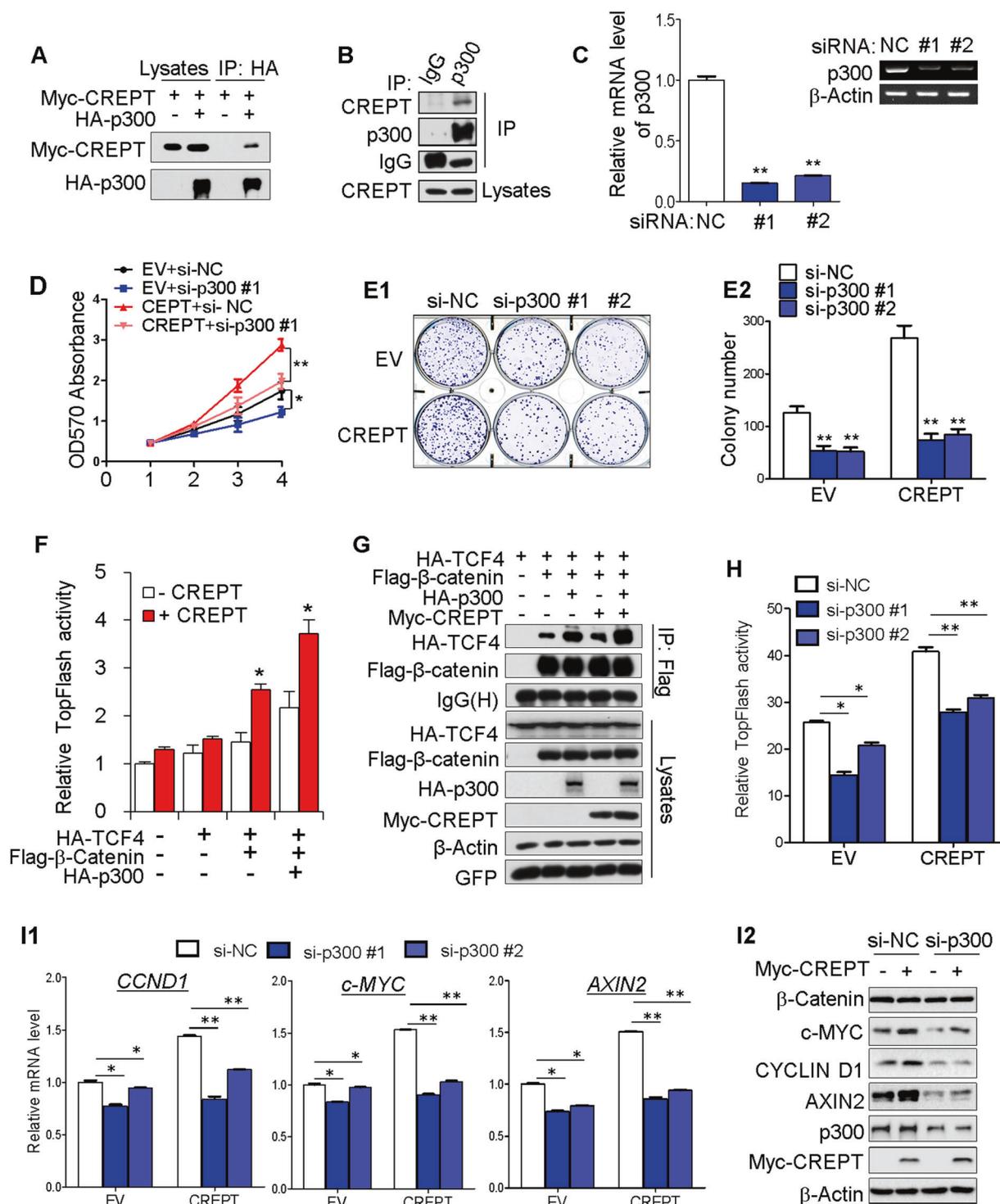
p300 belongs to histone acetyltransferase and regulates transcription via histone acetylation and chromatin remodeling, thus giving an epigenetic tag for transcriptional activation [35, 36]. In this regard, the level of active histone acetylation marker H3K27ac and H4Ac and repressive histone marker H3K9me3 was examined in the promoter of Wnt target gene *c-MYC*, under CREPT overexpression and knockdown by quantitative ChIP-PCR. Our result showed that CREPT enhanced the level of H3K27ac and H4Ac (Fig. 7h) and reduced level of H3K9me3 in the *c-MYC* promoter region (Fig. 7i), indicating that p300 recruited by CREPT on Wnt/ β -catenin downstream target genes and control the transcription efficiency through directly regulated histone modifications.

CREPT is positively correlated with Wnt signaling pathway in CRC patients

To further prove the clinical relevance of CREPT in CRC patients, we checked the protein expression of CREPT, β -catenin, and C-MYC in our cohort of CRC patients by IHC staining. Pearson correlation analysis revealed that the expression of CREPT significantly correlated with the expression of β -catenin, and C-MYC in CRC clinical samples ($P < 0.001$, Fig. 8a). Next, we performed Gene Set Enrichment Analysis (GSEA) analysis in CRC patients from TCGA based on RNA-seq data, revealing that CREPT mRNA expression was significantly positively correlated with WNT signaling and three WNT signaling-related pathways of cell cycle, MYC, and VEGF (Fig. 8b). Moreover, we verified mRNA expression levels of key WNT signaling target genes in CRC (refer to *Wnt homepage*: <https://web.stanford.edu/group/nusselab/cgi-bin/wnt/>) from TCGA CRC RNA-seq data (Fig. 8c1). We found that *CCND1*, *c-MYC*, *AXIN2*, *CLDN1*, *MET*, *FGF18*, *VEGFA*, and *MSL1* were significantly positively correlated with *CREPT* mRNA expression (Fig. 8c2). These results indicated that CREPT promotes WNT signaling in CRC patients.

Discussion

In this study, we found CREPT expression was frequently upregulated in colon cancer cell lines and primary CRC tissues. High level of CREPT was associated with poor



disease-free survival for patients with CRC. Copy number gain of CREPT is one of the main regulatory mechanisms of CREPT upregulation in CRC. CREPT was located on chromosome 20q11.23, which is one of the most frequently amplified regions in CRC [16, 17]. Gene amplification is thought to promote overexpression of genes favoring tumor development [6–9]. CREPT copy number gain or

amplification was observed in 48.28% (56/116) CRC cases, indicating that amplification of CREPT is a common event in colorectal tumorigenesis. Importantly, we revealed that copy number gain of CREPT was positively associated with its mRNA overexpression in 116 CRC cases ($P < 0.0001$), suggesting that increased CREPT copy number contributes

◀ **Fig. 6** p300 was a novel co-partner of CREPT and participated in CREPT-mediated tumorigenesis. **a** Co-immunoprecipitation showed the interaction between CREPT and p300. Anti-HA antibody was used to immunoprecipitate HA-tagged p300 from whole cell extracts prepared from HEK293T cells co-expressing Myc-CREPT and HA-p300. **b** Endogenous CREPT was capable of interacting with endogenous p300 in SW480 cells. **c** p300 knocking down efficiency was examined by real-time PCR and semi-quantitative PCR in DLD1 cells. **d** DLD1 cell growth rate in p300 knockdown was slower after CREPT overexpression. **e** Colony-formation assay indicated p300 knockdown cells showed decreased clonogenicity after CREPT overexpression in DLD1 cells. **f** Co-transfection of CREPT and p300 further enhanced the transcriptional activity of β -catenin/TCF4 complex by dual-luciferase reporter assays. **g** The β -catenin-TCF4 complex formation was significantly enhanced to the highest degree when cells were co-transfected with CREPT and p300. **h** The transcriptional activity of β -catenin/TCF4 complex was decreased in p300 knockdown DLD-1 cells with CREPT overexpression. **i** The mRNA (**i1**) and protein expression (**i2**) of WNT/ β -catenin signaling downstream targets *CCND1*, *c-MYC*, and *AXIN2* were decreased in p300 knockdown DLD-1 cells with CREPT overexpression

to the upregulation of CREPT, which might play an important oncogenic role in CRC.

CRC patients greatly vary in clinical outcome depending on growth status and aggressiveness of the tumors. Currently, TNM stage is the most popularly used as clinical prognostic indicator of disease outcome. However, a part of CRC patients at the early stages of TNM still suffered from disease recurrence. Therefore, additional prognostic biomarkers are requested for better risk assessment. In this regard, we examined the influence of CREPT upregulation on clinical outcomes of 152 CRC patients. High level of CREPT was significantly associated with poor disease-free survival especially at the early stage of patients with CRC (Fig. 1g). Multivariate Cox regression analyses indicated that upregulated CREPT could be regarded as a new independent prognostic marker for CRC recurrence (Table 1).

We previously demonstrated that CREPT was associated with transcriptional complexes not only in promoter regions but also in gene bodies and near mRNA 3'-ends [10]. On one hand, CREPT serves as RNA polymerase II (RNAPII) carboxyl-terminal domain (CTD) scaffolds to coordinate RPAP2-mediated dephosphorylation of phospho-S5, which might be a prerequisite for further association of transcription elongation/processing factors [18, 19]. On the other hand, CREPT was capable of binding to the promoter of *CCND1* as well as its 3'-end, thereby promoting loop formation to bring RNAPII physically close to the transcription start site and enhancing RNAPII recycling from the termination to the promoter region [10]. We also reported that the main oncogenic pathway regulated by CREPT is Wnt/ β -catenin signaling pathway [20]. However, the structure analysis of CREPT showed that CREPT consists of RPR

domain and CCT domain, but lacks common catalyse domain, such as kinase, E3 ligase and acetyltransferase etc. The function of CREPT should thus rely on other functional factor(s). As expected, p300, a novel CREPT interacting protein, was identified as a functional co-player of CREPT in colon cancer by immunoprecipitation and mass spectrometry. Our results showed that besides RNAPII transcriptional complexes, CREPT also coordinated with β -catenin and p300 to control Wnt pathway and its target genes including *CCND1*, *c-MYC*, and *AXIN2*. CREPT and p300 synergized to stimulate Wnt signaling and promote tumor cell proliferation and metastasis in colon cancer cells both in vivo and in vitro. As evidenced by p300 knockdown assay in CREPT-overexpressing cells, CREPT-mediated oncogenic functions and Wnt signaling activation were alleviated by p300 knockdown, suggesting that p300 acted as an essential co-partner of CREPT. More and more evidence demonstrated that p300 and p300/CBP coactivator family members play critical roles in regulating Wnt/ β -catenin pathway through directly binding to β -catenin [22–24, 32–34]. As acetyltransferases, p300 and p300/CBP coactivator family members catalyze the acetylation of β -catenin, thereby improving β -catenin stability, increasing the affinity of β -catenin for TCF4 and leading to efficient transcription [24, 33, 34]. The acetylation of Lys345 on β -catenin by p300 increased the affinity of β -catenin for TCF4 [24]. The cooperation between p300 and β -catenin was greatly dampened by the K345R mutation, implying that acetylation of β -catenin plays an important role in efficient activation of WNT/ β -catenin transcription. Interestingly, acetylation of Lys345 on β -catenin was strongly enriched in the colon cancer cells with hyperactivating Wnt/ β -catenin signaling [24]. p300 was required for β -catenin-mediated neoplastic transformation in colon cancer [23]. In our study, immunoprecipitation result showed that CREPT promoted the association of β -catenin with p300 and enhanced β -catenin acetylation. The transcriptional activity of β -catenin/TCF4 complex and the binding affinity of β -catenin for TCF4 were increased to the higher degree after co-transfection of CREPT and p300. Moreover, the degradation rate of β -catenin was slowed down in the presence of CREPT after cycloheximide treatment, implying that CREPT affected the level of β -catenin via a post-translational mode due to enhanced β -catenin acetylation. Therefore, one of the key mechanisms by CREPT is that it coordinates with p300 to enhance the interaction between p300 and β -catenin, causing acetylation of β -catenin and robust activation of Wnt/ β -catenin pathway. Moreover, p300 is also a histone acetyltransferase. It acted not only on β -catenin but also on chromatin directly. We found that CREPT modulated the level of active histone acetylation marker H3K27ac and H4Ac and repressive histone marker

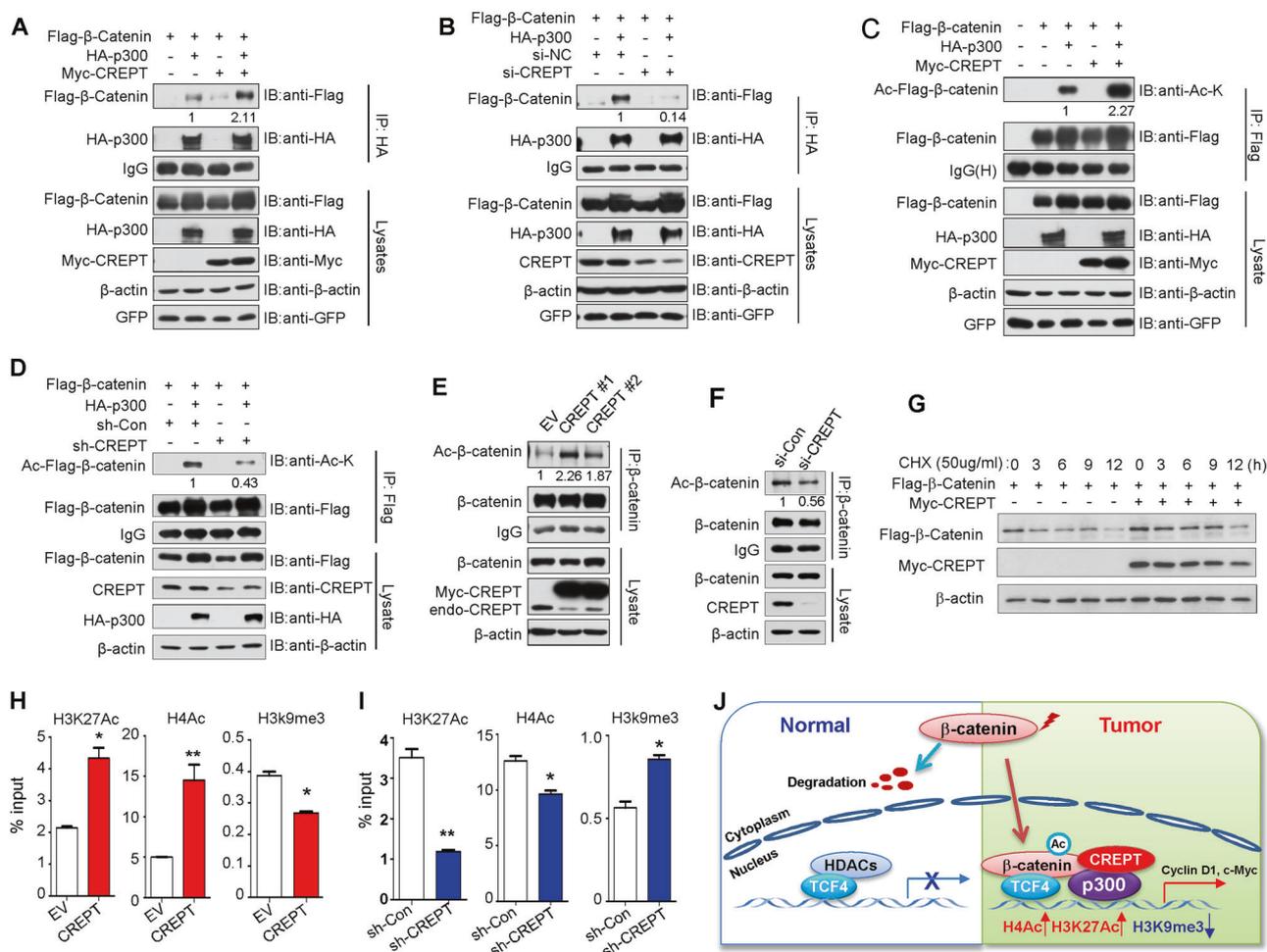


Fig. 7 p300 promoted acetylation and stability of β -catenin and regulated the epigenetic changes on the promoter of Wnt target genes. **a** CREPT enhanced the interaction between p300 and β -catenin. **b** Depletion of CREPT diminished the interaction between p300 and β -catenin. **c** The acetylation level of ectopic β -catenin was greatly enhanced in DLD-1 cells co-transfected with CREPT and p300. **d** CREPT knockdown in SW480 cells led to reduced acetylation level of ectopic β -catenin induced by p300. **e** CREPT overexpression caused increased acetylation level of endogenous β -catenin in DLD-1 cells. **f** The acetylation level of endogenous β -catenin was reduced in CREPT knockdown SW480 cells. **g** CREPT reduced the rate of β -catenin

degradation after cycloheximide addition. **h** CREPT upregulated the level of H3K27ac and H4ac and downregulated the level of H3K9me3 on the c-MYC promoter region in DLD-1 cells. The relative fold enrichment of H3K27ac, H4ac, and H3K9me3 at the c-MYC promoter region was determined by ChIP analysis using chromatin prepared from CREPT-overexpressing and control DLD-1 cells. **i** CREPT depletion led to decreased level of H3K27ac and H4ac and elevated level of H3K9me3 in the c-MYC promoter region in SW480 cells. **j** Proposed mechanistic model of oncogenic function of CREPT in colorectal cancer

H3K9me3 in the promoter of Wnt target genes. Collectively, this study revealed the mechanisms underlying CREPT in regulating Wnt signaling pathway: CREPT recruited p300 to enhance the acetylation and stability of β -catenin and thus activating Wnt signaling pathway.

In summary, we have identified a novel functional oncogene *CREPT* overexpressed in CRC. Upregulated CREPT was associated with poor disease-free outcome. Copy number gain of *CREPT* contributes to its upregulation in CRC. CREPT promoted CRC growth and invasion/migration ability through coordinating with a novel interacting partner p300. CREPT and p300 synergized to

stimulate Wnt/ β -catenin signaling and promoted tumorigenesis in two ways (Fig. 7j). On one hand, CREPT enhanced the interaction between p300 and β -catenin and promoted p300-mediated acetylation of β -catenin, leading to increased β -catenin-TCF4 complex formation on Wnt target genes. On the other hand, p300 recruited by CREPT directly regulated the histone modifications on the promoter of Wnt target genes. Collectively, CREPT plays a pivotal oncogenic role in colorectal carcinogenesis through promoting Wnt/ β -catenin pathway via cooperating with p300. CREPT may serve as a prognosis biomarker for CRC patients.

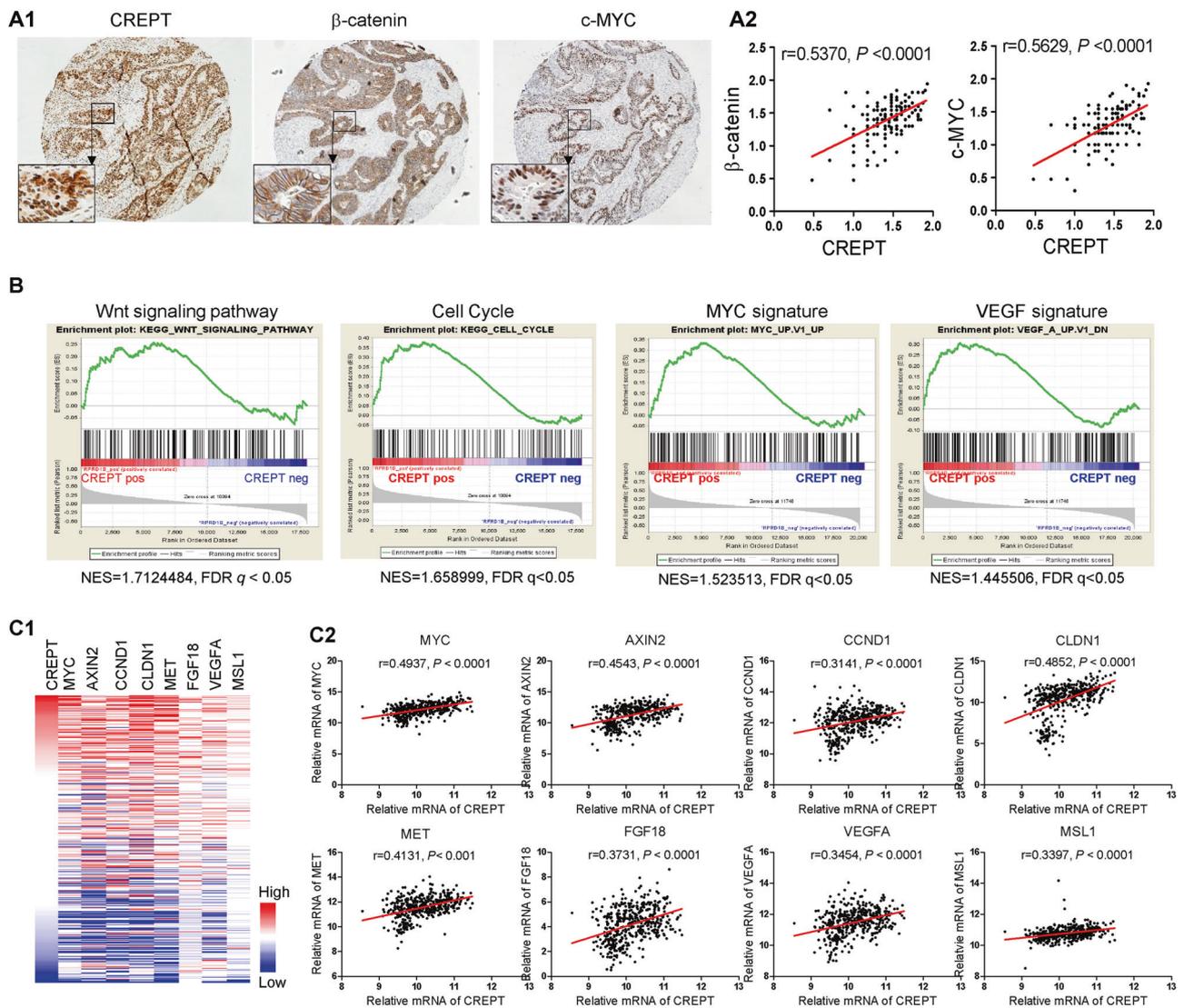


Fig. 8 CREPT was positively correlated with Wnt signaling pathway in CRC patients. **a** Representative IHC pictures to show staining of CREPT, β -catenin and c-MYC using same lot of TMA (**a1**). Correlation of CREPT with β -catenin or c-MYC was performed according to log₁₀ of H score of individual protein staining (**a2**). **b** GSEA results

using TCGA colorectal cancer RNA sequencing data indicated CREPT was positive correlated with Wnt signaling. **c** Correlation of CREPT with Wnt-targeted genes. **c1** Heatmap showed expression of CREPT and Wnt-targeted genes in TCGA. **c2** Correlation analysis of CREPT with Wnt-targeted genes in TCGA

Materials and methods

Plasmids and reagents

Myc-CREPT, Flag- β -catenin, HA-p300, HA-TCF4, Top-Flash, Fop-Flash constructs were used in this study. Anti-cyclinD1 (A-12), anti-Myc (9E10), anti-HA (F-7), anti- β -catenin (E-5), anti-Ac-lysine (7F8), and anti-GFP (FL) antibodies and protein G-agarose beads (sc-2002) were from Santa Cruz Biotechnology, Inc (Dallas, Texas, USA). Anti-c-Myc (9402), anti-AXIN2 (2151) used for western blot was purchased from Cell Signaling Technology, Inc (Danvers, MA). Anti-Flag (F1804), anti- β -Actin (AC-15), Crystal Violet (C3886), DMSO (D2650), and MTT

(M5655) were purchased from Sigma-Aldrich (St Louis, MO). Anti-c-MYC (ab32072) for IHC, anti-H3K27ac (ab4729), anti-H3K9me3 (ab8898) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-H4ac (06-866) antibody was from Millipore (Hong Kong, China). Anti- β -catenin (M3539) for IHC was from Dako/Agilent Technologies (USA). Anti-CREPT antibody was generated by our lab. siRNAs were synthesized by Shanghai GenePharma Co. Ltd, China. The oligo sequence information as: si-p300 #1: GGACUACCCUAUCAAGUAATT; si-p300 #2: CAUCACGGGUAUACAAAUAATT; si-CREPT: GCAAGAACGAAGUGUUAUTT; siRNA negative control (NC): UCUCGAAGUCACGUTT. siRNAs were transfected into cells using Lipofectamine™ RNAiMAX

Transfection Reagent (13778150) twice. FastStart Universal SYBR Green Master used for real-time PCR was purchased from Roche Applied Sciences (Indianapolis, IN, USA). Puromycin (A1113803) and Hygromycin B (10687010) was from ThermoFisher Scientific (Waltham, MA). Cycloheximide (CHX) was from AMRESCO Inc. (Ameresco, Ohio).

Cell lines

Human CRC cell lines (CaCO2, Colo205, DLD-1, HCT116, HT29, LoVo, SW1116, SW480, and SW620) and one immortalized colorectal epithelial cell line (NCM460) were from the American Type Culture Collection (Manassas, VA) and were cultured as described in previous study [37]. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Cells were kept at 37 °C in a 5% CO₂-containing atmosphere. Human colonic epithelial cells (HCEC) 1CT and 2CT were a gift from Prof. Jerry W. Shay (University of Texas Southwestern Medical Center, Dallas, Texas, USA) and were cultured in the conditions previously reported [21]. For generating CREPT stable overexpression or knockdown cell lines, cells were infected with lentivirus and then selected by puromycin at different concentration: 2 µg/ml (DLD-1), 1.5 µg/ml (HCT116), 2 µg/ml (HT29), 4 µg/ml (SW480) or by Hygromycin B (400 µg/ml) for HCEC 1CT and 2CT.

Clinical samples

Thirteen primary paired CRC samples (tumor sample and adjacent non-tumorous sample) randomly selected from Prince of Wales Hospital (Hong Kong) were used for western blot detection. A tissue microarray including 152 cases of CRC patient samples from Prince of Wales Hospital was used for immunohistochemistry staining. In total, 116 CRC tissues obtained from the Beijing University Cancer Hospital (China) were used for copy number variant assay [38]. All patients signed informed consent before specimen collection. The Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee and Human Ethics Committee of Beijing University Cancer Hospital approved the ethics individually.

Animal assay

For xenograft model, 5×10⁶ cells were subcutaneously injected into the right or left dorsal flank of each 4-week-old male Balb/c nude mice, respectively. Each experimental group consisted of five mice. Tumor volume was measured every 2 days for 4 weeks and calculated using the formula $V = (W^2 \times L)/2$ (V = volume, L = length, W = width). For

the metastasis model, 2×10⁶ cells were intravenously injected through the tail vein of nude mouse (4-week old, 5 mice/group). All mice were killed at 6 weeks after injection. The lungs from each mouse were embedded in paraffin for hematoxylin and eosin staining. All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Copy number assay

Gene copy number variation was performed using a TaqMan copy number assay (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, genomic DNA was extracted from CRC samples as templates of PCR. DNA copy number was examined by real-time PCR using a specific Taqman probe against CREPT (Hs01601842_cn) and a reference probe targeted to RNase P (#4403326). Each sample was detected with four replicates to generate the reliable copy number calls. The result was analyzed using Applied Biosystems® CopyCaller® Software (<http://www.appliedbiosystems.com/absite/us/en/home/support/software/real-time-pcr/copycaller.html>).

Cell proliferation analysis

MTT assay was used for cell proliferation detection. 2000 cells/well were seeded into a 96-well plate. The absorbance at 570 nm was measured using Multiskan™ GO Microplate Spectrophotometer (ThermoFisher Scientific). Three independent experiments were performed.

Colony formation

For anchorage-dependent growth, 1000 cells were seeded into a six-well plate. For anchorage-independent growth in soft agar, 7000 cells were inoculated in cell culture medium containing 0.35% agar and seeded in six-well plate with medium containing 0.7% agar. Cells were grown for 2 weeks (anchorage-dependent) or 4 weeks (anchorage-independent). Cell clones were counted and presented as mean ± standard deviation (SD) from three individual experiments.

Cell migration and invasion assay

BD Falcon Cell Culture Inserts (#353097) purchased from BD (Franklin Lakes, New Jersey, USA) were used for migration assay. For invasion assay, BD BioCoat™ Matrigel™ Invasion Chamber (BD, #354480) was used according to the manufacturer's instructions. The number of invaded cells was counted under a phase contrast microscope. Cells in five different fields of each well were averaged.

RNA sequencing

HCT116 cells overexpressing CREPT or empty vector were harvested for total RNA extraction. RNA sequencing and analysis were performed by Beijing Novogene Technology Co. Ltd.

Protein half-lives assay

To detect the half-life of the β -catenin protein, Flag- β -catenin was transfected into HEK293T cells alone or with Myc-CREPT. After 24 h of transfection, cells were treated with 50 μ g/ml cyclohexanone (CHX) and further cultured for indicated time course. Cells lysates were examined using western blotting.

Co-immunoprecipitation and western blotting

Co-immunoprecipitation was performed as previously reported [39]. Specifically, for transient transfection interaction assays, Flag- β -catenin, HA-TCF4, HA-p300 and Myc-CREPT plasmids were transfected into HEK293T cells according to the assay design. For endogenous interaction assays, the nuclear fraction of SW480 cells was used for immunoprecipitation

Luciferase assay

HEK293T, DLD-1, or SW480 cells were transiently transfected with the indicated plasmids using Lipofectamine 2000. Luciferase activity was measured after 24 h of transfection using a Dual-Luciferase reporter assay system (E1910, Promega, Madison, WI). The luciferase activity was normalized by firefly against Renilla luciferase activity and presented as mean \pm SD.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as previously described [20]. Briefly, sonicated DNA was immunoprecipitated with histone modification markers H3K27Ac, H4Ac, and H3K9me3 antibodies. Normal IgG was used as negative control. ChIPed DNA was examined by real-time PCR to evaluate histone modification status on the *c-MYC* promoter, using primers of TCF4 binding site: 5'-TTGCTGGGTTATTTAATCAT-3' and 5'-ACTGTTTGACAAACCGCATCC-3' [40]. The ChIP-qPCR data was normalized using Percent Input Method.

Statistical analysis

All experiments were repeated at least three times. Data were presented as mean \pm standard deviation. Correlation

analysis of copy number and mRNA was performed using linear regression. Significant differences between groups were determined using the Student's *t* test. The difference between the two groups in cell growth curve and tumor growth rate of nude mice were determined by repeated-measures analysis of variance. The H-score of CREPT as a weighted sum of the intensity of IHC tumor cell nuclei was calculated as reported [41]. Survival analysis was calculated using Kaplan–Meier analysis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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