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CREPT Accelerates Tumorigenesis by Regulating the Transcription of Cell-Cycle-Related Genes

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SUMMARY

Tumorigenesis is caused by an uncontrolled cell cycle and the altered expression of many genes. Here, we report a gene *CREPT* that is preferentially expressed in diverse human tumors. Overexpression of CREPT accelerates tumor growth, whereas depletion of CREPT demonstrates a reversed effect. CREPT regulates cyclin D1 expression by binding to its promoter, enhancing its transcription both in vivo and in vitro, and interacting with RNA polymerase II (RNAPII). Interestingly, CREPT promotes the formation of a chromatin loop and prevents RNAPII from reading through the 3′ end termination site of the gene. Our findings reveal a mechanism where CREPT increases cyclin D1 transcription during tumorigenesis, through enhancing the recruitment of RNAPII to the promoter region, possibly, as well as chromatin looping.

INTRODUCTION

Tumor development is highly related to uncontrolled cell growth (Hanahan and Weinberg, 2000), a process where both oncogenes and tumor suppressors are involved (Gordon et al., 2005; Vogelstein and Kinzler, 2004). Oncogenes are upregulated or mutated with gain of function, whereas tumor suppressors are downregulated or lost in most tumors (Yamasaki and Pagano, 2004). Oncogenes encode growth factors, receptors, signal transducers, and nuclear proteins including transcriptional factors (Polsky and Cordon-Cardo, 2003; Zhang et al., 2005a, 2005b, 2005c), among these, cell-cycle-related proteins such as cyclin D and cyclin-dependent kinase (CDK)s have been demonstrated to play a major role in tumorigenesis (Johnson and Walker, 1999; Osborne et al., 2004; Semczuk and Jakowicki, 2004; Sherr, 1996).

Cell-cycle-related proteins precisely regulate cell proliferation. CDK4/6 and CDK2 pair with their cyclins (cyclin D, E) to control cell progression through G1 to S phase (Johnson and Walker, 1999; Lee and Yang, 2003; Sherr, 1996). Cyclin D/CDK4 or cyclin D/CDK6 complex forms in early G1 phase and functions during the whole G1 to S phase transition (Lee and

Significance

Many genes have been attributed to tumorigenesis; however, additional unknown genes are to be characterized in human tumors. In this study, we found a gene named *CREPT* that is highly expressed in human tumors and is correlated with short survival time of cancer patients. We revealed a mechanism where CREPT promotes cell proliferation by enhancing transcription of *CYCLIN D1*. CREPT prevents RNAPII from "reading through" and possibly promotes the recycling of RNAPII to the promoter of genes via the formation of a chromatin loop. We provided evidence that tumor cells with a high level of CREPT use "antiterminator" instead of "torpedo" model to accelerate transcription of *CYCLIN D1*. Our study provides insights on the mechanisms of tumorigenesis.

Yang, 2003), and phosphorylates the retinoblastoma protein (RB), which activates the transcription factor E2F. The cyclin E/CDK2 complex forms in mid to late G1 phase and is important for S phase entry (Obaya and Sedivy, 2002). Activities of these cyclins/CDKs can be regulated by CKIs (CDK inhibitors, such as $p15^{INK4b}$, $p16^{INK4a}$, $p21^{CIP1/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$), which act as tumor suppressors, at the protein level (Lee and Yang, 2003; Obaya and Sedivy, 2002). The G1/S transition and progression in the S phase also requires the cyclin A/CDK2 complex. Furthermore, cyclin A2 and cyclin B1 form a complex with CDK1 and function in the G2/M transition.

Several oncoproteins positively regulate cyclins and CDKs but negatively regulate CKIs in tumor development (Felsher, 2003; Polsky and Cordon-Cardo, 2003; Zhang et al., 2005a, 2005b, 2005c). Great efforts have been put on the identification and characterization of these proteins (Felsher, 2003; Hunter and Pines, 1994; Massagué, 2004). To date, different screenings have been performed and numerous oncogenes have been found (Dickins et al., 2005; Gordon et al., 2005); however, additional unknown genes are likely involved in tumor development and it is important to identify and characterize novel genes that regulate tumorigenesis (Bonetta, 2005).

In a search for genes related to tumors, we performed a database homology screen using a recently isolated gene (*p15rs*), which is functionally linked to $p15^{INK4b}$ (Liu et al., 2002) and inhibits cell proliferation (Wu et al., 2010), and identified a gene *CREPT* (cell-cycle-related and expression-elevated protein in tumor). Here, we demonstrate the role of CREPT in tumorigenesis.

RESULTS

CREPT Is a Homolog of *Rtt103* and Highly Conserved across Species

Similarity searches of the NCBI protein database with a recently reported protein p15RS (Liu et al., 2002) identified a gene with no reported function. We named this gene *CREPT* (cell-cycle-related and expression-elevated protein in tumor) based on our later observation that this gene is related to the cell cycle and highly expressed in tumors (GenBank accession numbers DQ372938, DQ372939, DQ372940). Human (h) *CREPT* encodes a protein of 326 amino acids with a high similarity to p15RS (Figure S1A available online). CREPT contains an RPR domain (regulation of nuclear pre-mRNA, or CID, CTD-interacting domain) and is highly conserved across species (Figures S1B and S1C). In yeast, the homologous gene is called *Rtt103* (Scholes et al., 2001; Tong et al., 2001). A phylogenetic analysis indicates that two genes, *CREPT* and *p15RS*, in mammals, birds, fish, and *Arabidopsis* are homologs of yeast *Rtt103*, whereas in nematode, fly, and frog only one homolog is predicted (Figures S1B and S1C).

CREPT Is Highly Expressed in Human Tumor Tissues

To investigate the role of CREPT in human diseases, we screened samples from patients with cancers using a monoclonal antibody we generated (Figure S1D). We found that CREPT is more highly expressed in tumor tissues from colon cancer patients (Figure 1A), whereas in the paired noncancer tissues it is hardly (patients 1, 3, and 7) or weakly (patients 2, 4, 5, and 6) detected (Figure 1A, top panel). High levels of CREPT protein are in concordance with its mRNA levels in the tumor tissues (Figure 1A, bottom panel).

Elevated expression of CREPT is not limited just to the colon cancer, as we demonstrated that CREPT mRNA expression is elevated in other cancers including lung, liver, breast, prostate, stomach, uterine endometrium, and cervix cancers (Table 1). Patient samples (eight patients for each cancer are shown in Figure S1E) demonstrate that CREPT expression is dramatically enhanced in all cancer tissues compared to the paired noncancer tissues. In many cases, we could not detect the expression of CREPT in noncancer tissues but observed high levels in cancer tissues. In some cases, we detected relative high levels of CREPT expression in noncancer tissues, but the levels were always lower than those in the paired cancer sections (e.g., colon cancer patient 1, breast cancer patient 7, liver cancer patients 2 and 6 in Figure S1E).

Immunohistochemistry (IHC) analyses with an anti-CREPT antibody showed strong expression of CREPT in tumor regions compared to paired nontumor regions in the same patients (Figure 1B). The results also demonstrated that CREPT is highly expressed in the nucleus of tumor cells (Figure 1B, enlarged images). The nuclear localization was confirmed by overexpression of GFP-CREPT (Figure S1F) or immunostaining with an anti-CREPT antibody along with DAPI in HeLa and HEK293T cells (Figure S1G). Clearly, few CREPT positive staining cells can be observed in the nontumor regions, whereas most of the cells were negatively stained (Figure 1B).

In total, we examined 347 cases by RT-PCR and 466 cases by IHC analyses for patients with eight different types of cancers. Significantly elevated levels of CREPT mRNA or protein were found in 83.3% or 86.5%, respectively, in tumor compared to nontumor tissues from cancer patients (Table 1). Interestingly, a survival analysis of 117 stomach cancer patients indicated that positive CREPT staining was significantly correlated with shorter survival time of the patients after surgery and treatment (Figure 1C). CREPT is also highly expressed in most tumor cell lines examined (Figure S1H).

CREPT Promotes Cell Proliferation In Vitro

Elevated expression of CREPT in tumor cells suggests a role in cell proliferation. Since cells maintain high proliferation ability during embryonic development, we sought to examine the level of CREPT in different stages of the mouse embryos and young mice. An RT-PCR and a northern analysis indicated that CREPT was highly expressed during early stages of mouse embryonic development (Figure S1I). CREPT expression was observed in different normal tissues from mice after birth but remained at low levels in adult mouse tissues (Figure S1J). An IHC analysis showed that 10 days after birth tissues that highly expressed CREPT had correlated high levels of cyclin D1 and Ki67, two cell proliferation markers (Figure S1K). These results suggest that CREPT plays a critical role in cell proliferation during embryogenesis and mouse development.

To address whether CREPT affects cell proliferation, we stably overexpressed or depleted CREPT in MGC803 cells. The results showed that cells overexpressing CREPT proliferated more rapidly than the mock cells (Figure 2A), whereas cells

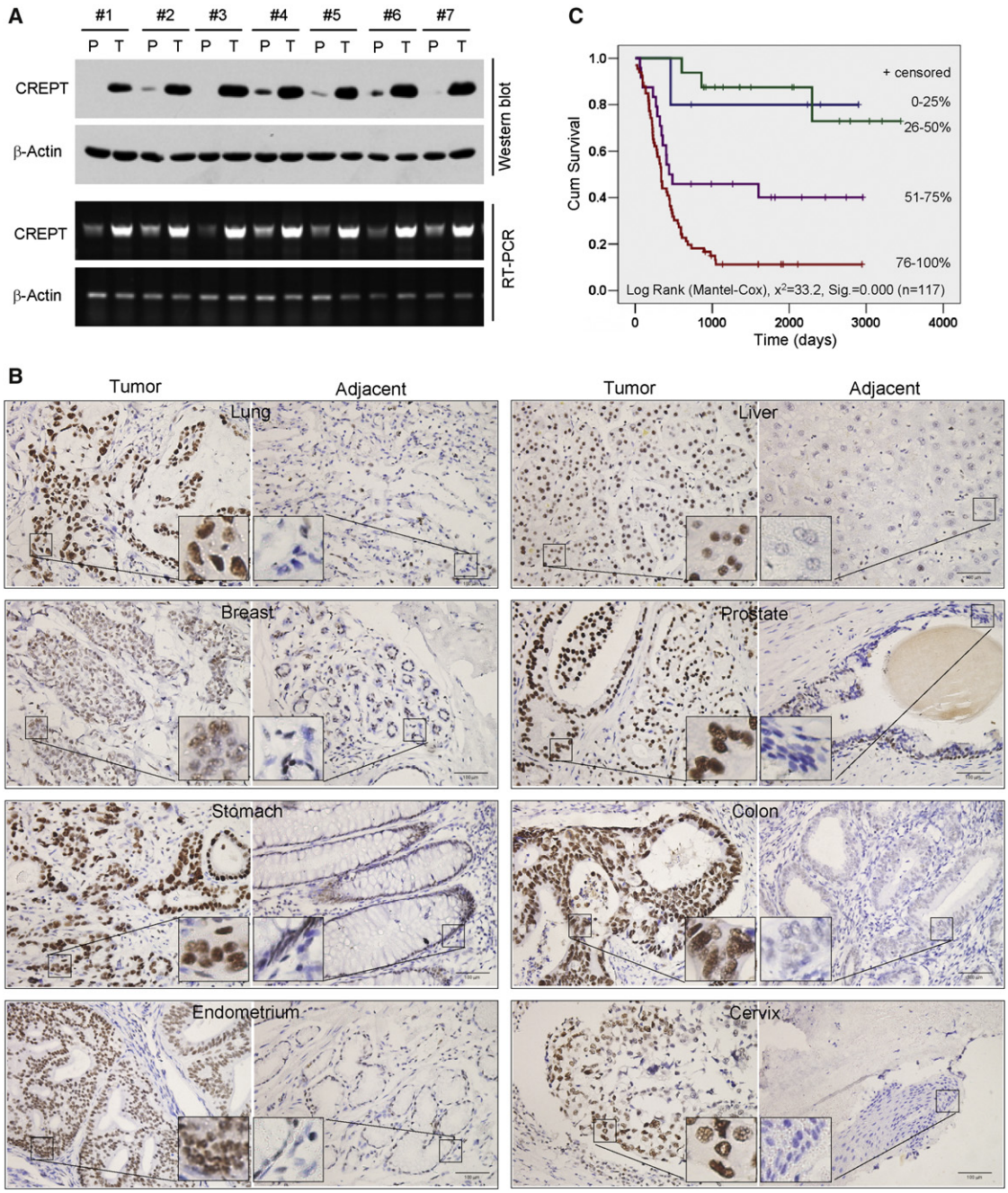

Figure 1. CREPT Is Highly Expressed in Tumor Tissues

(A) CREPT is highly expressed in colon cancer. RT-PCR and western blotting were performed in colon cancer samples. P refers to paired nontumor tissue and T tumor tissue from the same patient.

(B) CREPT is highly expressed in different tumor tissues. Immunohistological staining assays were performed with an anti-CREPT antibody (DAB staining, scale bars, 100 μm).

(C) Expression of CREPT correlates with the survival time of stomach cancer patients. A tissue array analysis was done in 117 cases of patients with the survival information. The percentage of CREPT-positive cells (%) was determined by counting five random views of each sample at 400-fold magnification.

See also Figure S1.

with depleted CREPT grew more slowly than the mock-si cells (Figure 2B). Consistently, overexpression or depletion of CREPT in HepG2 (Figure 2C) and HeLa (Figure S2A) cells led to accelerated or inhibited cell proliferation. In addition to HepG2 and HeLa cells, which are of tumor origin, we also observed similar results in a tetracycline induced CREPT-overexpression system in NIH 3T3 cells (Figure S2B), which are of fibroblast origin and expresses low endogenous CREPT

Table 1. Enhanced Expression of CREPT in Different Tumors Assessed by RT-PCR and Immunohistochemistry

	Upregulated Expression[a] (%)	
Tumor Organs	RT-PCR	Immunohistochemistry
Lung	57.1 (8/14)	75.9 (22/29)
Liver	86.9 (186/214)	73.8 (118/160)
Breast	75.0 (21/28)	77.1 (27/35)
Prostate	100.0 (8/8)	100.0 (8/8)
Stomach	70.0 (14/20)	88.3 (128/145)
Colon	75.0 (15/20)	80.9 (55/68)
Uterus endometrium	79.0 (15/19)	79.2 (19/24)
Uterine cervix	91.7 (22/24)	89.7 (26/29)
Total	83.3 (289/347)	86.5 (403/466)

[a] Upregulated expression was defined as those the expression of CREPT in tumor side is greater than that in paired nontumor side. The numbers in parentheses refer to number of tumors showing increased expression/total number of tumors.

(Figure S1I). Taken together, these results demonstrated that CREPT functions as a positive regulator of cell proliferation in both tumor-originated (epithelial) and nontumor-originated (fibroblastic) cells.

CREPT Promotes Tumorigenesis In Vitro and In Vivo

Since CREPT is highly expressed in tumors and promotes cell proliferation, we speculated that CREPT might enhance tumorigenesis. To examine this hypothesis, we first performed a colony formation assay in soft agar. The result showed that HepG2 cells stably overexpressing CREPT produced more colonies, whereas cells depleted of CREPT yielded fewer colonies than mock cells (Figure 2D). Similar results were observed with HeLa cells (Figures S2C and S2D).

To investigate whether CREPT accelerates tumorigenesis in vivo, we injected cells into nude mice and observed tumor growth. The result showed that CREPT-overexpressing cells generated larger tumors than control cells, whereas CREPT-depleted cells formed smaller tumors (Figures 2E and 2F). In addition, tumor appearance time (defined as the day when the tumors become palpable after injection) was shortened to 6 days when CREPT was overexpressed, whereas depleting CREPT expression delayed tumor appearance by additional 4.5 days (Figure 2G). Furthermore, we observed that tumors originating from cells with CREPT overexpression showed higher malignancy and much stronger proliferation ability as indicated by HE staining (Figure S2E) and immunostaining with an anti-PCNA antibody (stronger PCNA expression in CREPT overexpressed cells) (Figure S2F).

To address whether CREPT causes the transformation of cells, we overexpressed CREPT in tetracycline inducible NIH 3T3 cells. A soft agar experiment showed that cells with overexpressed CREPT formed colonies (Figure S2G). Consequently, NIH 3T3 cells overexpressing CREPT formed tumors in nude mice, whereas control cells had no ability to form tumors (Figure S2H). These data suggested that CREPT promotes tumor growth in vitro as well as cell transformation in NIH 3T3 cells.

CREPT Alters the Cell Cycle

A FACS analysis was performed to determine whether CREPT enhances cell growth and promotes tumorigenesis via alteration of the cell cycle. The size of the cell population in the S phase is significantly increased with overexpression of CREPT 12 hr after release from synchronization, whereas that in the G1 phase remains at a low level at this same time point in MGC803 cells (Figure 3A; Figure S3A). In contrast, the reversed effect was observed when CREPT was depleted (Figure 3B; Figure S3B). The proportion of cells in the G2/M phase was either slightly decreased or unchanged when CREPT was overexpressed (Figure 3A) or depleted (Figure 3B). Similar results were observed with HeLa (Figure S3C) and HepG2 (Figure S3D) cells. Furthermore, when CREPT was overexpressed in NIH 3T3 cells, we also observed more cells in the S phase (Figure 3C). By determining the length of each phase, we found that the overexpression of CREPT shortened the cell cycle in HeLa cells (Figure S3E). These results indicate that CREPT alters the cell cycle by promoting the G1 to S phase transition.

CREPT Regulates Expression of Genes Controlling the Cell Cycle

Given that the cell cycle was altered by the overexpression or depletion of CREPT, we addressed whether CREPT affects the expression of cell-cycle-related genes. Since CREPT affected the G1 to S phase transition (Figures 3A and 3B), we focused our attention on genes for activators (CDK4/6/cyclin D1 and CDK2/cyclin E/A), inhibitors ($p15^{INK4b}$/16^{INK4a} and $p21^{CIP1/WAF1}$/27^{KIP1}), as well as downstream genes such as RB, E2F1, and PCNA during cell-cycle regulation. An RT-PCR analysis indicated that the cyclin D1, CDK6, and CDK4 mRNA levels increased dramatically when CREPT expression was induced by the withdrawal of tetracycline in the NIH 3T3 cells (Figure 3D; Figure S3F). In contrast, cyclin A, $p21^{CIP1/WAF1}$, and $p27^{KIP1}$ remained constant and $p15^{INK4b}$, $p16^{INK4a}$, and RB decreased. Both cyclin E and CDK2 were also increased when CREPT was overexpressed (Figure 3D; Figure S3F). Similar results were observed when CREPT was overexpressed in HeLa cells (Figure S3G, left two lanes) and the reverse trend for the cell-cycle-related gene expression was observed when CREPT was depleted by an siRNA in HeLa cells (Figure S3G, right two lanes). Among these genes, we found that the *CYCLIN D1* gene, in particular, responded to the CREPT alteration quickly (Figure 3D). These data suggested that CREPT regulates expression of cell-cycle-related genes.

CREPT Enhances Gene Transcription Specifically

To examine whether changes in mRNA levels of genes that CREPT regulated are at the transcription level, we performed nuclear run-on experiments. The result indicated that transcription of CDK4/6, cyclin D1, CDK2, and cyclin E was enhanced when CREPT was overexpressed and almost completely abolished when CREPT was depleted (Figure 4A). In contrast, transcription of $p15^{INK4b}$, $p16^{INK4a}$, and RB was blocked by CREPT overexpression, and this inhibition was released when CREPT was depleted. These data suggest that CREPT regulates the expression of CDK4/6, cyclin D1, CDK2, and cyclin E at the transcriptional level. Moreover, the regulation is gene specific

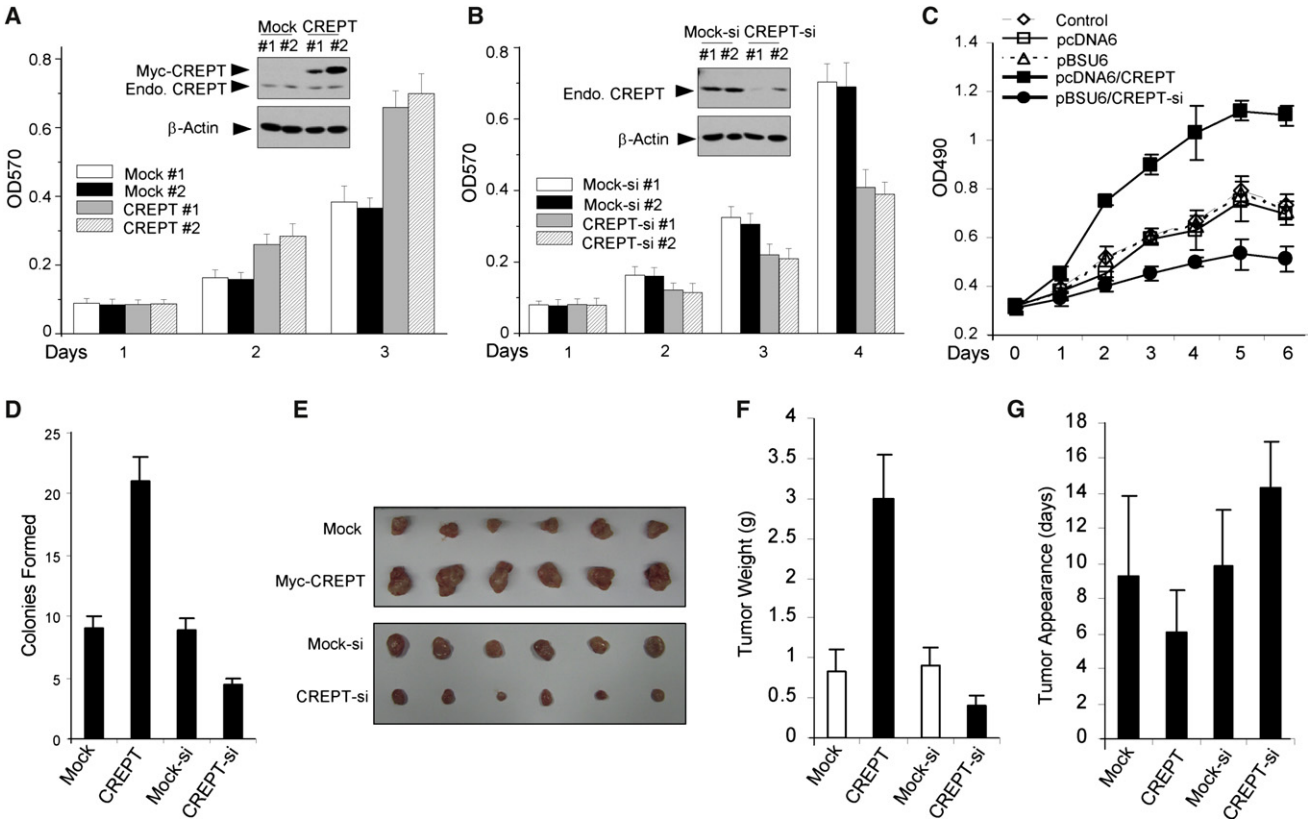

Figure 2. CREPT Promotes Cell Proliferation and Tumorigenesis

(A) Overexpression of CREPT enhances cell growth. Cell lines originating from the MGC803 cell line (a stomach cancer cell line) were established with stable overexpression of CREPT (western blot panels) and measured by an MTT assay. Endo. CREPT indicates the endogenous expression of CREPT. Error bars represent ±SD.

(B) Depletion of CREPT suppresses cell growth. MGC803 cells were stably transfected with an siRNA targeting CREPT.

(C) CREPT accelerates cell growth in HeLa cells. Cell proliferation was examined by MTT experiments in HeLa cells with stable overexpression (pcDNA/CREPT) or depletion (pBS/U6/CREPTi) of CREPT.

(D) CREPT promotes colony formation. One thousand cells from indicated cell lines based on HepG2 cells were seeded into soft agar. Colonies were stained with crystal violet and counted. Bars represent ±SD from three independent experiments.

(E–G) CREPT promotes tumor growth. Six nude mice were injected subcutaneously with 5×10^6 cells/mouse for each of the indicated stable cell lines. Results are shown as isolated tumors (E), tumor weights (F), and tumor appearance times (G). si refers to siRNA.

See also Figure S2.

because cyclin A, $p21^{CIP1/WAF1}$, and $p27^{KIP1}$ transcriptions were not affected (Figure 4A).

To investigate how CREPT activates the transcription of targeted genes, we selected the *CYCLIN D1* promoter (Herber et al., 1994) as this gene quickly responded to the elevation of CREPT expression (see Figure 3D). Assays of the luciferase reporter gene linked to the native *CYCLIN D1* promoter (Figure S4A) showed that overexpression of CREPT enhanced the luciferase activity, whereas depletion of CREPT decreased the activity (Figure 4B; Figure S4B, also Figures S4C and S4D). A nuclear run-off experiment with the *CYCLIN D1* promoter-luciferase reporter indicated that addition of nuclear extracts from CREPT-overexpressing cells increased transcription in vitro, whereas, in the presence of nuclear extracts from CREPT-depleted cells, no transcripts were detected (Figure 4C). In another nuclear run-off experiment using purified GST-CREPT protein (Figure S4E) together with nuclear extracts from HeLa cells, we observed that addition of GST-CREPT protein increased transcription in a dosage dependent manner in vitro (Figure 4D). Taken together, these data suggest that CREPT enhances the transcriptional activity of the *CYCLIN D1* promoter both in vivo and in vitro.

CREPT Interacts with RNAPII Physically

To reveal factors involved in transcription regulation by CREPT, we performed an immunoprecipitation (IP) experiment in HEK293T cells overexpressing Myc-CREPT. A mass spectrometry analysis identified RNAPII as an interacting protein with Myc-CREPT (Figure 5A). The interaction of CREPT with RNAPII occurred at the RPR domain as demonstrated by an IP experiment with Flag-tagged CREPT (Figure 5B), which is consistent with the observation that the RPR domain of yeast Rtt103 interacts with RNAPII (Kim et al., 2004). Furthermore, reciprocal IP analyses indicated that endogenous CREPT and RNAPII interacted (Figures 5C and 5D) and purified GST-CREPT protein pull-down RNAPII (Figure 5E), suggesting that CREPT

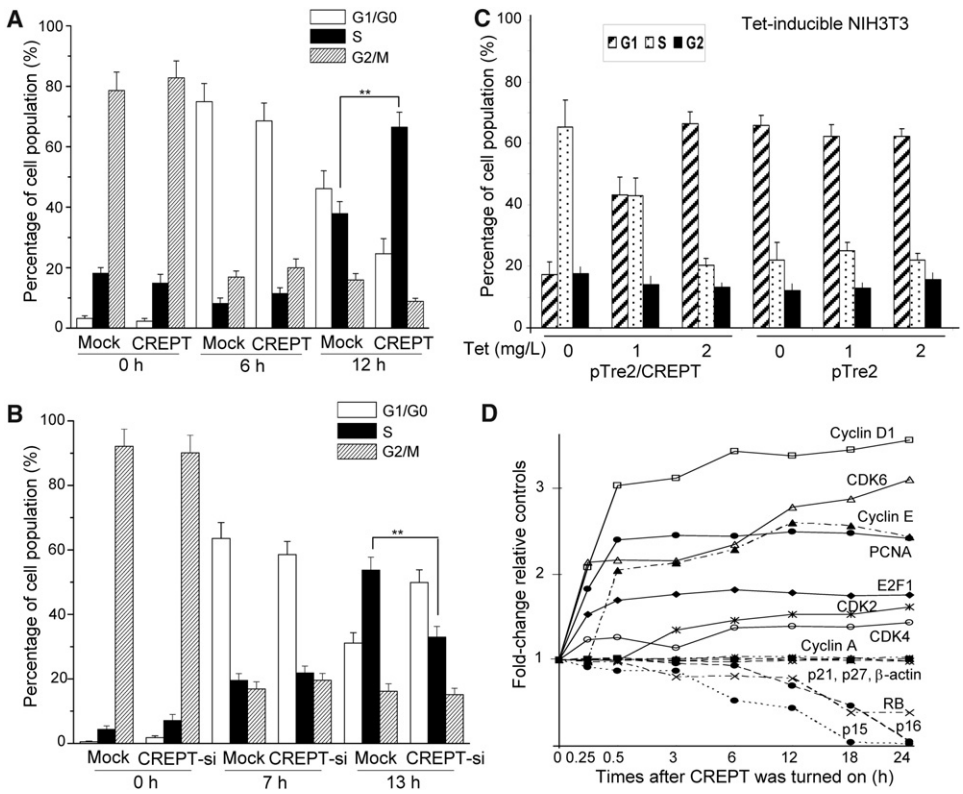

Figure 3. CREPT Alters the Cell Cycle and Cell-Cycle-Related Gene Expression

(A and B) Overexpression (A) and depletion (B) of CREPT in MGC803 cells alters the proportion of cells in the G1 and S phase. Cells were stained by propidium iodide at the indicated time after release from synchronization with nocodazole (200 ng/ml) for 18 hr. Sizes of cell populations averaged from three independent experiments with standard deviations. **$p < 0.01$.

(C) CREPT alters the cell cycle in a Tet-off system. A stable cell line was established for CREPT expression in NIH 3T3 cells under the control of tetracycline (Tet) off system. Ectopical CREPT expression is regulated by the withdrawal of Tet. pTRE2 is an empty vector. Cells were synchronized by starvation for 24 hr.

(D) CREPT alters the expression of cell-cycle-related genes. A quantitative representation of the time response to changes of gene expression as a consequence of ectopically expressed CREPT from a Tet-controlled expression system in NIH 3T3 cells. The complete withdrawal of Tet (Ectopic CREPT expression is turned on) was considered to be time point 0 and used for calculating fold change. The results are presented as fold changes relative to controls where ectopic CREPT is off.

See also Figure S3.

interacts with RNAPII in vivo and in vitro. These results imply that CREPT regulates the transcription of cyclin D1 via directly interacting with RNAPII.

CREPT Promotes RNAPII Occupancy on the *CYCLIN D1* Gene

To examine whether CREPT functions with RNAPII in the regulation of transcription of cyclin D1, we first addressed whether CREPT is involved in the *CYCLIN D1* promoter complex. Chromatin immunoprecipitation (ChIP) assays using different primers across the *CYCLIN D1* gene (Figure 6A) showed that promoter region B of *CYCLIN D1* was strongly precipitated down by an anti-CREPT antibody and that overexpression of CREPT enhanced the precipitation significantly, whereas depletion of CREPT abolished precipitation (Figures 6B and 6C). Although most of the other regions were not precipitated, region F, just before the poly(A) cleavage site, was precipitated in the presence of overexpressed CREPT, similar to the result for promoter region B (Figures 6B and 6C). These results suggest that CREPT crosslinks strongly to the *CYCLIN D1* promoter region, and also to a region related to transcription termination at the 3′ end of the gene.

Next, we analyzed the influence of CREPT on RNAPII binding density across the *CYCLIN D1* gene. A ChIP experiment indicated that strong binding of RNAPII to the promoter region B occurred in the presence of overexpressed CREPT, but only weak binding with the depletion of CREPT (Figures 6D and 6E), suggesting that CREPT enhances RNAPII binding to the *CYCLIN D1* promoter. However, CREPT did not affect RNAPII binding to regions C, D, and E of the *CYCLIN D1* gene, implying CREPT does not affect transcription elongation. Intriguingly, CREPT enhanced RNAPII binding to region F, a region before the poly(A) site, and reduced RNAPII binding to region G after the poly(A) site. In contract, depletion of CREPT resulted in less binding of RNAPII in regions B and F, but increased RNAPII binding to region G (Figures 6D and 6E). These results suggest that CREPT stops RNAPII from moving beyond the poly(A) site.

The enhanced binding of RNAPII in both the promoter (B) and termination (F) regions suggested that CREPT might promote recycling of RNAPII from the terminator to the promoter region.

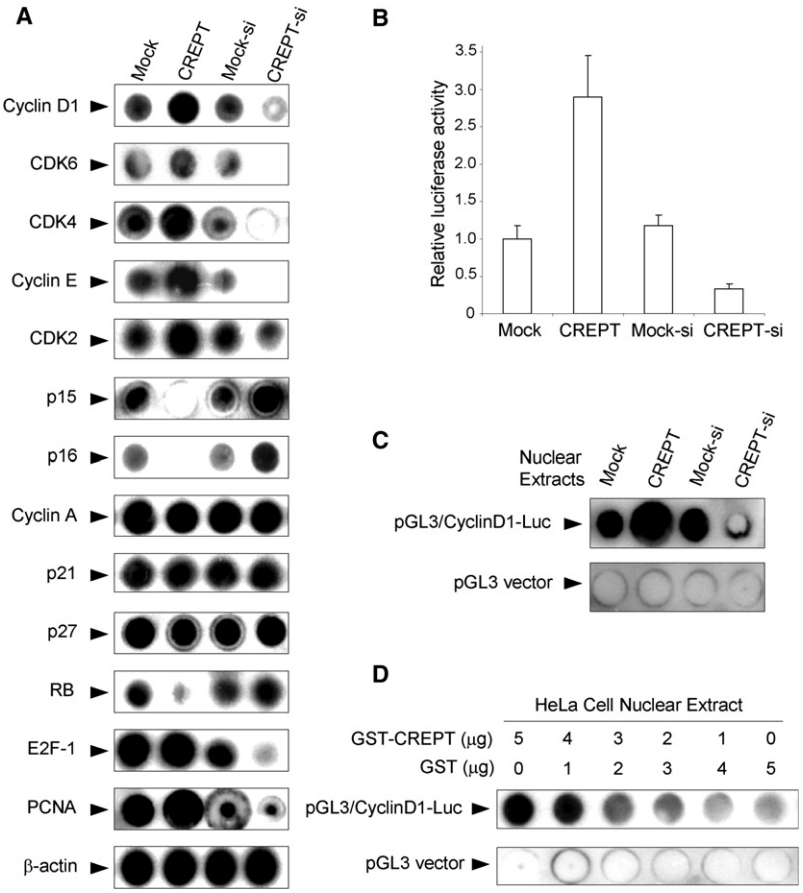

Figure 4. CREPT Accelerates *CYCLIN D1* Transcription

(A) CREPT regulates transcription of cell-cycle-related genes. Nuclear run-on assays, showing the transcriptional level of indicated genes in HepG2 cells where CREPT was stably overexpressed or depleted, were performed.

(B) CREPT activates transcription of *CYCLIN D1*. A luciferase reporter linked with the full-length native promoter of the *CYCLIN D1* gene was used for the luciferase assay. Results were normalized with internal controls and presented as averages with standard deviations from three repeats.

(C) CREPT enhances the transcriptional activity of the *CYCLIN D1* promoter. A nuclear run-off assay was performed with templates linearized from a luciferase reporter linked with a short fragment of the native *CYCLIN D1* promoter (186 bp). Nuclear extracts were from HEK293T cells transfected with indicated plasmids for overexpression or depletion of CREPT. Transcripts were dotted onto membranes and detected with a labeled probe from the luciferase reporter.

(D) CREPT activates transcription of CYCLIN D1 in vitro. Purified GST-CREPT proteins were added to the nuclear extract from HeLa cells and a nuclear run-off assay was performed using the same templates as in (C).

See also Figure S4.

To examine this hypothesis, we used a chromosome conformation capture (3C) experiment (Dekker et al., 2002) to test whether CREPT promotes RNAPII to drive the *CYCLIN D1* gene forming a chromatin loop (Figure 6F). For the 3C experiment, we chose primers that do not crossreact and have high specificity to observe loop formation. Indeed, a strong loop band was observed in cells when CREPT was overexpressed and the band was diminished when CREPT was depleted (Figures 6G and 6H). To avoid possible random ligation during the 3C experiment, we used DNA-protein complexes that were precipitated down by an anti-RNAPII antibody (8WG16) and performed the 3C experiment with and without ligation in combination with NcoI digestion. The results showed that the anti-RNAPII antibody precipitated down a strong cycled band when CREPT was overexpressed (Figure 6I). These data indicate that CREPT promotes loop formation to bring RNAPII physically close to the transcription start site, implying that CREPT may enhance RNAPII recycling from the terminator to the promoter region during transcription of the *CYCLIN D1* gene.

CREPT Regulates the Transcription of the *CYCLIN D1* Gene in Tumors

We have proposed a mechanism where CREPT enhances RNAPII binding to the *CYCLIN D1* promoter and terminator regions thereby driving the *CYCLIN D1* gene to form a chromatin loop. To examine whether CREPT functions to promote the chromatin loop formation in tumors, we investigated 12 cases of colon cancers. A western blot analysis showed a correlation of expression of CREPT and cyclin D1 in the tumor tissues where in most patients, elevated expression of CRPET is coupled with high levels of cyclin D1 (Figure 7A, patients 7 and 8 being the exceptions). The positive correlation of CREPT, cyclin D1, and Ki67 expression was confirmed, by IHC experiments, in these patient samples (Figure 7B), similar to that observed for several other cancers including stomach, lung, prostate, endometrium, liver, and breast cancers (Figure S5). Next, we performed a ChIP assay with tumor samples from three patients (2, 4, and 12) with the results showing that CREPT binds strongly to regions B and F of the *CYCLIN D1* gene (Figure 7C). These results are consistent with the results from cells overexpressing CREPT (Figure 6D), with RNAPII strongly binding to regions B and F and weakly to region G of the *CYCLIN D1* gene in tumor tissues (Figure 7D). Finally, we addressed whether the *CYCLIN D1* gene forms a chromatin loop in tumors. A 3C experiment showed a strong band in tumor tissues (Figure 7E), suggesting that a chromatin loop is formed in the cells of tumor tissues. All of the data indicate that CREPT promotes chromatin looping of the *CYCLIN D1* gene in tumor cells, which results in an increased binding of RNAPII to both the promoter and 3′ end regions.

DISCUSSION

Many genes are known to be associated with tumor formation; however, additional genes remain to be identified and characterized (Gordon et al., 2005). Here, we report a gene *CREPT* that is highly expressed in different tumors and is an oncogene

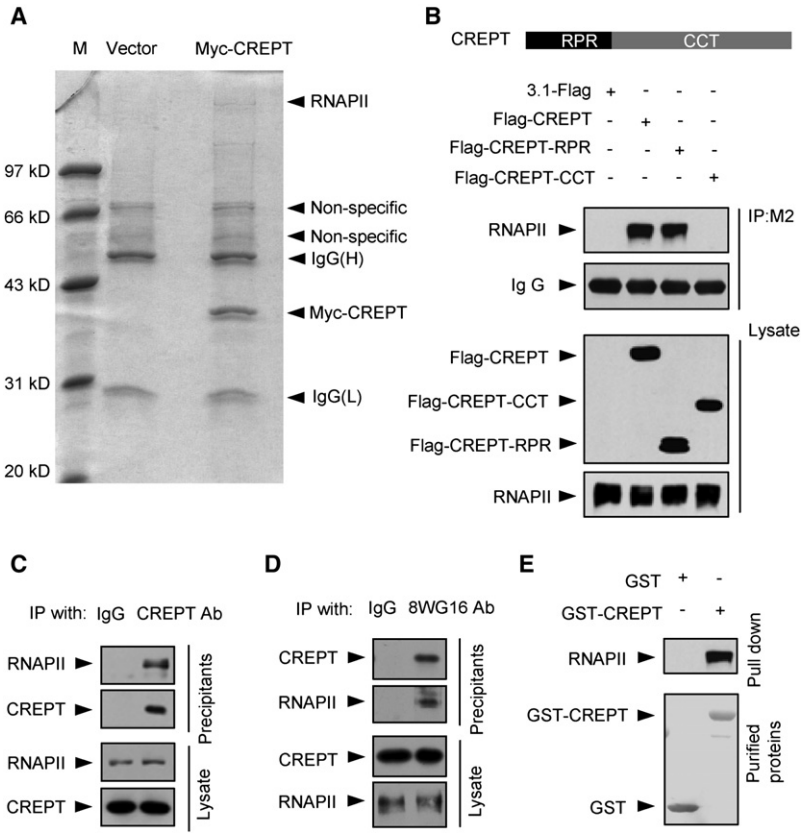

Figure 5. CREPT Interacts with RNAPII

(A) A mass spectrometry analysis of proteins associated with CREPT. HEK293T cells were transfected with pCDNA 3.1/Myc or Myc-CREPT and cell lysates were immunoprecipitated using an anti-Myc antibody. Precipitants were analyzed by an SDS-PAGE gel and stained with Coomassie blue. Bands were cut out and identified by a mass spectrometry analysis.

(B) CREPT interacts with RNAP II. Schematic illustration of CREPT and its deletions are presented on the top panel. RPR domain and CCT (coiled coil terminus) are shown. HEK293T cells were transfected with Myc-tagged full-length and two deletions of CREPT. Cell lysates were immunoprecipitated using an anti-Myc antibody and the precipitants were detected with an anti-RNAP II antibody (8WG16).

(C and D) Endogenous CREPT interacts with RNAP II in vivo. IP experiments were performed with an antibody against CREPT under the normal condition (C) and reciprocally with an antibody against RNAPII (8WG16) under fixation with formaldehyde (4%) (D).

(E) CREPT interacts with RNAP II in vitro. A GST pull-down assay was performed with purified GST or GST-CREPT protein.

based on the observations that CREPT accelerates the malignant cell growth and tumorigenesis. An anti-CREPT antibody clearly defined tumor boundaries, although a few positive staining cells were observed occasionally in the paired nontumor sections. These results suggest that CREPT could be used as a marker to diagnose tumors.

Cell-cycle alteration is a major feature of tumorigenesis (Johnson and Walker, 1999), which occurs by shortening the G1 phase (Hall and Peters, 1996; Hunter and Pines, 1994; Sherr, 1996). In this study, we observed that CREPT increased the proportion of cells in the S phase and, since no major change in the G2/M phase cell populations was observed when CREPT was overexpressed, we concluded that CREPT accelerates the G1 to S phase transition, and thus a shorter G1 phase, similar to the effects of many other oncogenes (Massague, 2004; Sherr, 1996). Consistently, we found that CREPT enhanced the expression of cell-cycle-related genes. Among the genes functioning during the G1 and S phase transition, we observed that the *CYCLIN D1* gene quickly responded to changes in CREPT expression; therefore, we used *CYCLIN D1* as an example to study the molecular mechanisms by which CREPT promotes cell proliferation.

CREPT contains an RPR domain, which is predicted to function in the 3′ end processing of mRNA (Doerks et al., 2002) and is a homolog of the yeast *Rtt103* gene. Previous studies implicated Rtt103 function in Ty1 transposition (Scholes et al., 2001) and in cell viability and growth (Tong et al., 2001). The Rtt103 protein was found in RNAPII complexes associated with other factors (Rat1, Pcf11) (Kim et al., 2004). Similar to the polyadenylation factors Rna14, Rna15, and Pcf11, Rtt103 was reported to strongly bind near the 3′ end of genes and to help Rat1 in degrading mRNA downstream of poly(A) site cleavage (Kim et al., 2004), supporting the "torpedo" model in the termination of transcription (Buratowski, 2009; Connelly and Manley, 1989; Moore and Proudfoot, 2009; Proudfoot, 1989). Previous IP experiment and structure-based analyses indicated that Rtt103 preferred to bind to Ser2P CTD of RNAPII (Kim et al., 2004; Lunde et al., 2010), downstream of the poly(A) site at the 3′ end of genes in yeast. To our surprise, we observed that CREPT bound not only to the region BEFORE the poly(A) site, but also to the promoter region of the *CYCLIN D1* gene. Interestingly, CREPT promotes RNAPII binding to both the promoter and the termination region before the poly(A) site and decreases RNAPII binding to the region after the poly(A) site. Together with our results from the 3C experiments, we propose that CREPT helps RNAPII recycle from the termination site to the promoter region of the *CYCLIN D1* gene through a chromatin loop (Figure 6F). CREPT binding to the 3′ end of the gene appears to prevent RNAPII from "reading through," and allows RNAPII to be recruited back to the promoter. The function of CREPT in binding to the 3′ end of *CYCLIN D*1 gene revealed in this study supports the allosteric (antiterminator) model for transcription termination (Luo and Bentley, 2004; Moore and Proudfoot, 2009), a model that explains the recycling of RNAPII from the termination to the promoter site (Mapendano et al., 2010).

Gene looping has been observed in the regulation of gene transcription in both yeast (O'Sullivan et al., 2004) and mammalian cells (Perkins et al., 2008; Tan-Wong et al., 2008). In this study, we observed that CREPT promotes loop formation at *CYCLIN D1*. We envisioned that this loop formation might

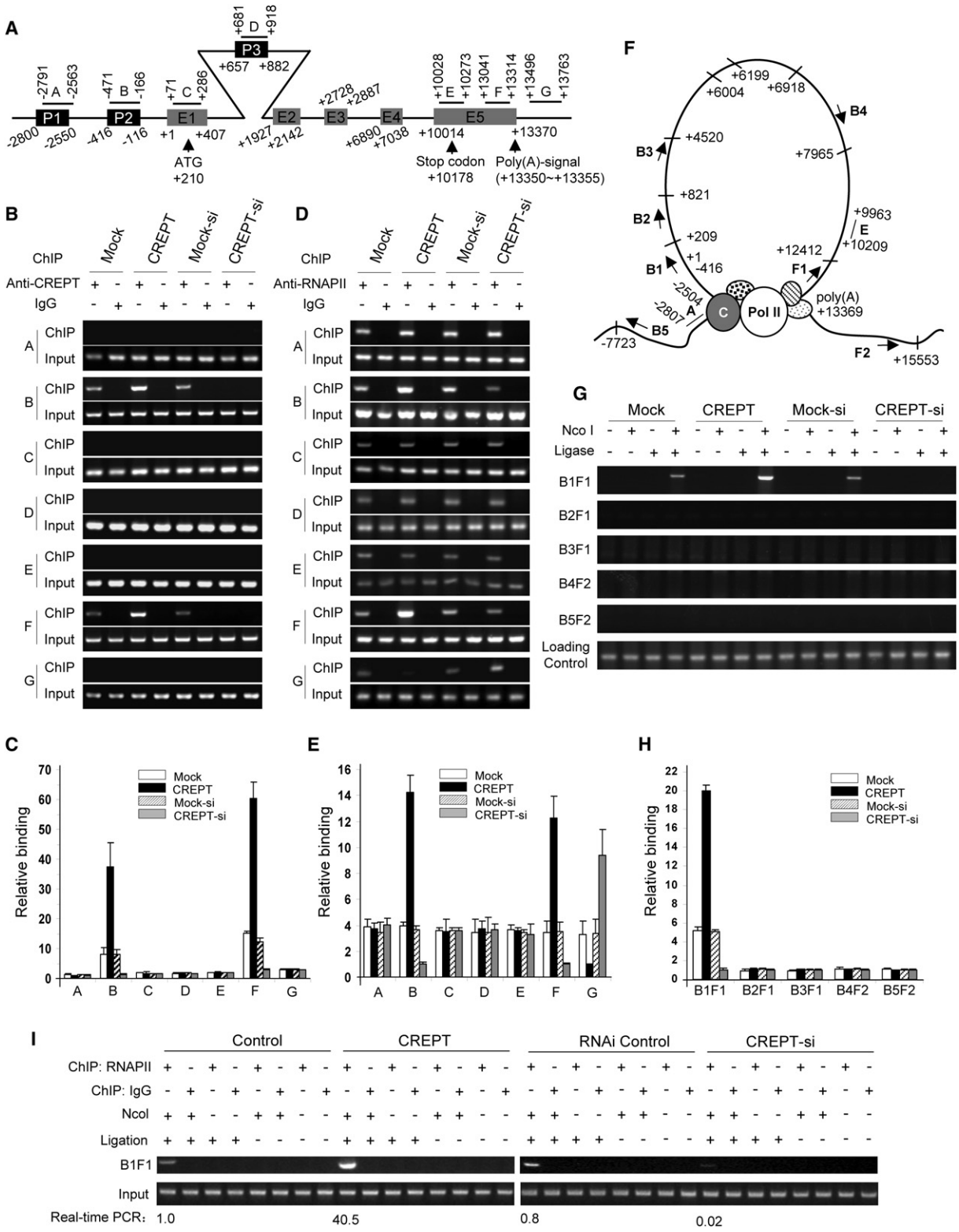

Figure 6. CREPT Promotes RNAPII Recycling in the *CYCLIN D1* Gene

(A) A graphic representation of the *CYCLIN D1* genomic structure. P1, P2, and P3 are predicated promoter regions. The P2 promoter has been functionally defined as the site of initiation of transcription of the *CYCLIN D1* gene. E1 to E5 represent exons. Numbers indicate the nucleotides counting from the primary transcription start site as +1. Fragments detected by PCR are shown as A to G.

facilitate the recycling of RNAPII and accelerate transcription. However, we could not exclude the possibility that CREPT has independent functions at the promoter region, as an activator, and the terminator. It is possible that CREPT may function directly in the promoter region to enhance the initiation of transcription and thereafter promote loop formation. We cannot exclude the possibility that the loop is required for enhanced transcription. It is possible that the loop functions as an enhancer, as has been observed for the estrogen receptor positive enhancer of the *CYCLIN D1* gene in breast cancer (Eeckhoute et al., 2006). In this study, we conclude that the loop formation is coupled with transcription initiation, possibly acting as an enhancer, as we found that CREPT associates with RNAPII.

Yeast Rtt103 appears to only function as a factor in transcriptional termination (Kim et al., 2004) as the RPR domain of Rtt103 binds to the Ser2P CTD of RNAPII in yeast (Lunde et al., 2010). Here, we observed that CREPT binds to both the promoter and the 3′ end of the *CYCLIN D1* gene. CREPT appears to help RNAPII stop at the poly(A) site, functioning as a terminator for the gene transcription, a role conceivable since yeast Rtt103 has been shown to function as a termination factor. However, the role of CREPT at the 5′ end of a gene, in the regulation of the gene transcription, seems different from that of its yeast ortholog Rtt103. A difference in the roles of CREPT appears not only in the binding to the promoter, where yeast Rtt103 does not, but also in the way it facilitates termination. We demonstrated that knocking down CREPT resulted in readthrough of RNAPII past the 3′ end of the *CYCLIN D1* gene, whereas yeast Rtt103 was shown to help Rat1 and Rai1 increase the degradation of nuclear mRNA after RNAPII reads through the poly(A) site (Kim et al., 2004). Interestingly, CREPT and p15RS are both homologs of Rtt103 in mammals. Our previous study indicated that p15RS functions as an intrinsic inhibitor of Wnt signaling mediated transcription (Wu et al., 2010), a function different from the role of CREPT as an oncoprotein observed in this study. p15RS (Wu et al., 2010) and CREPT together, therefore, may provide more regulatory functions to mammalian cells than are provided by Rtt103 to yeast (Kim et al., 2004).

Intriguingly, we observed that CREPT is highly expressed in human tumors. When CREPT is depleted, tumor cells grow slowly, an observation that echoes the role of Rtt103 in maintaining yeast viability and growth (Tong et al., 2001). The role of CREPT in cell-cycle regulation is attributed to the specific regulation of cell-cycle-related genes. We used *CYCLIN D1* as a model to demonstrate a mechanism where CREPT regulates gene transcription via interacting with RNAPII at both the termination and promoter regions. Consistent with cell experiments, we observed that cells from human tumors have a strong chromatin loop in the *CYCLIN D1* gene. Of importance, we found that RNAPII together with CREPT accumulate in the promoter region (region B) and region (F) before the poly(A) site but released from DNA after the poly(A) site (see binding in region G) (Figure 7) in human tumors. In the absence of excess CREPT, others have shown that RNAPII accumulates in the region after the poly(A) site of the *CYCLIN D1* gene (Eeckhoute et al., 2006) and RNAPII release generally occurs after the poly(A) site (Lian et al., 2008), observations that supports the torpedo model of transcription termination (Buratowski, 2009; Connelly and Manley, 1989; Moore and Proudfoot, 2009; Proudfoot, 1989). In this study, we observed that CREPT promotes the accumulation of RNAPII in the F region, a region just before the poly(A) site. Interestingly, when CREPT was depleted it appears that RNAPII reads through the poly(A) site, the situation that has generally been observed by others (Eeckhoute et al., 2006; Lian et al., 2008). Our results, therefore, describe conditions where both the torpedo and the antiterminator models are involved in the termination of gene transcription (Kim et al., 2004; Moore and Proudfoot, 2009). Cells appear to use the antiterminator mechanism to accelerate transcription of *CYCLIN D1* when CREPT is overexpressed in tumors and the torpedo model to maintain a lower level of cyclin D1 expression when CREPT is low as found in normal or slowly proliferating cells. This study illustrates a case where a gene can use different models for transcription termination (Buratowski, 2009).

Many questions remain concerning the function of CREPT. For example, what causes tumor cells to express CREPT at such high levels? How does CREPT enhance RNAPII binding to the *CYCLIN D1* promoter? Does CREPT enhance transcription termination by mechanisms similar to other RPR domain containing proteins (Kim et al., 2004)? How does CREPT specifically regulate gene expressions at the global genome level? Do other specific factors regulate the activity of CREPT? Answering these questions will help our understanding of the mechanisms of cell-cycle regulation, transcription control, and tumorigenesis. In conclusion, we reported that *CREPT* is a highly conserved

(B and C) CREPT crosslinks to a promoter region and the pre-poly(A) cleavage site in the *CYCLIN D1* gene. ChIP analyses were performed in MGC803 cells under stable overexpression or depletion of CREPT. Precipitation was performed using an anti-CREPT antibody. Results from PCR (B) and real-time PCR (C) using indicated primers are presented. Error bars represent ±SD from three repeats. Input indicates the PCR products from cell lysates without immunoprecipitation. Mouse IgG was used as a negative control in the precipitation experiment.

(D and E) CREPT enhances RNAPII crosslinking to the promoter and the pre-poly(A) cleavage site and decreases binding to the post-poly(A) cleavage site of the *CYCLIN D1* gene. ChIP analyses were performed in MGC803 cells under stable overexpression or depletion of CREPT using an anti-RNAPII antibody (8WG16) with indicated primers for PCR (D) and real-time PCR (E) analyses. Input indicates the PCR products from cell lysates without immunoprecipitation. Mouse IgG was used as a negative control in the precipitation experiment. Error bars represent ±SD from three repeats.

(F) Diagram of the primers used in the 3C assay of the human *CYCLIN D1* genomic region and a model of the chromatin loop of the *CYCLIN D1* gene by CREPT and RNAPII. Vertical lines indicate the Nco I restriction endonuclease sites, short arrows mark the positions of primers used in the 3C assay (labeled B1-5 and F1-2). C indicates CREPT and Pol II for RNAPII.

(G and H) CREPT promotes the formation of a chromatin loop at the *CYCLIN D1* gene. Products of amplifications with various primers with purified 3C DNA is shown in (G). Region E of *CYCLIN D1* is used as a loading control. Real-time PCR analyses of the products of the 3C assay are shown in (H). Error bars represent ±SD from three repeats.

(I) Specificity of the chromatin loop formation is shown by a ChIP-3C experiment. PCR analyses were performed on RNAPII-containing precipitated complexes. Real-time PCR result is shown as relative fold increases based on the level of the control normalized to the input.

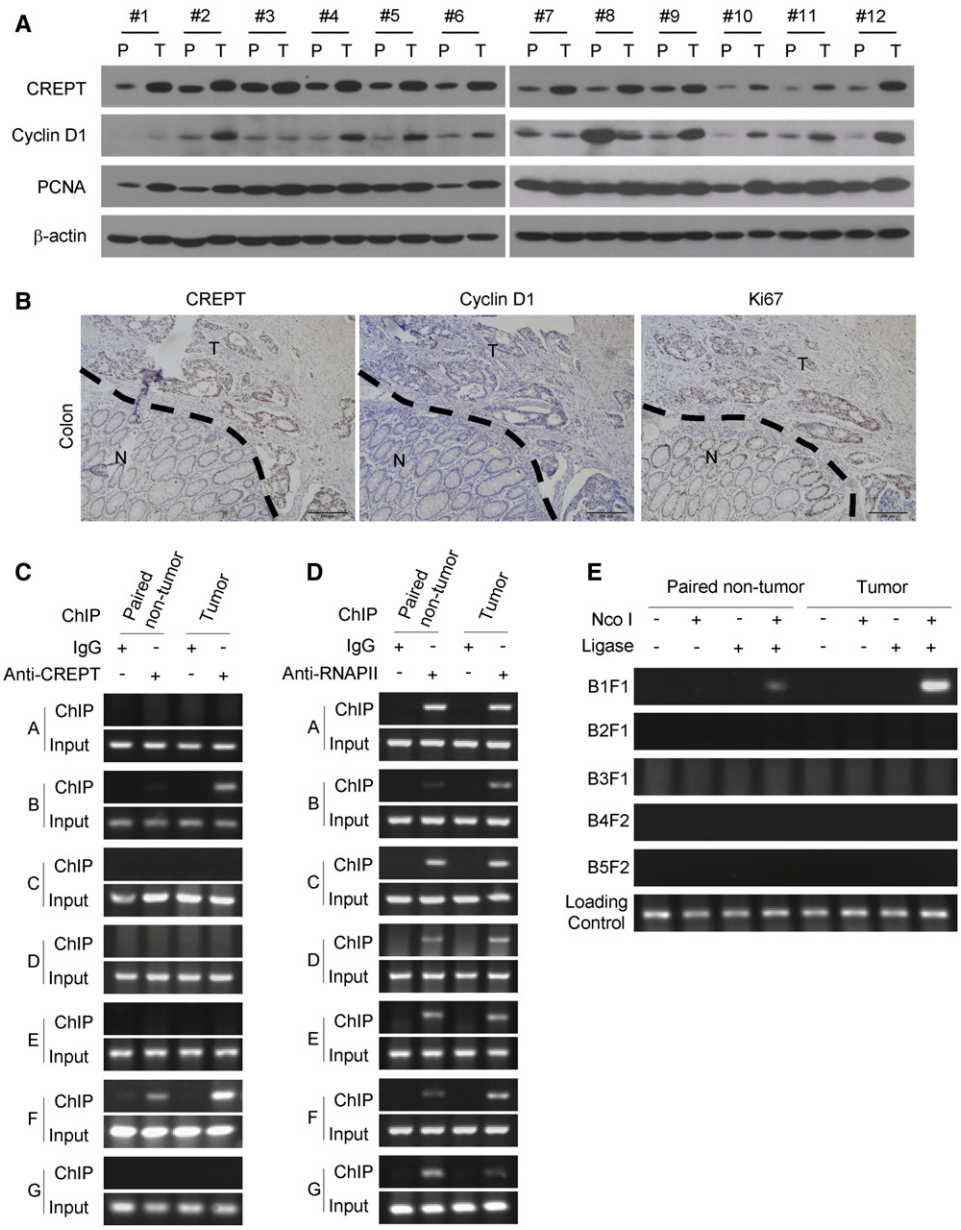

Figure 7. CREPT Promotes Chromatin Looping of the *CYCLIN D1* Gene in Tumors

(A) Correlation expression of cyclin D1, PCNA, and CREPT in colon cancers. A western blot was performed for samples from colon cancer patients. P refers to paired nontumor tissue and T refers to tumor tissue. # refers to the patient number.

(B) CREPT, cyclin D1, and Ki67 are elevated simultaneously in human colon tumor tissues. IHC was performed with colon cancer samples and a representative image (scale bar, 200 μm) is shown.

(C) CREPT binds to both the promoter and the 3′ end region of the *CYCLIN D1* gene in tumors. ChIP experiments were performed in tumor or paired nontumor tissues from three patients (1, 3, and 7).

(D) RNAPII strongly binds to both the promoter and the 3′ end region of the *CYCLIN D1* gene in tumors. ChIP experiments were performed as in (C).

(E) The *CYCLIN D1* gene forms stronger chromatin loops in tumors. 3C experiments were performed in the tumor and paired nontumor tissues from three patients. See also Figure S5.

oncogene that is highly expressed in tumors and accelerates tumor development. We reveal that the mechanism by which CREPT enhances tumor growth is by directly regulating cyclin D1 expression at the transcriptional level. We believe that CREPT should be another target for tumor diagnosis and therapy development.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies

Expression plasmids for human CREPT are pEGFPN1/Flag-CREPT, pGEX/5X-2/ GST-CREPT, pcDNA6/CREPT, pcDNA3.1/Myc-CREPT, pCMV/HA-CREPT, and pTre2/CREPT, which were constructed in our lab. The plasmid pBS/U6/CREPT-si was constructed according to a previous protocol (Sui

et al., 2002). The target sequence by an siRNA (CREPT-si), GGACCTGAATTC ACTAGAGA, is identical in human and mouse. Antibodies against Flag (M2), RNAPII (8WG16), Cyclin D1, PCNA, Ki67, and β-actin were purchased from Santa Cruz Biotechnology and Covance Research. An anti-CREPT antibody (3E10) was raised in our lab (see Figure S1D).

Human Tumor Specimens and Staining

Cancer tissues were collected in the Third People's Hospital of Qidong, Qi Dong Tumor Hospital, Wuxi 101 Hospital, the Chinese University of Hong Kong and the Chinese PLA General Hospital in China. Tissues were kept and stained according to routine protocols (Rong et al., 2006). The tissue collection procedure with informed consent was approved by the Ethic Affair Committee of the Third People's Hopspital of Qidong, the Ethic and Health Committee of Qi Dong Tumor Hospital, The Ethic Committee of Wuxi 101 Hospital, the Clinical Research Ethics Committee of the Chinese University of Hong Kong, and the Clinical Ethic Committee of the Chinese PLA General Hospital.

Western Blot, Immunoprecipitation, PCR, and RT-PCR

Experiments were performed according to protocols in the lab (Wu et al., 2010). CREPT cDNA was amplified using the primers: (forward) 5′-TATAGG TACCATGTCCTCCTTCTCTGAG-3′ and (reverse) 5′-TATACTCGAGCTAGT CAGTTGAAAACAGGTC-3′. Semiquantitative PCR products were quantified by Imagequant software (Amersham Pharmacia Biotechnology).

Cell Culture, Transfection, and Assays from Stable Cell Lines

Cells used for cell-cycle, growth, and luciferase experiments were cultured according to previous studies (Wu et al., 2010). Tumor formation assays were done according to a previous study (Rong et al., 2006) following the institutional guidelines and regulations on the animal health and ethics, approved by the animal health and ethics committee in Tsinghua University. All the experiments were performed in triplicate.

Chromatin Immunoprecipitation Assay

Cells were fixed at 37°C for 10 min with 1% formaldehyde for crosslinking (Wu et al., 2010). Sonication was done at 4°C for 30 s at level 2 (Ultrasonic Processor, Sonics) to yield fragments from 100 to 400 bps. Information of primers for PCR amplifications can be found in the Supplemental Information). The specificity of the primers to the different regions of the *CYCLIN D1* gene was examined and no crossreaction bands were observed. The efficiency of PCR in the chromatin immunoprecipitation (ChIP) experiments was greater than 20% when compared with the input. Real-time PCR was performed to quantify the data.

Nuclear Run-on and Run-off Assays

For nuclear run-on assays, nuclei prepared from the indicated cells were incubated in 2 × Transcription Mix, 10 × Nucleotide Mix, and 500 μCi [^{32}P]-dUTP at 37°C for 30 min. Labeled mRNA was hybridized to cDNA probes predotted onto membranes. For nuclear run-off assays, linearized pGL3/cyclin D1-Luc was incubated with purified GST-CREPT and HeLa cell nuclear extracts, or with nuclear extracts from the indicated cell lines. Transcript products were dotted on membranes and hybridized with [^{32}P]-CTP-labeled Luc cDNA probe.

Chromosome Conformation Capture Assay

Chromosome conformation capture (3C) assays were conducted essentially as described by Dekker et al. (2002) with some modifications (see Supplemental Information). Real-time PCR was performed to quantify the results.

ACCESSION NUMBERS

The GenBank accession numbers for human, murine, and chicken CREPT mRNAs are DQ372938, DQ372939, and DQ372930, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.12.016.

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