Published in final edited form as:

Leukemia. 2015 June ; 29(6): 1379–1389. doi:10.1038/leu.2014.350.

Replication factor C3 is a CREB Target Gene that Regulates Cell Cycle Progression through Modulation of Chromatin Loading of PCNA

Hee-Don Chae, **Bryan Mitton**, **Norman J. Lacayo**, and **Kathleen M. Sakamoto***

Division of Hematology/Oncology, Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305

Abstract

CREB (cAMP Response Element Binding protein) is a transcription factor overexpressed in normal and neoplastic myelopoiesis and regulates cell cycle progression, although its oncogenic mechanism has not been well characterized. Replication Factor C3 (RFC3) is required for chromatin loading of proliferating cell nuclear antigen (PCNA) which is a sliding clamp platform for recruiting numerous proteins in DNA metabolism. *CREB1* expression, which was activated by E2F, was coupled with *RFC3* expression during the G1/S progression in the KG-1 acute myeloid leukemia (AML) cell line. There was also a direct correlation between the expression of *RFC3* and *CREB1* in human AML cell lines as well as in AML cells from patients. CREB interacted directly with the CRE site in *RFC3* promoter region. CREB knockdown inhibited primarily G1/S cell cycle transition decreasing expression of RFC3 as well as PCNA loading onto chromatin. Exogenous expression of RFC3 was sufficient to rescue the impaired G1/S progression and PCNA chromatin loading caused by CREB knockdown. These studies suggest that RFC3 may play a role in neoplastic myelopoiesis by promoting the G1/S progression and its expression is regulated by CREB.

Keywords

Acute Myelogenous Leukemia; CREB; RFC3; PCNA; Cell Cycle

Introduction

Acute myeloid leukemia (AML) is a genetically and phenotypically heterogeneous malignancy with a poor survival rate.[1] While oncogenic steps involved in myeloid leukemogenesis remain largely uncharacterized, several lines of evidence suggest that the transcription factor CREB (cAMP response element-binding protein) may play an important

***Correspondence to**: Kathleen M. Sakamoto, M.D., Ph.D. Division of Hematology/Oncology Department of Pediatrics Stanford University 269 Campus Drive, CCSR 1215C Stanford, California 94305-5162 kmsakamo@stanford.edu Telephone: 650-725-7126 Fax: 650-723-6700.

Conflict-of-interest disclosure: Drs. Sakamoto, Mitton, Lacayo and Chae have no conflicts of interest to disclose related to this research.

role in the pathogenesis of AML and other cancers.[1-4] CREB is a 43 kDa-basic/leucine zipper transcription factor that regulates gene expression through growth-factor-induced phosphorylation at Ser133, promoting its association with the co-activator, CREB-binding protein (CBP).[3] CREB regulates a number of critical cellular functions, including cell division and apoptosis, and alterations in CREB function fundamentally change diverse cellular outcomes.[3] We have previously observed that CREB is typically overexpressed in AML and ALL patients, and this aberrant expression is related to poor prognosis in AML patients.[2]

Transgenic mice overexpressing CREB in myeloid cells did not develop AML in spite of inducing myeloproliferative disease (MPD/MDS), suggesting that overexpression of CREB alone may not be sufficient to cause myeloid leukemogenesis.[5] A 'second hit', such as Sox4 signaling was sufficient for myeloid leukemogenesis in cooperation with CREB.[6] However, the set of specific CREB target genes that may be associated with leukemogenesis remains uncharacterized. Given that CREB is a critical regulator of the cell cycle,[2-5] we wished to define CREB-driven processes that may underlie the rapid proliferation rates of cancer cells.

Replication factor C (RFC) is a heteropentameric primer-recognition protein complex involved in DNA replication and DNA repair processes. This complex functions to load proliferating cell nuclear antigen (PCNA), a ring-shaped homo-trimer clamp loaded on chromatin to provide a sliding platform for various proteins involved in DNA replication, repair, chromatin assembly, and cell cycle control[7-10] onto the 3'-ends of nascent DNA strands.[11-13] PCNA is expressed at a high level particularly in most tumor cells.[7] RFC consists of one large subunit (RFC1) and four small subunits (RFC2-5). Recently, the RFC3 subunit has been reported to have oncogenic activity being amplified in esophageal adenocarcinoma and other epithelial cancer cells.[14] RFC3 is a 38kDa subunit with an ability to bind preferentially to primed single-stranded DNA and PCNA.[15] RFC3 knockdown inhibited proliferation and anchorage-independent growth of cancer cells.[14] Disruption of RFC3-PCNA complex induced by 9-*cis*-retinoic acid (RA)-activated retinoid X receptor α (RXR α) resulted in growth inhibition of RA-sensitive breast cancer and embryonic cells through suppression of S-phase entry.[16]

Here we provide evidence that *RFC3* may be a critical factor in in promoting leukemogenesis through aberrant PCNA loading onto chromatin and G1/S progression, and that CREB directly regulates its expression throughout the cell cycle. These data provide new insight into CREB-driven regulation of the cell cycle in AML cells, and may contribute to leukemogenesis associated with CREB overexpression.

Materials and Methods

Cell culture, synchronization, and cell cycle analysis

KG-1, HL-60, and U937 human acute myeloid leukemia cells were cultured at 37°C with 5% CO_2 in Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum plus 1% penicillin/streptomycin/L-glutamine. For cell cycle analysis experiments, KG-1 cells were first synchronized at prometaphase using a modified thymidine plus nocodazole block.[17] Briefly, KG-1 cells

were treated with 2 mM thymidine (Sigma, St. Louis, MO, USA) for 30h, washed with PBS and released from G1/S block in fresh media for 4h. The cells were then incubated with 300 nM nocodazole (Sigma) for 13h. The prometaphase synchronized cells were washed with PBS and released from the mitotic block by the addition of normal serum-containing media. To inhibit cyclin-dependent kinases (CDK), cells were treated with AT7519 (2 or 10 μM, Selleckchem, Houston, TX, USA) for 16 hours.

For cell proliferation assays, 1×10^5 KG-1 cells were seeded in 12-well plates. Viable cells were counted using trypan blue exclusion method using a Vicell Cell Counter (Beckman Coulter, Brea, CA, USA).

Lentiviral vector construction and Transduction

Lentiviral vectors expressing CREB shRNAs have been described previously.[18] Lentiviral vectors expressing RFC3 shRNA (NM_181558.2-415s21c1) and luciferase shRNA were purchased from Sigma. To create the pCDH-phosphoglycerate kinase-1 (PGK)-x-CMV-mCherry lentiviral vector, the cytomegalovirus (CMV) promoter and elongation factor-1 alpha (EF1)-GFP expression cassette in the pCDH-CMV-x-EF1-GFP backbone (System Bioscience, Mountain View, CA, USA) were replaced with PGK promoter from the MGP retroviral vector[19] and the CMV-mCherry expression cassette from the pHAGE2-CMV-mCherry lentiviral vector, respectively. FLAG-RFC3 was generated by RT-PCR using cDNA from KG-1 cells and the following primers; (forward primer with FLAG sequence) 5'-ACGCTAGCATGGATTACAAGGATGACGACGATAAGAGCCTCTGGGTGGACAAG TAT-3', (reverse primer) 5'-ACGGATCCTCAGAACATCATGCCTTCCAATC-3'. The amplified PCR fragments were cloned in pCDH-PGK-x-CMV-mCherry lentiviral vector at the SwaI site downstream of the PGK promoter. All constructs were verified by DNA sequencing. VSV-G pseudotyped lentiviral particles were produced by transient transfection of HEK293 cells by calcium phosphate transfection method.[20] Lentivirus supernatants were purified and concentrated by ultracentrifugation on a sucrose (10%) cushion. After ultracentrifugation for 2h at 24,000 rpm in a Sorvall swinging bucket rotor (SureSpin 630; Thermo Scientific, Waltham, MA, USA), the lentivirus pellets were resuspended in PBS. Titers of recombinant lentivirus were determined by infecting HEK293 cells using a serial dilution. Cells were infected with lentivirus using Retronectin-precoated plates. Lentivirus-infected cells were isolated using a FACS Aria (BD Biosciences, San Jose, CA, USA) or selected by culturing the cells with puromycin (Sigma) at 2 μg/mL for at least 4 days.

The efficacy of knockdown of endogenous CREB, RFC3 and exogenous RFC3 transcripts expression were assessed by qRT-PCR, and Western blot analysis, respectively.

Immunoblotting

Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCL, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate), containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and phosphatase inhibitor cocktail 2 (Sigma). Cell lysate was resolved on 12% SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were

probed with antigen specific antibodies. The following antibodies were used in western blot analyses: anti-CREB (X-12), anti-PCNA (FL261), anti-β-tubulin (H-235, Santa Cruz Biotechnology, Santa Cruz, CA); ant-RFC3 (PA1-27673, Thermo Scientific; N1C3, GeneTex, Irvine, CA); anti-FLAG (M2, Sigma); anti-cyclin A2 (BF683), anti-cyclin B1 (4138), anti-cyclin E1 (HE12, Cell Signaling Technology, Danvers, MA, USA). Primary antibodies bound to the membranes were detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Cell Signaling Technology) and visualized with enhanced chemiluminescence system (Advansta, Menlo Park, CA, USA).

Flow Cytometry Analysis

For cell-cycle analysis, cells were fixed in 70% cold-ethanol for at least 1 hour at −20°C. Fixed cells were incubated in propidium iodide (PI) staining buffer (PBS containing RNase A (50 μg/ml), 0.1% sodium citrate, and PI (50 μg/ml)) for 30 minutes at RT. Cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences). Cell-cycle distribution was determined using the FlowJo software (TreeStar, Ashland, OR, USA).

For the flow cytometry analysis of chromatin-bound PCNA, cells were treated with a detergent containing hypotonic buffer (Hypotonic lysis buffer: 10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, 0.5% Nonidet P-40, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 (Sigma)) for 10 min at 4°C, and fixed with 1% paraformaldehyde for 5 min at RT, and then post-fixed in 70% ethanol at −20°C.[11] Fixed cells were washed and incubated with anti-PCNA (FL261) antibody (Santa Cruz) in PBS with 1% BSA (1:100 dilution) for 2 hours. Cells were washed and immunostained with anti-rabbit IgG antibody conjugated with Alexa Fluor 647 with DAPI (1ug/ml, Sigma) for 30 min. Cells were analyzed for cell-cycle distribution of chromatin-bound PCNA on a DxP10 FACScan (BD Biosciences/Cytek Development, Fremont, CA, USA) using the FlowJo software.

RNA extraction and quantitative reverse transcription PCR (qRT- PCR)

Total RNA was extracted from cells using Aurum total RNA mini kit (BioRad, Hercules, CA, USA) according to the manufacturer's instructions and reverse transcribed to generate complementary DNA (cDNA) with iScript cDNA Synthesis Kit (BioRad). PCR was carried out on a CFX384 Real-time PCR system (BioRad) using IQ™ SYBR® Green Supermix (BioRad). The β-actin or 7SL lnc RNA was used as a control gene. Gene-specific PCR primers were chosen from our previous studies or designed using the Primer3 software (http://primer3plus.com). Relative expression levels were determined with the $2^{-\Delta\Delta CT}$ Livak method.[21]

Primer sequences (5′ to 3′); *7SL*scRNA F: ATCGGGTGTCCGCACTAAGTT, 7SL RNA R: CAGCACGGGAGTTTTGACCT[22]; *CCNE1* F: AGGACGGCGAGGGACCAGTG, *CCNE1* R: TTTGCCCAGCTCAGTACAGGCAGC[23]; *CCNA1* F: TACACCAGCCACCTCCAGACAC, *CCNA1* R: CCTCCACAGCTTCAAGCTTTTG[23]; *CCNB1* F: TTTGCACTTCCTTCGGAGAGC, *CCNB1* R: AAGGAGGAAAGTGCACCATGTC[24]; *ACTB* F: GGACTTCGAGCAAGAGATGG , *ACTB* R: AGCACTGTGTTGGCGTACAG; *PCNA* F: GGCGTGAACCTCACCAGTAT, *PCNA* R: TTCTCCTGGTTTGGTGCTTC; *RFC3* F:GCCTGCAGAGTGCAACAATA,

RFC3 R:TCAAGGAGCCTTTGTGGAGT; *CREB* F:GTATATTGCCATTACCCAGGGAG, *CREB* R: CTGCTGCATTGGTCATGGT

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out with SimpleChIP® Plus Enzymatic Chromatin IP Kit (Cell Signaling) based on the manufacturer's protocol. KG-1 cells were cross-linked with 1% formaldehyde at room temperature for 10 min and then incubated with 0.125 mM glycine for 5 min. Cross-linked chromatin was digested by Micrococcal nuclease and then sonicated. Chromatin immunoprecipitations were undertaken with antibodies against CREB (48H2, Cell Signaling), E2F1 (3742, Cell Signaling), Histone H3 (positive control; Cell Signaling) or normal IgG (negative control). Immunoprecipitated DNA was analyzed by PCR reactions with primers specific for the human *RFC3* promoter (SA BioScience, Valencia, CA, USA) or *CREB1* promoter (F: 5'-ATGGGGCATATTTCCAGGGG-3', R: 5'-CTGGGGAAGAAGGTCTGCTG-3'). PCR products from the ChIP assay were resolved on an agarose gel. The input DNA represented 2% of total chromatin.

Statistical analysis

Correlation between the CREB1 and RFC3 mRNA expression was determined by Pearson correlation and linear regression analyses using Prism software (GraphPad Software, La Jolla, CA, USA). Unless otherwise stated, all experiments were performed in unique triplicates (n = 3). Statistical significance was determined by Student's t-Test, and data with a p-value less than 0.05 was considered statistically significant.

Results

CREB regulates Cell Cycle Progression in AML cells

Given the previously described role of CREB in the proliferation and survival of AML cells, we wished to investigate the underlying molecular mechanism by which CREB regulates cell cycle progression in KG-1 AML cells. To examine the effects of CREB knockdown in KG-1 cells, we infected the cells with lentivirus expressing GFP and CREB shRNAs or GFP alone (control) as reported previously.[18, 25] Consistent with previous results,[18, 25] KG-1 cells with CREB knockdown showed significantly reduced proliferative capability compared to the control KG-1 cells (cell counts after 4d with 1×10^5 seeding: $34.18\times10^5 \pm 1.2\times10^5$ vs. $19.21\times10^5 \pm 0.54\times10^5$ vs. $14.52\times10^5 \pm 0.46\times10^5$, for control cells vs. CREB shRNA-1 vs. CREB shRNA-2, respectively (mean ± SEM, n=3, $p< 0.01$)) without inducing apoptosis.

To examine whether delay or arrest in specific phases of the cell cycle was responsible for growth inhibition by CREB shRNAs, we performed cell cycle analysis. Control and CREB knockdown KG-1 cells were arrested at prometaphase using a thymidine/nocodazole block to achieve synchronization as described in materials and methods. Following release from mitotic block, cells were analyzed at 4-hour intervals by measuring DNA content by flow cytometry. Our results showed that G1 to S phase transition as assessed by % S phase was significantly impeded by CREB knockdown at 8 and 12 hours after mitotic release (S phase (%), control vs. CREB-knockdown #2, 8 h post-release: 53.29 ± 0.54% vs. 23.57 ± 1.69%; 12 h post-release: 66.92 ± 0.63% vs. 45.16 ± 0.50%, mean ± SEM, n=3, $p< 0.01$) (Figure 1a

and b). However, we did not detect any changes in the G2/M to G1 transition (0-4 hours) as a result of CREB knockdown. Taken together, our data suggest that CREB knockdown inhibits primarily G1/S cell cycle transition in KG-1 cells after mitotic release.

Cell cycle progression is driven by the sequential activation of cyclin-dependent kinases (CDKs). Cyclins are expressed at specific phases of cell cycle to regulate CDK activity in an orderly manner (Cyclin E at late G1 phase, cyclin A at S phase and cyclin B at M phase).[26, 27] PCNA is required for DNA replication, and its expression is increased in the late G1 to S phase.[28] Since CREB knockdown affected the G1/S transition of KG-1 cells, we investigated whether CREB-knockdown might alter temporal expression of these cell cycle regulatory genes [*Cyclin E1* (*CCNE1*), *PCNA*, *Cyclin A2* (*CCNA2*) and *Cyclin B1* (*CCNB1*)] after release from mitotic arrest by quantitating their mRNA levels using qRT-PCR. Our results indicated that there were no significant changes in the expression levels of these cell cycle regulatory genes at the mRNA level upon CREB knockdown (Figure 1c). CREB knockdown KG-1 cells demonstrated a normal increase in the levels of *CCNE1* and *PCNA* mRNAs in the early G1/S transition stage at 8 hours post-release, as well as increased *CCNA2* and *CCNB1* mRNA expression levels in the middle of the S phase (12 hours post-release) and the G2/M phase (16 hours post-release), respectively (Figure 1c). Expression levels of cyclin E1, cyclin A2, cyclin B1 and PCNA proteins were assessed to affirm whether expression of these genes at the mRNA level correlated with that at protein levels throughout the cell cycle progression. Our results showed that the expression patterns at the protein and mRNA levels were concordant. Cyclin E1 and cyclin A2 increased at protein level during S phase (8-16 hours post-release). Cyclin B1 protein level also increased in mitosis reaching a maximum level when cells were arrested at prometaphase (0 hour). In contrast, PCNA protein expression levels were not changed during cell cycle progression, although PCNA mRNA levels started to increase at the G1/S transition phase (Figure 1c). Importantly, the temporal expression profiles of cyclin E1, cyclin A2, cyclin B1 and PCNA during the cell cycles were not altered by CREB knockdown (Figure 1d).

Expression of *CREB1* was significantly knocked-down with the CREB-specific shRNA throughout the cell cycle (Figure 2). Consistent with the results shown in Figure 1a, these data showed that cell cycle progression kinetics were not altered by CREB knockdown once the cells progressed out of the G1/S transition stage.

RFC3 is a direct transcriptional target for CREB

In an effort to seek novel CREB-responsive target genes associated with the G1/S progression in AML cells, we analyzed a previous microarray dataset for CREB knockdown K562 chronic myeloid leukemia cells (Gene Expression Omnibus (GEO) accession GDS3487).[25] *RFC3*, a 38 kDa subunit of the RFC complex involved in DNA replication and repair processes[29] was markedly down-regulated by CREB knockdown in K562 cells. We verified the dependency of the *RFC3* expression on CREB. RFC3 expression was decreased by approximately 60% in CREB knockdown KG-1 cells at both protein (Figure 2a) and mRNA (Figure 2b) expression levels, respectively. Next, the temporal relationship of *RFC3* expression during the cell cycle was assessed by qRT-PCR in KG-1 cells at various time points after release from mitotic arrest. Interestingly, expression levels of *RFC3* and *CREB1*

mRNAs were coupled with cell cycle phases; expression levels of *RFC3* and *CREB1* were reduced in the G1 phase of the cell cycle (4 hours post-release), and then rose as cells entered the S phase (Figure 2c). Induction of *RFC3* in the S phase was abrogated in CREB knockdown KG-1 cells (Figure 2c, lower panel).

A sharp increase in *RFC3* and *CREB1* mRNA expression at the G1/S progression (Figure 2c) suggested that *RFC3* and *CREB1* might be target genes of E2F, a key transcriptional regulator of the G1/S progression. Moreover, analysis of *RFC3* and *CREB1* promoters revealed putative CRE and E2F binding sites sequences (Figure 2d). We next performed ChIP assay to determine whether CREB and E2F directly interact with the *RFC3* and *CREB1* promoters *in vivo*. Using anti-CREB or anti-E2F1 antibodies, ChIP assay results demonstrated that CREB directly interacted with the CRE sites in both *RFC3* and *CREB1* promoter regions. Though there were two potential E2F binding sites in the *RFC3* promoter region, ChIP assays provided no evidence for E2F1 binding to the *RFC3* promoter, whereas E2F1 could directly act on the *CREB1* expression (Figure 2e).

Thus, E2F1 binding to *CREB1* promoter, as well as expression of *CREB1* in S phase, suggests that *CREB1* expression is turned on by E2F during the G1/S progression. CDKs regulate E2F transcription factors through Rb.[26, 27] To further investigate the CDK-E2F function on the expression of *CREB1* and *RFC3*, we assessed the *CREB1* and *RFC3* mRNA levels after treatment of a pan-CDK inhibitor AT7519.[30] AT7519 inhibited expression of *CREB1* and *RFC3* (Figure 3a) as well as well-known E2F target genes such as *CCNE1, CCNA2* and *CCNB1* (Figure 3b) in KG-1 cells. These results indicate that *CREB1* overexpression, a potentially important prognostic marker in leukemia patients, may be associated with dysregulated CDK-E2F activity in leukemia.

Co-regulated expression of *RFC3* and *CREB1* in AML cells

To examine the generalizability of these results, similar experiments were performed using the HL-60 and U937 AML cell lines. Consistent with findings in the KG-1 AML cell line, CREB-knockdown inhibited *RFC3* mRNA expression levels in the U937 and HL-60 AML cell lines as assessed by qRT-PCR (Figure 4a).

We also extended these studies to primary human AML patient samples in order to examine the potential relationship between CREB and RFC3 expression levels. The relative expression levels of *RFC3* and *CREB1* mRNA in diagnostic samples from AML patients were compared to those from healthy individuals. As shown in Figure 4b, there was a significant correlation between the expression levels of *CREB1* and *RFC3* in human AML patient samples ($n = 19$, Pearson correlation coefficient $r=0.6628$, $p = 0.002$). We next performed ChIP assay to determine whether CREB regulated *RFC3* expression at a transcriptional level in primary human AML cells. CREB bound the *RFC3* promoter in primary AML cells as manifested by amplified *RFC3* promoter region including putative CRE site when the anti-CREB antibody immunoprecipitated DNA was subjected to PCR (Figure 4c). These data suggest that overexpressed CREB up-regulates *RFC3* expression at a transcription level in AML patients, serving as an important pro-growth driving signal in AML cells.

RFC3 requires CREB for chromatin loading of PCNA in G1/S progression

Since CREB knockdown inhibited both RFC3 expression and cell proliferation, we investigated whether RFC3 was involved in AML cell proliferation. To examine effect of RFC3 on KG-1 cell proliferation, we compared growth rates of the cells whose RFC3 was knocked down with specific shRNA and control cells expressing luciferase shRNA. Western blotting results showed that RFC3 shRNA blocked RFC3 protein expression almost completely (Figure 5a). We found that RFC3 knockdown resulted in significant growth suppression of KG-1 cells (Figure 5b) without affecting cell viability. We then assessed effect of RFC3 knockdown on cell cycle progression by analyzing the DNA contents in synchronized cells using flow cytometry. We first tried to synchronized cells at mitosis by using thymidine-plus-nocodazole block for a clearer resolution in G1/S progression. However, less than a half of RFC3 knockdown KG1 cells accumulated at G2/M phase even after 28 h post release from thymidine block into medium with nocodazole (Figure 5c). We monitored cell cycle progression every 4 hours after release from thymidine-induced G1/S arrest by analyzing DNA content by flow cytometry. Treatment of KG1 cells with 2 mM thymidine for 30 h resulted in a majority of the control and RFC3 knockdown cells arresting in G1/S boundary (Figure 5c and d). Following release from G1/S arrest, most of control cells exited S phase and entered G2/M phase after 16 h. In contrast, around 50% of RFC3 knockdown KG1 cells were retained in S phase with prominent G1/S boundary peak even after 28 h post-release from thymidine block (% cells in S phase for control vs. RFC3-knockdown: 29.38.19 ± 1.09% vs. 61.81 ± 1.39% at 16 h post-release, and 6.09 ± 0.11% vs. 48.44 ± 0.03% at 28 h post-release; % cells in G2/M phase: 62.92 ± 1.03% vs. 34.13 ± 1.92% at 16 h post-release, and 88.68 ± 1.52% vs. 46.44 ± 0.61% at 28 h post-release. mean ± SEM, n=3, $p < 0.01$) (Figure 5c and d). This clearly indicates that RFC3 plays a key role in promoting the S phase entry and cell cycle progression.

Next, we investigated whether exogenous expression of RFC3 rescued the impaired G1/S phase transition caused by CREB knockdown using KG-1 CREB knockdown cells transduced with lentiviral vectors expressing FLAG-tagged RFC3 and mCherry or mCherry alone as a control. The degree of CREB knockdown and RFC3 expression were assessed by qRT-PCR (Figure 6a) and immunoblotting (Figure 6b). Exogenous expression of RFC3 restored cellular RFC3 levels in CREB knockdown cells but did not affect CREB expression (Figure 6a and b). We found that exogenous expression of RFC3 in KG-1 CREB knockdown cells completely rescued impaired G1/S progression as evidenced by % S phase at 9h after release from mitotic arrest (% cells in S phase for control vs. CREB-knockdown vs. CREB-knockdown with low level of exogenous RFC3 expression vs. CREB-knockdown with high level of exogenous RFC3 expression: 57.80 ± 1.16% vs. 38.97 ± 0.45% vs. 60.40 ± 0.69% vs. 62.24 ±1.06% at 9 h post-release, and 62.66 ± 0.47% vs. 48.12 ± 0.60% vs. 64.48 ± 0.74% vs. 67.70 ± 1.15% at 12 h post-release. mean ± SEM, n=3, $p < 0.01$) (Figure 6c and d).

The PCNA trimer plays fundamental roles in DNA replication as a sliding clamp platform for recruiting numerous proteins, including DNA polymerase δ and ε.[7-10] There are two forms of PCNA; a detergent-soluble unbound form and a detergent-insoluble chromatin-bound form in S phase.[31] PCNA is required to be loaded onto chromatin by the pentameric

clamp loader RFC complex for its function.[7-10] We examined the impact of CREB/RFC3 on chromatin loading of PCNA at indicated times after release from mitotic arrest with a thymidine/nocodazole dual block. The chromatin-bound PCNA population dramatically increased as cells entered late G1 or S phase (8 hours post-release) in control cells (Figure 7a and b). We found that CREB knockdown reduced chromatin-bound PCNA levels. However, exogenous expression of RFC3 in CREB-knockdown KG1 cells rescued impaired chromatin loading of PCNA in G1/S progression (control vs. CREB knockdown vs. CREB knockdown with exogenous RFC3 expression: 66.87 ± 0.90 vs. 24.77 ± 0.99 vs. 79.17 ± 0.12, n=3, $p < 0.01$ at 8 hours post-release, mean ± SEM, n=3, $p < 0.01$) (Figure 7a and b). These data show that CREB knockdown inhibits S phase entry by decreasing chromatin-bound PCNA levels in KG-1 cells.

Discussion

CREB is overexpressed in acute leukemia and enhances cellular proliferation and survival of myeloid cells.[2, 5] In this study, we sought to identify CREB target genes and underlying molecular mechanism of CREB-associated leukemogenesis. We conclude that CREB controls chromatin loading of PCNA during G1/S progression by activating RFC3 expression, providing a direct link between CREB expression levels and cell cycle progression in AML cells.

RFC3 expression is activated as cells enter S phase in KG-1 cells (Figure 2c), consistent with previous data in budding yeast (Gene Expression Omnibus (GEO) accession GDS2318)[32] and in NPrEC epithelial cells (GEO accession GDS3354).[33] Our data show that the CDK-E2F axis, a well-defined transcriptional activation pathway for G1/S progression,[26, 27, 34] regulates *CREB1* expression in a cell cycle phase-dependent manner. In this signaling pathway, Rb is initially phosphorylated by Cyclin D-CDK4/6, which activates *Cyclin E* and *CDK2* expression for G1/S progression. Rb is further phosphorylated by Cyclin E-CDK2, inducing *Cyclin A* expression for complete phosphorylation of Rb and S phase progression.[26, 27, 34] *Cyclins A* and *D* themselves have been reported to be targets of CREB, implying cross-talk within this pathway.[35, 36] Given activation of *Cyclin D* expression by CREB and central role of Cyclin D/CDK4/6 for S phase entry, we predicted that *Cyclin D* would be a CREB- regulated gene in G1/S progression. However, expression levels of the E2F-regulated genes *Cyclin E1*, *Cyclin A2*, *PCNA* and *Cyclin B1* were identical in CREB knockdown and control KG-1 cells (Figure 1c). Thus, CREB knockdown does not appear to inhibit E2F activity in G1/S progression. Finally, exogenous expression of RFC3 rescued impaired G1/S progression in CREB knockdown cells (Figure 5c), and CREB directly bound to a CRE site in the *RFC3* promoter (Figure 2e), suggesting *RFC3* is a direct target gene of CREB in G1/S progression in KG-1 cells.

The molecular mechanism(s) leading to CREB overexpression in AML cells has not been fully characterized; our previous work showed that CREB is overexpressed in the majority of AML patients, and that this is associated with a poor prognosis even after adjustment for other known negative prognostic factors.[1-4] Thus, defining the mechanism leading to CREB overexpression would represent a step forward in understanding leukemogenesis of high-risk disease. Two possible mechanisms have been proposed: increase of *CREB1* gene copy

number through chromosomal duplication and down-regulation of miR-34b CREB-targeting miRNA.[5, 37] Our data provide another possibility, as *CREB1* expression can be activated by CDK-E2F pathway (Figure 2e and 3). Previous reports have shown that tumor cells acquire aberrant cellular proliferation activity secondary to deregulation of the CDK-E2F axis.[26, 38] Analysis of the expression of *CREB1* during the cell cycle progression of synchronized NPrEC epithelial cells (GEO accession GDS3354)[33] revealed that the expression level of *CREB1* rises with *RFC3* as the cells enter the S phase. Moreover, we found that E2F1 directly interacted with the E2F site in the *CREB1* promoter region *in vivo* by ChIP assays (Figure 2e). These data suggest that that *CREB1* expression might be upregulated in acute leukemias by deregulated CDK-E2F activity.

The clamp-loading function of the RFC complex for PCNA is prerequisite for DNA metabolism, including DNA replication and repair.[7-10] Chromatin loading of PCNA during G1/S progression was inhibited by CREB downregulation, and exogenous expression of RFC3 restored the chromatin-bound PCNA levels in CREB knockdown cells (Figure 7), suggesting CREB controls chromatin loading of PCNA for G1/S progression via RFC3. Inhibition of RFC3 and PCNA blocks cellular proliferation of cancer cells.[14, 16, 39, 40] Furthermore, RFC3 knockdown represses DNA synthesis and anchorage-independent growth of cancer cells.[14, 16] Fission yeast *rfc3* mutants have defects in DNA replication and DNA damage checkpoint,[40] and an inhibitor of chromatin loading of PCNA have an inhibitory effect on tumor cell growth.[39] Therefore, targeted inhibition of RFC3/PCNA might represent a new strategy for drug development against CREB overexpressing acute leukemia.

Taken together, our results suggest that RFC3 is a novel downstream oncogenic target of activated CREB, as a critical factor for aberrant chromatin loading of PCNA during G1/S progression in AML cells.

Acknowledgments

This research was supported by NIH R01 HL75826 (K.M.S.), and by the American Cancer Society Greeley & Seattle Gala/Friends of Rob Kinas Postdoctoral Fellowship and the Stanford University Dean's Post-Doctoral Fellowship program (B.M.).

Sorting was performed on an instrument in the Shared FACS Facility obtained using NIH S10 Shared Instrument Grant S10RR025518-0. We thank Dr. Young-June Kim for helpful comments and discussion on the manuscript.

References

1. Frohling S, Scholl C, Gilliland DG, Levine RL. Genetics of myeloid malignancies: pathogenetic and clinical implications. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005; 23:6285–6295. [PubMed: 16155011]
2. Crans-Vargas HN, Landaw EM, Bhatia S, Sandusky G, Moore TB, Sakamoto KM. Expression of cyclic adenosine monophosphate response-element binding protein in acute leukemia. Blood. 2002; 99:2617–2619. [PubMed: 11895805]
3. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nature reviews Molecular cell biology. 2001; 2:599–609. [PubMed: 11483993]
4. Siu YT, Jin DY. CREB--a real culprit in oncogenesis. The FEBS journal. 2007; 274:3224–3232. [PubMed: 17565603]

5. Shankar DB, Cheng JC, Kinjo K, Federman N, Moore TB, Gill A, et al. The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. Cancer cell. 2005; 7:351–362. [PubMed: 15837624]
6. Salemiz Sandoval CK, Cho Er-Chieh, Cho Michelle, Bies Juraj, Manara Elena, Accordi Benedetta, Landaw Elliot M. Wolff Linda, Pigazzi Martina, Sakamoto Kathleen M. Sox4 cooperates with CREB in myeloid transformation. Blood. 2012 in press.
7. Stoimenov I, Helleday T. PCNA on the crossroad of cancer. Biochemical Society transactions. 2009; 37:605–613. [PubMed: 19442257]
8. Prosperi E. The fellowship of the rings: distinct pools of proliferating cell nuclear antigen trimer at work. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2006; 20:833–837. [PubMed: 16675840]
9. Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. Cell. 2007; 129:665–679. [PubMed: 17512402]
10. Maga G, Hubscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. Journal of cell science. 2003; 116:3051–3060. [PubMed: 12829735]
11. Scovassi AI, Prosperi E. Analysis of proliferating cell nuclear antigen (PCNA) associated with DNA. Methods Mol Biol. 2006; 314:457–475. [PubMed: 16673899]
12. Erzberger JP, Berger JM. Evolutionary relationships and structural mechanisms of AAA+ proteins. Annual review of biophysics and biomolecular structure. 2006; 35:93–114.
13. Kim J, MacNeill SA. Genome stability: a new member of the RFC family. Current biology : CB. 2003; 13:R873–875. [PubMed: 14614842]
14. Lockwood WW, Thu KL, Lin L, Pikor LA, Chari R, Lam WL, et al. Integrative genomics identified RFC3 as an amplified candidate oncogene in esophageal adenocarcinoma. Clin Cancer Res. 2012; 18:1936–1946. [PubMed: 22328562]
15. Majka J, Burgers PM. The PCNA-RFC families of DNA clamps and clamp loaders. Progress in nucleic acid research and molecular biology. 2004; 78:227–260. [PubMed: 15210332]
16. Maeng S, Kim GJ, Choi EJ, Yang HO, Lee DS, Sohn YC. 9-Cis-retinoic acid induces growth inhibition in retinoid-sensitive breast cancer and sea urchin embryonic cells via retinoid X receptor alpha and replication factor C3. Mol Endocrinol. 2012; 26:1821–1835. [PubMed: 22949521]
17. Whitfield ML, Sherlock G, Saldanha AJ, Murray JI, Ball CA, Alexander KE, et al. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Molecular biology of the cell. 2002; 13:1977–2000. [PubMed: 12058064]
18. Cheng JC, Kinjo K, Judelson DR, Chang J, Wu WS, Schmid I, et al. CREB is a critical regulator of normal hematopoiesis and leukemogenesis. Blood. 2008; 111:1182–1192. [PubMed: 17975014]
19. O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. Proceedings of the National Academy of Sciences. 2009; 106:7113–7118.
20. Salmon P, Trono D. Production and titration of lentiviral vectors. Current protocols in human genetics / editorial board, Jonathan L Haines [et al]. 2007 Chapter 12: Unit 12 10.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]
22. Galiveti CR, Rozhdestvensky TS, Brosius J, Lehrach H, Konthur Z. Application of housekeeping npcRNAs for quantitative expression analysis of human transcriptome by real-time PCR. RNA. 2010; 16:450–461. [PubMed: 20040593]
23. Chaves-Perez A, Mack B, Maetzel D, Kremling H, Eggert C, Harreus U, et al. EpCAM regulates cell cycle progression via control of cyclin D1 expression. Oncogene. 2013; 32:641–650. [PubMed: 22391566]
24. Lavine JA, Raess PW, Davis DB, Rabaglia ME, Presley BK, Keller MP, et al. Overexpression of pre-pro-cholecystokinin stimulates beta-cell proliferation in mouse and human islets with retention of islet function. Mol Endocrinol. 2008; 22:2716–2728. [PubMed: 18845673]
25. Pellegrini M, Cheng JC, Voutila J, Judelson D, Taylor J, Nelson SF, et al. Expression profile of CREB knockdown in myeloid leukemia cells. BMC cancer. 2008; 8:264. [PubMed: 18801183]
26. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer. 2009; 9:153–166. [PubMed: 19238148]

27. Ji JY, Dyson NJ. Interplay Between Cyclin-Dependent Kinases and E2F-Dependent Transcription. Contemp Cancer Res. 2010:23–41.
28. Takasaki Y, Deng JS, Tan EM. A nuclear antigen associated with cell proliferation and blast transformation. J Exp Med. 1981; 154:1899–1909. [PubMed: 6172535]
29. Ashton NW, Bolderson E, Cubeddu L, O'Byrne KJ, Richard DJ. Human single-stranded DNA binding proteins are essential for maintaining genomic stability. BMC molecular biology. 2013; 14:9. [PubMed: 23548139]
30. Squires MS, Feltell RE, Wallis NG, Lewis EJ, Smith DM, Cross DM, et al. Biological characterization of AT7519, a small-molecule inhibitor of cyclin-dependent kinases, in human tumor cell lines. Molecular cancer therapeutics. 2009; 8:324–332. [PubMed: 19174555]
31. Tsurimoto T. PCNA binding proteins. Frontiers in bioscience : a journal and virtual library. 1999; 4:D849–858. [PubMed: 10577396]
32. Pramila T, Miles S, GuhaThakurta D, Jemiolo D, Breeden LL. Conserved homeodomain proteins interact with MADS box protein Mcm1 to restrict ECB-dependent transcription to the M/G1 phase of the cell cycle. Genes & development. 2002; 16:3034–3045. [PubMed: 12464633]
33. Bakshi S, Zhang X, Godoy-Tundidor S, Cheng RY, Sartor MA, Medvedovic M, et al. Transcriptome analyses in normal prostate epithelial cells exposed to low-dose cadmium: oncogenic and immunomodulations involving the action of tumor necrosis factor. Environmental health perspectives. 2008; 116:769–776. [PubMed: 18560533]
34. Henneke G, Koundrioukoff S, Hubscher U. Multiple roles for kinases in DNA replication. EMBO reports. 2003; 4:252–256. [PubMed: 12634841]
35. Shankar DB, Cheng JC, Sakamoto KM. Role of cyclic AMP response element binding protein in human leukemias. Cancer. 2005; 104:1819–1824. [PubMed: 16196046]
36. Sandoval S, Pigazzi M, Sakamoto KM. CREB: A Key Regulator of Normal and Neoplastic Hematopoiesis. Adv Hematol. 2009; 2009:634292. [PubMed: 19960054]
37. Pigazzi M, Manara E, Baron E, Basso G. miR-34b targets cyclic AMP-responsive element binding protein in acute myeloid leukemia. Cancer research. 2009; 69:2471–2478. [PubMed: 19258499]
38. Chen HZ, Tsai SY, Leone G. Emerging roles of E2Fs in cancer: an exit from cell cycle control. Nat Rev Cancer. 2009; 9:785–797. [PubMed: 19851314]
39. Tan Z, Wortman M, Dillehay KL, Seibel WL, Evelyn CR, Smith SJ, et al. Small-molecule targeting of proliferating cell nuclear antigen chromatin association inhibits tumor cell growth. Molecular pharmacology. 2012; 81:811–819. [PubMed: 22399488]
40. Shimada M, Okuzaki D, Tanaka S, Tougan T, Tamai KK, Shimoda C, et al. Replication factor C3 of Schizosaccharomyces pombe, a small subunit of replication factor C complex, plays a role in both replication and damage checkpoints. Molecular biology of the cell. 1999; 10:3991–4003. [PubMed: 10588638]

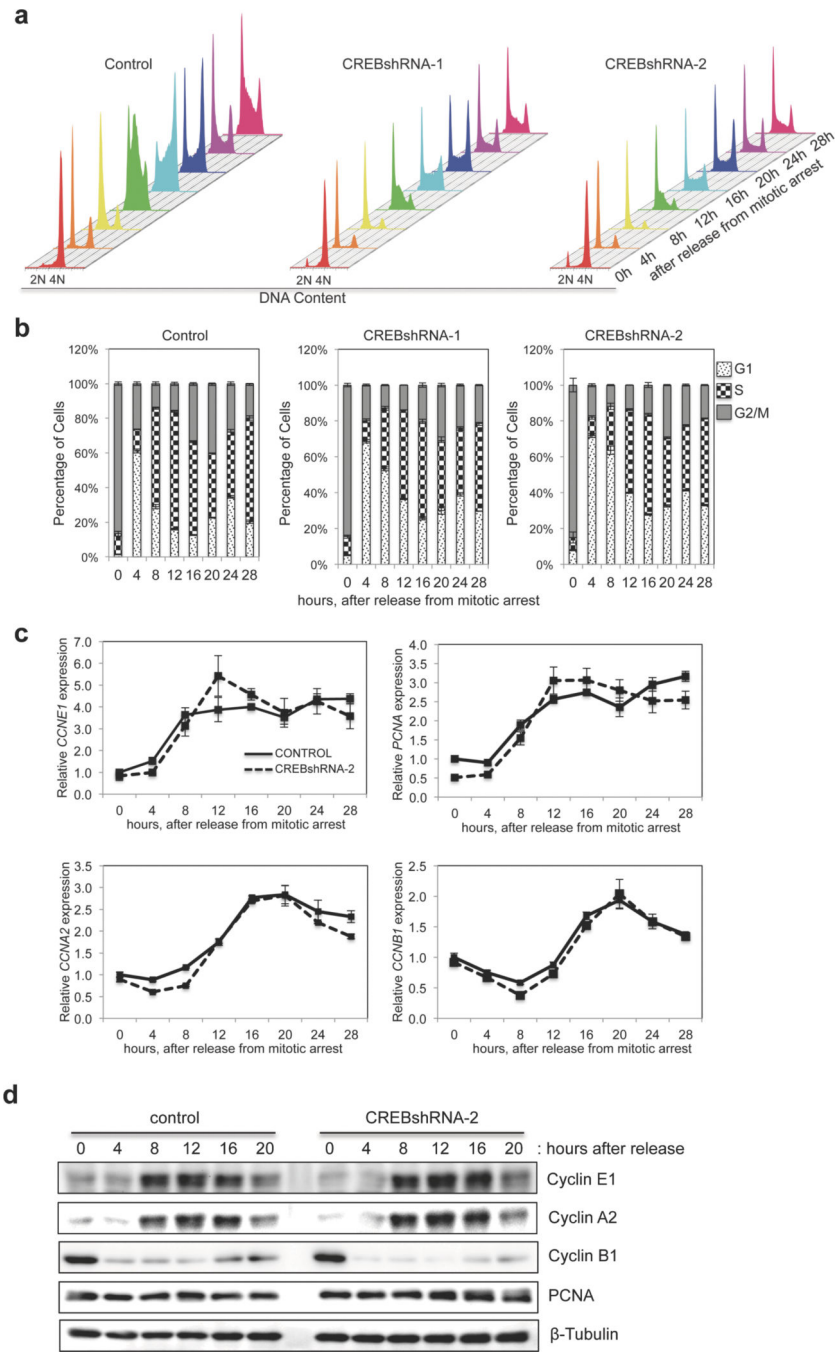

Figure 1.

Impaired G1/S progression in CREB knockdown KG-1 cells. (a) Cell cycle profile of control and CREB knockdown cells by flow cytometry. KG-1 cells were infected by CREB shRNA-expressing or control lentiviruses, and then transduced GFP-positive cells were sorted. Cells synchronized using a thymidine plus nocodazole block. Synchronized cells were released from the nocodazole block (mitotic arrest) and collected at the indicated times. DNA content was analyzed using propidium iodide staining and flow cytometry analysis. Cells started to enter S phase by 8h after release. 2N indicates G1 DNA content.

Plots are representative of three experiments with similar results. (b) Data represent the percentages of cell populations residing at each cell cycle stage calculated using FlowJo software and is expressed as mean ± SEM (n = 3). (c) Temporal expression patterns of *CCNE1, PCNA, CCNA2* and *CCNB1* genes in CREB knockdown KG-1 cells. CREB-knockdown and control KG1 cells were released from the mitotic arrest and harvested at indicated times. Relative mRNA expression of cyclin E1 (*CCNE1*), cyclin A2 (*CCNA2*), cyclin B1 (*CCNB1*) and *PCNA* genes were quantitated by qRT-PCR analysis. Expression of each gene was normalized against *β-actin* expression level. Relative expression levels are presented as fold induction above expression levels in control cells at 0 hours. Values are shown as mean ± SEM (n = 3). **, $p < 0.01$. (d) Cell extracts were prepared at the indicated times after release from mitotic arrest and protein expression levels of cyclin E1, cyclin A2, cyclin B1, PCNA and β-tubulin were analyzed by immunoblotting. β-tubulin was used as an internal control.

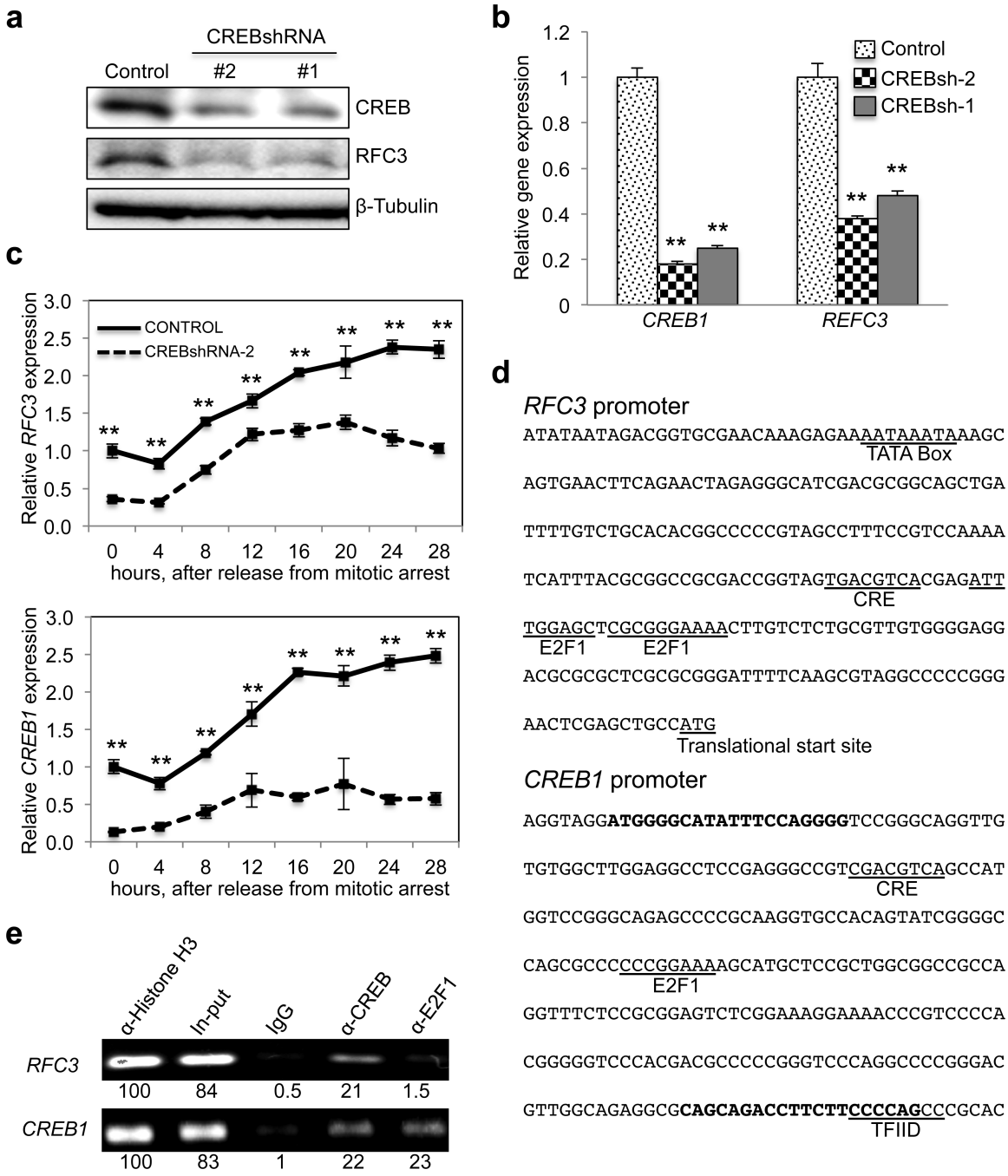

Figure 2.

RFC3 as a direct target gene of CREB. (a) Protein expression levels of CREB and RFC3 were analyzed in CREB knockdown and control KG-1 cells by immunoblotting. Total lysates were immunoblotted for CREB, RFC3 and β-Tubulin (loading control). A representative blot of at least three different experiments is shown. (b) *RFC3* mRNA expression levels were significantly decreased in CREB knockdown cells. *RFC3* and *CREB1* mRNA levels were determined by qRT-PCR and normalized against *β-actin* expression level. Relative expression levels are presented as mean ± SEM (n = 3). (c)

Temporal expression of *RFC3* and *CREB1* mRNAs. Expression levels of *RFC3* genes in synchronized cells were assessed by qRT-PCR. Values are indicated as mean ± SEM (n=3). **, $p < 0.01$. (d) Sequence of the human *RFC3* and *CREB1* promoter regions. Putative transcription factor binding sites are underlined. Sequences of PCR primers for *CREB1* ChIP are shown in bold type. (e) CREB binds to the *RFC3* promoter *in vivo*. ChIP assay was performed using normal rabbit IgG (negative control) or antibodies specific to Histone H3 (positive control), CREB, and E2F1 for demonstrating the in vivo binding of CREB and E2F1 to *RFC3* and *CREB1* promoters. *RFC3* and *CREB1* PCR primers were used to detect *RFC3* and *CREB1* promoter DNA fragments in chromatin immunoprecipitates, respectively. Two % of total in-put chromatin was used as a control. Relative PCR product levels are shown.

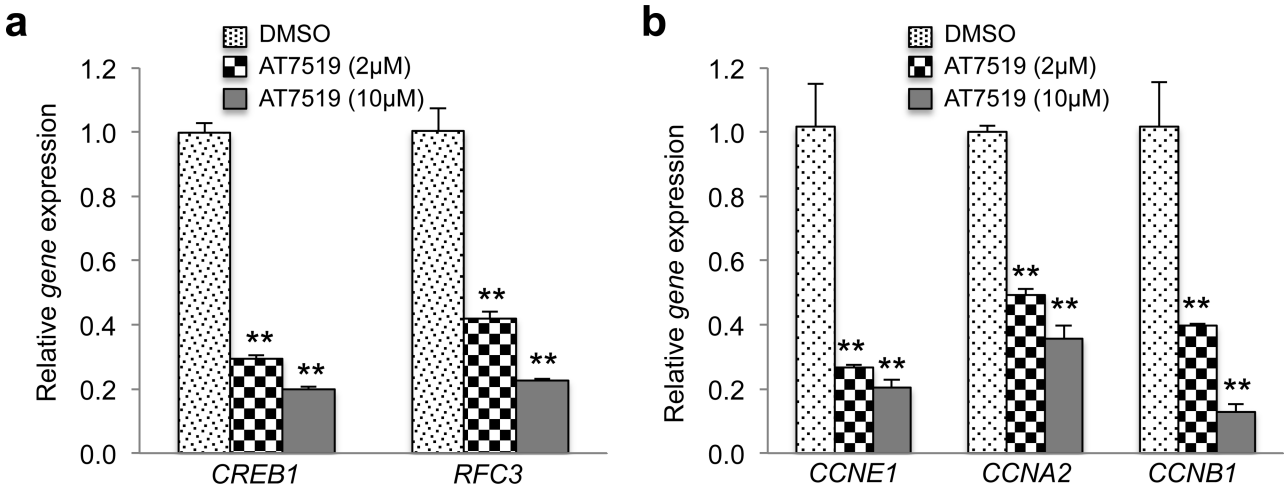

Figure 3.

Expression of *RFC3* and *CREB1* is dependent on CDK activity. KG-1 cells were cultured with or without AT7519 (2 or 10 μM) for 16 hours. (a) *RFC3* and *CREB1* mRNA levels were determined by qRT-PCR. (b) *CCNE1*, *CCNA2* and *CCNB1* mRNA levels were measured to assess CDK inhibition. Expression of each gene was normalized against *β-actin* expression levels. Data are graphed as mean ± SEM ($n = 3$). **, $p < 0.001$.

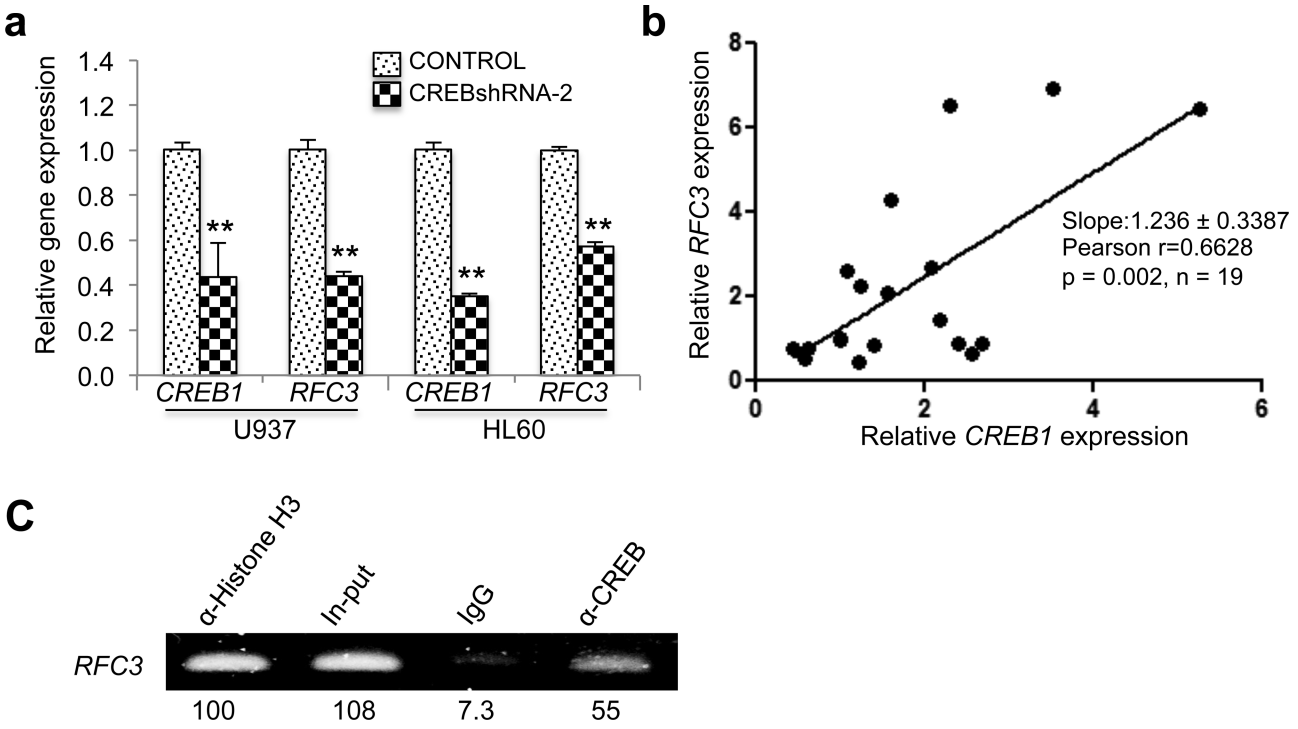

Figure 4.

Correlation between the expression levels of *RFC3* and *CREB1* in AML. (a) CREB knockdown inhibits *RFC3* expression in U937 and HL-60 AML cell lines. U937 and HL60 cells were transduced with lentiviral vector expressing CREBshRNA-2 or vector alone. Cells were sorted for GFP-positive cells, and then analyzed for mRNA expression levels of *CREB1* and *RFC3* by qRT-PCR. Expression of each gene was normalized against *β-actin* expression levels. Data are graphed as mean ± SEM ($n = 3$). **, $p < 0.001$. (b) Correlation between *RFC3* and *CREB1* mRNA expressions in diagnostic samples from AML patients (n = 19, Pearson r=0.6628, p = 0.002). The linear regression line is plotted and its slope is given. Relative mRNA expression levels of *CREB1* and *RFC3* were compared by qRT-PCR. Expression of genes was normalized against *β-actin* expression level. The values represent the ratio of fold change in gene expression from each sample relative to the average of three normal control samples. (c) Association of CREB with *RFC3* promoter region in primary human AML cells. ChIP assays were undertaken using human AML cells and normal rabbit IgG (negative control) or antibodies specific to Histone H3 (positive control) and CREB. DNA fragments spanning CRE consensus motif in *RFC3* promoter region were amplified from the immunoprecipitates by PCR using *RFC3* primers and displayed by gel electrophoresis. Relative PCR product levels are shown as arbitrary numbers by setting the level of DNA from anti-Histone H3 antibody immunoprecipitate as 100.

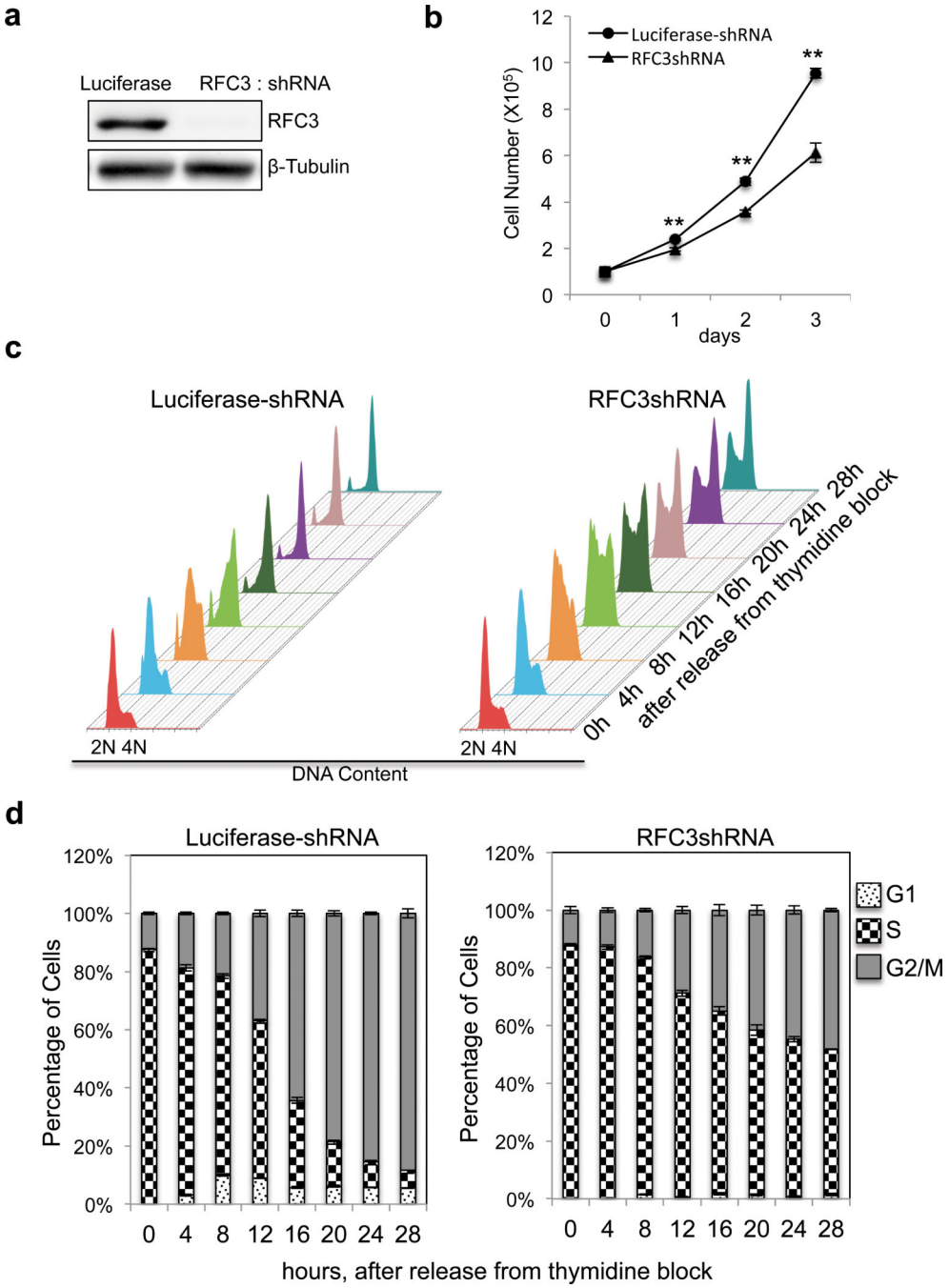

Figure 5.

RFC3 knockdown impairs the G1/S cell cycle progression. KG1 cells were transduced with pLKO.1 lentiviral vectors expressing RFC3 shRNA or luciferase shRNA, and then transduced cells were selected with puromycin. (a) Suppressed expression of RFC3 by corresponding specific shRNA was assessed at protein level. Total cell lysates were analyzed by immunoblotting for RFC3. β-tubulin was used as a loading control. (b) RFC3 knockdown inhibited proliferation of KG1 cells. A total of 1×10^5 cells were seeded in 12-well plates and the number of viable cells was counted for 3 days. Values represent mean ±

SEM (n = 3). **, $p < .01$. (c) Cell cycle profile of control or RFC3 knockdown KG1 cells released from thymidine block. Cells were synchronized at G1/S boundary with thymidine treatment (2 mM, 30 h), and then harvested at the indicated times after release into medium with nocodazole (300 nM). Cells were stained with PI following fixation with 70% cold ethanol, and then analyzed for DNA contents by flow cytometry. These data are representative plots from three experiments with similar results. (d) % cell populations at each cell cycle phase were calculated using FlowJo software and denoted as mean ± SEM (n = 3).

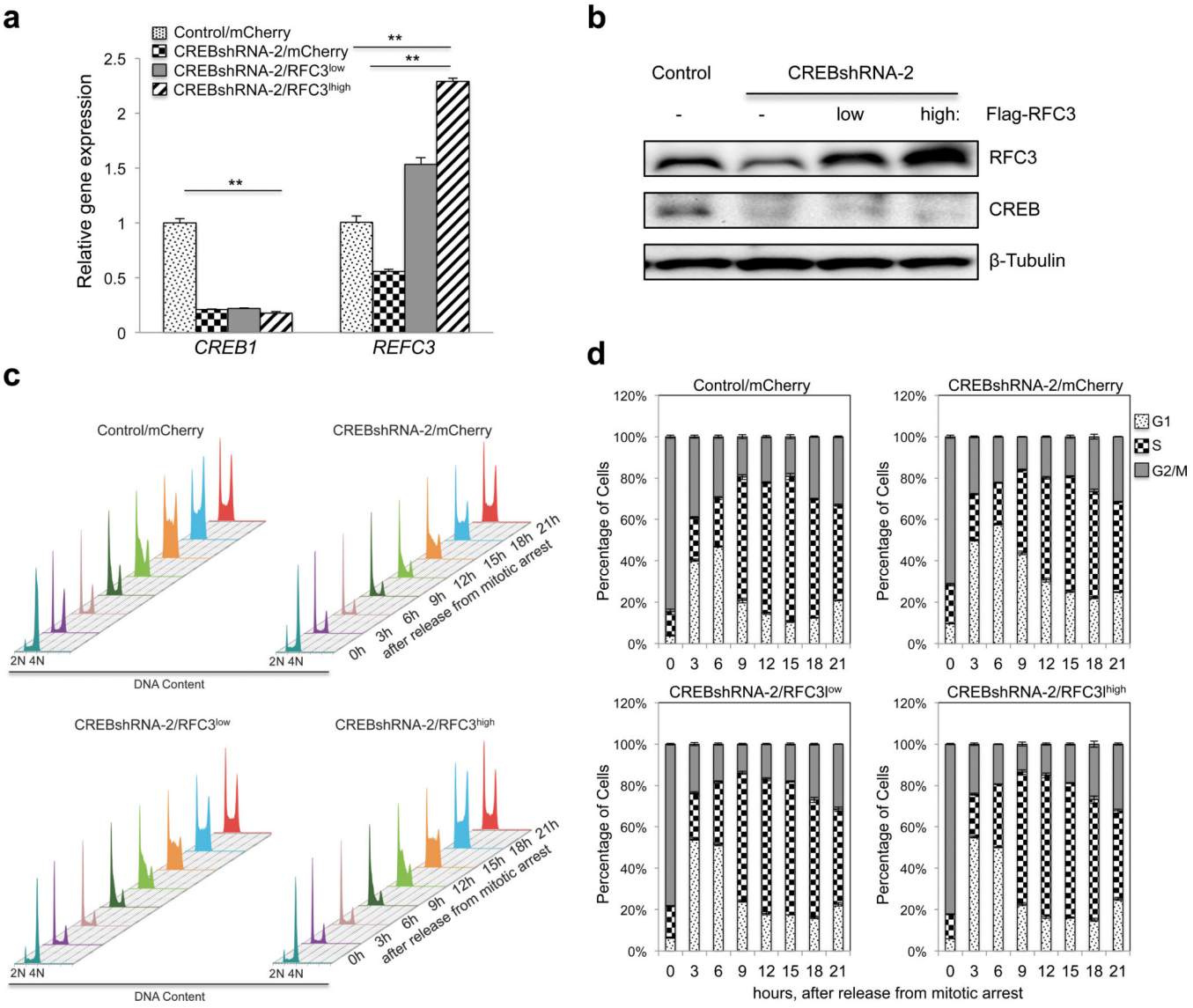

Figure 6.

Exogenous expression of RFC3 rescues the impaired G1/S progression in CREB knockdown KG-1 cells. Control or CREB knockdown (CREBshRNA-2) KG1 cells were transduced with lentiviral vectors expressing RFC3 and mCherry or mCherry alone. RFC3-high and low expressing cells were isolated based on mCherry levels. Expression levels of RFC3 and CREB were confirmed in mRNA levels by qRT-PCR (a) and protein levels by immunoblotting (b). mRNA expression levels of *CREB1* and *RFC3* were normalized against *β-actin* expression levels. Values are indicated as mean ± SEM ($n = 3$). **, $p < 0.01$. Cell lysates were analyzed for RFC3, CREB, and with β-tubulin as a loading control, by immunoblotting. A representative blot of at least three independent experiments is shown. (c) Exogenous RFC3 expression rescues the defective G1/S progression in CREB knockdown KG-1 cells. Cells were synchronized in mitosis by a thymidine/nocodazole dual block. Synchronized cells were released from the mitotic arrest and analyzed at the indicated times. DNA content was determined by flow cytometry analysis of propidium iodide stained

cells. Cells started to enter S phase by 9 hours after release. Plots are representative of three experiments with similar results. (d) Data represent the percentages of cell populations residing at each cell cycle stage calculated using FlowJo software as mean ± SEM (n = 3).

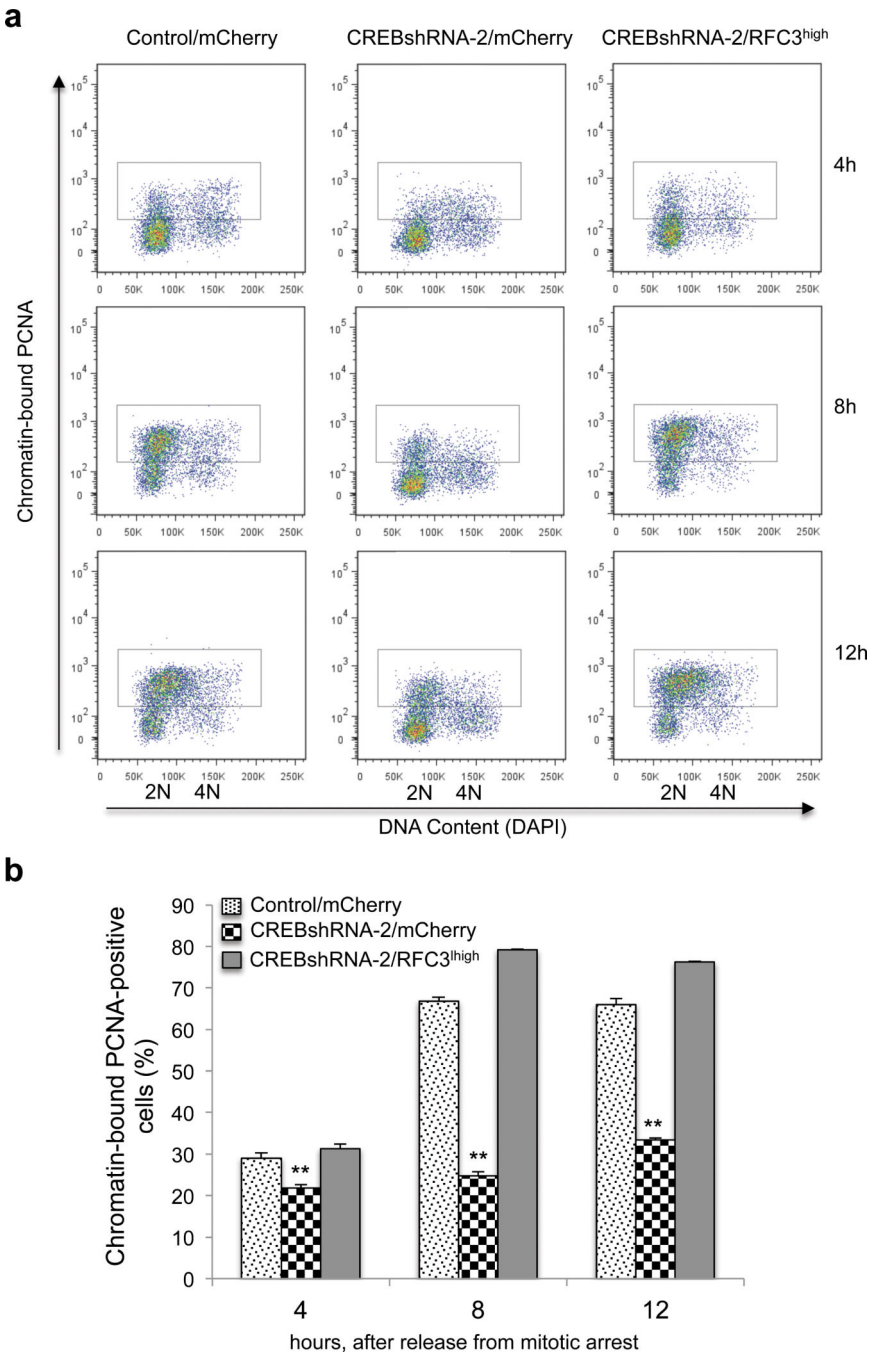

Figure 7.

CREB regulates loading of PCNA onto chromatin through RFC3. (a) Mitotic arrest KG-1 cells with a thymidine/nocodazole dual block were released and analyzed at the indicated times. Cells were extracted with NP-40 containing hypotonic buffer, fixed, then stained with anti-PCNA antibody and DAPI. Chromatin-bound PCNA and DNA content were determined by flow cytometry analysis. The region indicates chromatin-bound PCNA-positive populations. Plots are representative of three experiments with similar results. (b)

Data represent the percentages of chromatin-bound PCNA compartments calculated using FlowJo software as mean ± SEM (n = 3).