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

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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
role in the pathogenesis of AML and other cancers. 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). CREB regulates a number of critical cellular functions, including cell
division and apoptosis, and alterations in CREB function fundamentally change diverse
cellular outcomes. 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.

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. A ‘second hit’, such as Sox4
signaling was sufficient for myeloid leukemogenesis in cooperation with CREB. 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, 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 onto the 3'-ends of nascent DNA
strands. PCNA is expressed at a high level particularly in most tumor cells. 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. RFC3 is a 38kDa subunit with an ability
to bind preferentially to primed single-stranded DNA and PCNA. RFC3 knockdown
inhibited proliferation and anchorage-independent growth of cancer cells. 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.

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% CO₂ 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. Briefly, KG-1 cells

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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. Lentiviral vectors expressing RFC3 shRNA (NM_181558.2-415s2c1) 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 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' - ACGCTAGCATGGATTACAAGGATGACGACGATAAGAGCCTCTGGGTGGACAAGTAT-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. 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 by calcium phosphate transfection method. 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 DxFP10 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 Inc 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](http://primer3plus.com)). Relative expression levels were determined with the 2−ΔΔCT Livak method.21

Primer sequences (5′ to 3′); 7SLs cRNA F: ATCGGGTGTCCGCAGTTCATAGTT, 7SL RNA R: CAGCACGGGATTTTCTGACCT22, CCNE1 F: AAGGACGGCGAGGGACCAGTG, CCNE1 R: TTTGCCAGCTCAGTCACAGCAGC23; CCNA1 F: TACACCCAGCCACTTCAAGGACAGC, CCNA1 R: CCTCCACAGCTTCAAGGACAGTTC23; CCNB1 F: TTTGCCAGCTCAGTCACAGCAGC, CCNB1 R: AAGGAGAAGGCACCATAGTGC24; ACTB F: GGACTTCGAGCAAGGAGATGG, ACTB R: AGCACTGTGGCGCAGCTAG; PCNA F: GGCGTGAAACCTCAGGATAT; PCNA R: TTCTCCTGGGTGGTTGCTTCC; RFC3 F: GCCTGCAGTGGCAACAATA,
**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 glyceine 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’-CTGGGGGAAGAAGGTCTGCTG-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. Consistent with previous results, CREB knockdown showed significantly reduced proliferative capability compared to the control KG-1 cells (cell counts after 4d with 1x10^5 seeding: 34.18 \pm 1.2 x 10^5 vs. 19.21 \pm 0.54 \times 10^5 vs. 14.52 \pm 0.46 \times 10^5, for control cells vs. CREB shRNA-1 vs. CREB shRNA-2, respectively (mean \pm 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 \pm 0.54% vs. 23.57 \pm 1.69%; 12 h post-release: 66.92 \pm 0.63% vs. 45.16 \pm 0.50%, mean \pm 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 \[\textit{Cyclin E1 (CCNE1), PCNA, Cyclin A2 (CCNA2) and Cyclin B1 (CCNB1)}\] 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 \textit{CCNE1} and \textit{PCNA} mRNAs in the early G1/S transition stage at 8 hours post-release, as well as increased \textit{CCNA2} and \textit{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 \textit{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 processes29 was markedly down-regulated by CREB knockdown in K562 cells. We verified the dependency of the \textit{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 \textit{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 \textit{RFC3} and \textit{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 transcriptional 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 ± 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. There are two forms of PCNA; a detergent-soluble unbound form and a detergent-insoluble chromatin-bound form in S phase. 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)\(^3\)\(^2\) and in NPrEC epithelial cells (GEO accession GDS3354).\(^3\)\(^3\) Our data show that the CDK-E2F axis, a well-defined transcriptional activation pathway for G1/S progression,\(^2\)\(^6\),\(^2\)\(^7\),\(^3\)\(^4\) 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.\(^2\)\(^6\),\(^2\)\(^7\),\(^3\)\(^4\) Cyclins A and D themselves have been reported to be targets of CREB, implying cross-talk within this pathway.\(^3\)\(^5\),\(^3\)\(^6\) 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. 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. Analysis of the expression of CREB1 during the cell cycle progression of synchronized NPrEC epithelial cells (GEO accession GDS3354) 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 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. 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 chromat binding of PCNA for G1/S progression via RFC3. Inhibition of RFC3 and PCNA blocks cellular proliferation of cancer cells. Furthermore, RFC3 knockdown represses DNA synthesis and anchorage-independent growth of cancer cells. Fission yeast rfc3 mutants have defects in DNA replication and DNA damage checkpoint, and an inhibitor of chromatin loading of PCNA have an inhibitory effect on tumor cell growth. 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.

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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 $\beta$-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 $\beta$-tubulin were analyzed by immunoblotting. $\beta$-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 \textit{RFC3} and \textit{CREB1} in AML. (a) CREB knockdown inhibits \textit{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 \textit{CREB1} and \textit{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 \textit{RFC3} and \textit{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 \textit{CREB1} and \textit{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 \textit{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 \textit{RFC3} promoter region were amplified from the immunoprecipitates by PCR using \textit{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 ($n = 3$).