Medical Oncology (2019) 36:20
https://doi.org/10.1007/s12032-018-1240-2

ORIGINAL PAPER

A feedback regulation of CREB activation through the CUL4A and ERK signaling

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Received: 9 November 2018 / Accepted: 17 December 2018 / Published online: 21 January 2019

Abstract
CUL4A; an E3 ubiquitin ligase is involved in the degradation of negative regulators of cell cycle such as p21, p27, p53, etc., through polyubiquitination-mediated protein degradation. The functional role(s) of CUL4A proteins on their targets are well characterized; however, the transcriptional regulation of CUL4A, particularly at its promoter level is not yet studied. Therefore, in this study, using computational tools, we found cAMP responsive elements (CRE) at the locations of − 926 and − 764 with respect to transcription state site + 1 of CUL4A promoter. Hence, we investigated the role of CREB on the regulation of CUL4A transcription. Our chromatin immunoprecipitation (ChIP) data clearly showed increased levels of promoter occupancy of both CREB and pCREB on both CREs of CUL4A promoter. As expected, the expression of CUL4A increases and decreases upon the overexpression of and knocking down of CREB, respectively. Moreover, the inhibition of ERK pathway by U0126 not only reduces the CREB activation but also the CUL4A levels suggesting that CREB is the upstream activator of CUL4A transcription. The reduction of CUL4A levels upon the knocking down of CREB or by U0126 treatment increases the protein levels of CUL4A substrates such as p21 and p27. It is reported that CUL4A activates the ERK1/2 transcription and ERK1/2 pathway activates the CREB by phosphorylation. Based on our data and earlier findings, we report that CREB regulates the CUL4A levels positively which in turn activates the CREB through ERK1/2 pathway in the form of auto-regulatory looped mechanism.This suggests that CUL4A might be involved in proliferation of cancer cells by regulating the ERK1/2 and CREB signaling.

Keywords cAMP response element (CRE) · CREB · CUL4A · ERK1/2 · U0126 · Transcriptional regulation

Introduction

The regulated protein biosynthesis and degradation process is critical to maintain normal cellular process including cell proliferation and differentiation. A dysregulated pool of proteins lead to various disorders including cancers [1, 2]. There are two major proteolysis pathways that operate in eukaryotic system to maintain homeostasis, namely the lysosomal-mediated degradation system and ubiquitin mediated (Ub) proteasome system (UPS) [3–6]. The UPS is mediated by three enzymes, namely ubiquitin activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and ubiquitin-ligases (E3) and the majority of the target proteins are degraded through UPS pathway. Based on structural catalytic core domain, the E3 ubiquitin ligases are categorized into two major classes: HECT (Homologous to E6-AP C terminus) domain-containing E3 ligases and RING (really interesting new gene) E3 ligases [7].

The RING-based E3 ligases are evolutionarily conserved proteins which recruits a RING-based protein at one end to form a catalytic core and cullin-specific adaptor and/or substrate receptor at the other end and the whole complex is called as cullin-RING ubiquitin ligase (CRL) [8]. All cullins are positively regulated by an attachment of ubiquitin such as molecule NEDD8 (neural precursor cell expressed, developmentally downregulated) and negatively regulated by deneddylation process [8, 9]. There are eight cullin-based E3 ligases in human (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, and CUL7) and PARC [10]. CUL4 comprises of two closely related paralog proteins; namely CUL4A and CUL4B which shares 83% identity at amino acid level [11]. Both CUL4A

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and CUL4B interact with the common substrate adaptors such as DDB1 which selects the target proteins [12, 13]. Aberrant expression of CUL4A is observed in various types of cancers indicating its oncogenic potential [14–17]. CUL4A-based E3 ubiquitin ligases control several cellular processes (cell cycle, DNA replication, DNA repair, embryonic development, chromatin remodeling, etc.) through the degradation of target proteins. The well-studied targets of CUL4A are cell cycle inhibitors p27, p21, p53, PCNA, CDT1, c-Jun, HoxA9, H3 and CHK1 [18–26]. Though the functional role(s) of CUL4A E3 ligases is well documented in the literature, the regulation of CUL4A at transcriptional level is not yet investigated. Recent reports have shown that CUL4A regulates transcription of ERK1/2 by regulating its promoter methylation [27, 28] and the ERK1/2 signaling pathway is a major kinase pathway known to activate CREB [29, 30].

Cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) is a transcription factor that regulates several cellular processes including cell proliferation [31, 32]. Genome-wide analysis predicted that nearly 4000 gene promoters contain cAMP-responsive elements (CREs) where the CREB binds and regulate the transcription [33]. Phosphorylation of CREB at Ser133 position activates the CREB and this phosphorylation is mediated by kinases of different signaling pathways such as protein kinase A (PKA), protein kinase B (PKB/Akt), mitogen-activated protein kinases (MAPKs) and/or p90 ribosome S6 kinase (pp90RSK), etc. [32, 34]. The activated pCREB forms a complex with major transcriptional co-activator protein CREB-binding protein (CBP)/p300 which contain histone acetyl transferase activity (HAT) [35–37]. pCREB is deactivated by dephosphorylation of S133 residues by phosphatases such as protein phosphatase 1 (PP1) [38], protein phosphatase 2A (PP2A) [39] and protein phosphatase and tensin homolog (PTEN) [40] which inhibits CREB-mediated transcription. CUL4A, CREB and its related kinases and phosphatases are reported to be deregulated in various cancers [32, 41] suggesting the possible crosstalk of CUL4A with CREB signaling.

In this study, we provide experimental evidences that CREB binds to CUL4A promoter at CRE motifs and stimulate its transcription. Inhibition of ERK1/2 signaling, which inhibits CREB activation reduced the CUL4A expression significantly. This data shows the positive feedback loop of ERK1/2-mediated CREB activation through CUL4A transcriptional regulation.

Materials and methods

Cell culture and MEK inhibitor treatment

Hela (cervical cancer) and A549 (adenocarcinomic human alveolar basal epithelial cancer) cell lines were purchased from National Center for Cell Science (NCCS), Pune, INDIA and used. Cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin at 37 °C under 5% CO_2. U0126 (MEK inhibitor) was purchased from Cell Signaling Technology and used at different concentrations (5 µM and 10 µM). Total RNA and proteins were isolated from drug-treated and untreated cells at indicated time points and used in real-time qRT-PCR and western blot analysis, respectively.

Bioinformatics analysis of the promoter region

The human CUL4A promoter 5′-untranslated region and the human CUL4A mRNA sequence (Accession Number; NM_003589) were obtained from the National Center for Biotechnology Information (NCBI). JASPAR database (http://jaspar.binf.ku.dk/) was used for the prediction of putative binding sites on CUL4A promoter.

Plasmids and transfection

CREB-pcDNA3 clone was generated by PCR-amplified full-length CREB fragment using HEK293 cells cDNA as template in frame into pcDNA3 vector between EcoR1 and BamHI sites using the following primers; CREB-F: 5′-CGG AATTCATGACCATGGAATCTGGAGCCGAGA-3′ and R: 5′-CGGGATCCTTAATCTGATTTGTGGCAGTAA AGG-3′. Transient transfections were performed using the TurboFect transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's protocols. After 48 h of transfection, cells were harvested and used for experimental analysis.

RNA extraction, cDNA preparation and qRT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen) and cDNA was prepared using first-strand cDNA synthesis kit (Roche) according to the manufacturer's instruction. qRT-PCR was performed using LightCycler® 96 Real-Time PCR System with SYBR Green Master mix (Roche). The qRT-PCR reaction cycles were: 95 °C for 5 min, 35 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s with a final extension of 72 °C for 7 min. GAPDH was used as an internal control. The primers used were as follows: CUL4A 5′-AAGAGCAGGCAACAAAGAAGCCA-3′, 5′-TTGGCCAGTAGCCCATTGTGAGT-3′, GAPDH 5′-GAGTCA ACGGATTTGGTCG-3′, and 5′-TTGATT TTGGAGGGATCTCG3′. All experiments were performed in triplicates and the expression level was calculated as mean ± SD.

siRNA transfection

Approximately 2×10^5 HeLa and A549 cells were seeded and 16 h after plating, the cells were transfected with 20 picomolar CREB-specific siRNA or scramble siRNA (Santa Cruz, USA) using Lipofectamine RNAiMax (Invitrogen). After 48 h of transfection, cells were harvested and subjected to qRT-PCR and western blot analysis.

Immunoblotting

Cells were harvested, lysed in RIPA buffer; total protein was isolated and subjected to immunoblotting. The following antibodies were used in this study: anti-CREB, pCREB, pERK1/2, ERK1/2, p27 and GAPDH from Cell Signaling Technology, anti-p21 from Santa Cruz and anti-CUL4A was a gift from Dr. Pradip Raychaudhuri, UIC, Chicago, USA. The corresponding secondary antibodies conjugated with HRP were used and protein bands were detected using BM Chemiluminescence blotting substrate reagent (Roche).

Chromatin immunoprecipitation (ChIP) assay

The binding of endogenous CREB and pCREB on CUL4A promoter was evaluated by performing ChIP assay using a ChIP assay kit (Millipore) according to the manufacturer's instructions. The nuclei were isolated from HeLa cells, sonicated to shear the DNA (approximate average size was 100–500 bp), and chromatin was incubated overnight at 4 °C with anti-CREB, pCREB antibodies and rabbit IgG used

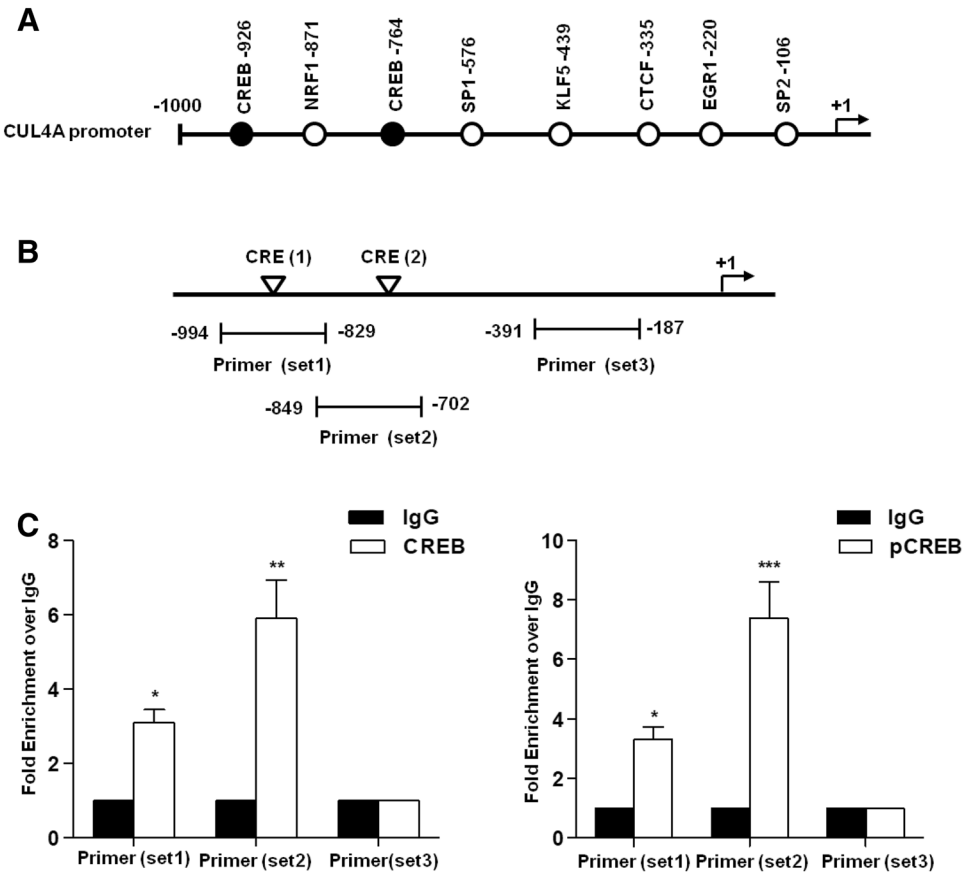

Fig. 1 The schematic representation of the human CUL4A gene promoter (**a**). Bioinformatic analysis of the position of transcription factor-binding sites in the CUL4A promoter by JASPAR database prediction. The +1 represents the transcription start site (TSS), binding sites of CREB is indicated in closed circle and other transcription factors are in open circles. The numbers on each transcription factors indicate the position of transcription factor-binding sites relative to TSS. (**b**) Binding of CREB on CUL4A promoter. Two CRE binding sites in the human CUL4A promoter region located at −926 and −756, the three sets of primers for the ChIP assay designed to encompass CRE-binding motif sites (−994 to −829 and −849 to −702) or not to encompass CRE-binding motif sites (−391 to −187) are indicated. (**c**) HeLa cells were subjected to ChIP assay using CREB and pCREB antibodies and measured the levels of CREB binding on CUL4A promoter by quantifying the promoter-bound DNA fragments using specific primers designed at specific region as shown in **b**. The fold change was calculated based on control IgG amplification. The experiment was carried out in triplicate and the average values with SD are shown. The statistical significance was analyzed by Student's *t* test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

as a control. Immunoprecipitated protein-DNA complexes were reverse crosslinked, chromatin-bound DNA was purified using purification kit (Thermo Fisher Scientific) and measured by qRT-PCR using the following primers; ChIP primer (set1), F-5′-TGGTAATGTGCGCCATGGGA-3′ and R-5′-CCGGTCCCCAGGTTCTCCCT-3′ ChIP primer (set2), F-5′-AGGGAGAACCTGGGGACCGG-3′ and R-5′-ACTGGACCCCAAACCCCTCA-3′ and ChIP primer (set3), F-5′-GAGGGTGTCCCGGCTCCCGA-3′ and R-5′-CGG ACTCCCCCTAAGAGATT-3′. Primer set 1 and 2 were designed to cover one CRE-binding motif on CUL4A promoter and primer set 3 does not contain CRE region.

Statistical analyses

Each experiment was repeated at least three times and the values were shown as mean ± SD. Graph generation and statistical analyses were performed using Graph Pad Prism software (v.6) and unpaired Student's *t* tests. *p* value of <0.05 was considered significant.

Results

CREB binds to CRE elements of CUL4A promoter and regulates its transcription

To investigate the transcriptional regulation of CUL4A particularly the potential transcription factor(s) responsible for the transcription of CUL4A, we employed computational tools and identified several putative positive transcription factors (Fig. 1a). Computational prediction identified two CREs (located at −926 and −764) in CUL4A promoter and it is shown schematically in Fig. 1a. It should be noted that the transcription factor CREB binds to CRE motifs and regulate the transcription of downstream genes.

To confirm the prediction of CREB binding on CREs of CUL4A promoter, the chromatin immunoprecipitation (ChIP) assay was performed using CREB and pCREB antibodies in HeLa cells and measured the CUL4A promoter-bound DNA fragments using specific primers as shown in Fig. 1b. Our ChIP data clearly shows that both CREB and pCREB bind on both the CREs of CUL4A promoter (Fig. 1c). We observed 3.1- and 5.9-fold of CREB occupancy

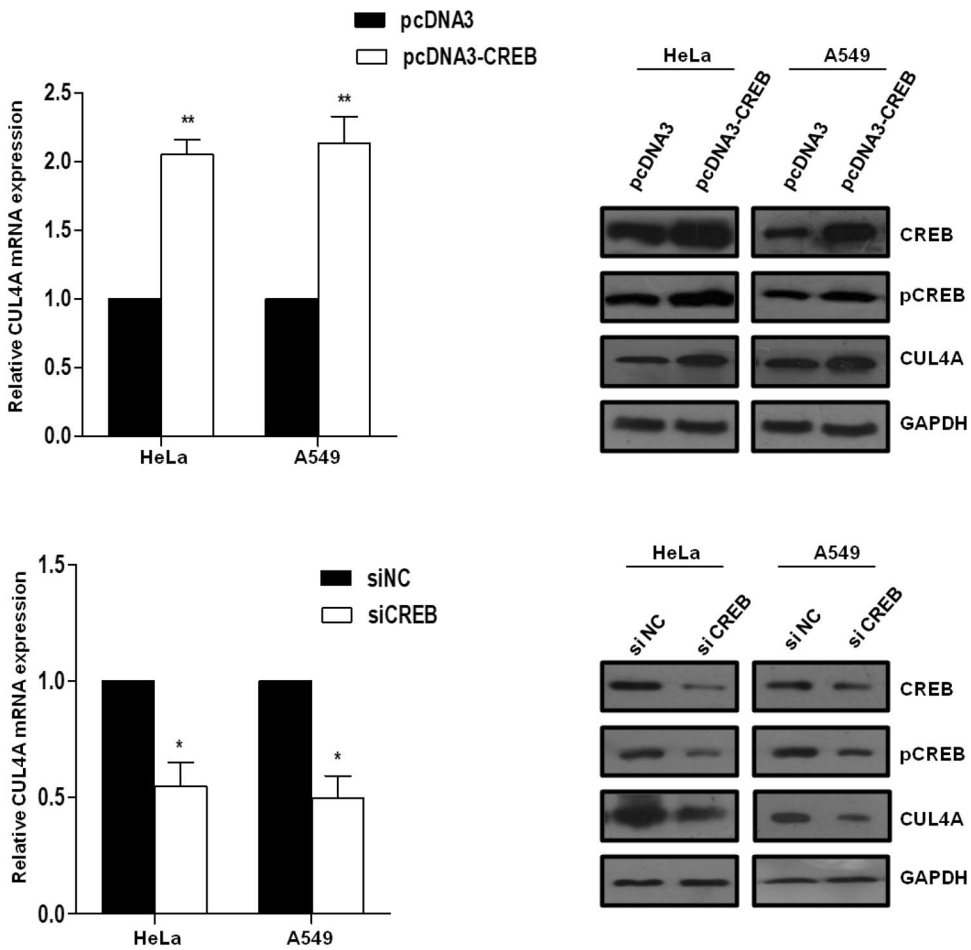

Fig. 2 Effects of CREB on the regulation of CUL4A expression. RNA and proteins were isolated from CREB overexpression and silencing condition (HeLa and A549 cells) and measured the CUL4A transcripts by qRT-PCR using specific primers and proteins by immunoblotting using specific antibodies as shown in the figure. The qRT-PCR experiment was carried out in triplicate and the average values with SD are shown. The statistical significance was analyzed by Student's *t* test. $^{*}p<0.05$, $^{**}p<0.01$

on CRE1 and CRE2 of CUL4A promoter, respectively, as compared to control IgG samples (Fig. 1c left panel). Similarly, the pCREB binds to CRE 1 and CRE2 by 3.3- and 7.4-fold, respectively (Fig. 1c right panel) indicating that the binding of pCREB on CRE2 might induce the CUL4A transcription. As expected, no changes were observed in the negative control region which lacks CRE motif (Fig. 1c).

CREB regulates CUL4A levels

To further investigate the role of CREB on CUL4A transcription, we analyzed the expression profile of CUL4A in HeLa and A549 cells both at transcript and protein level in CREB overexpression and CREB silencing conditions. Notably, the transcripts and proteins of CUL4A under these conditions correlate well with CREB expression; higher in overexpression and lower in silencing which suggest that CREB regulates the CUL4A transcription positively (Fig. 2). As expected, the protein levels of CREB and pCREB were higher in overexpression and lower in silencing conditions (Fig. 2).

Inhibition of ERK signalling reduces the CREB activation and CUL4A transcription

Finally, to prove the point that CREB activation is responsible for CUL4A transcription, we used U0126 inhibitor to block MEK pathway-mediated CREB activation and checked the expression of CUL4A. There is a significant reduction of CUL4A transcripts and proteins in drug-treated HeLa and A549 cells when compared to vehicle-treated cell (Fig. 3a, b). We also found no significant difference between untreated and vehicle-treated cells (data not shown). This indicates that the activation of CREB by MEK pathway stimulates the CUL4A expression, which in turn might regulate the MEK pathway positively in the form of feedback activation of CREB (data not shown). The protein levels of ERK, pERK, CREB, pCREB also showed significant reduction in

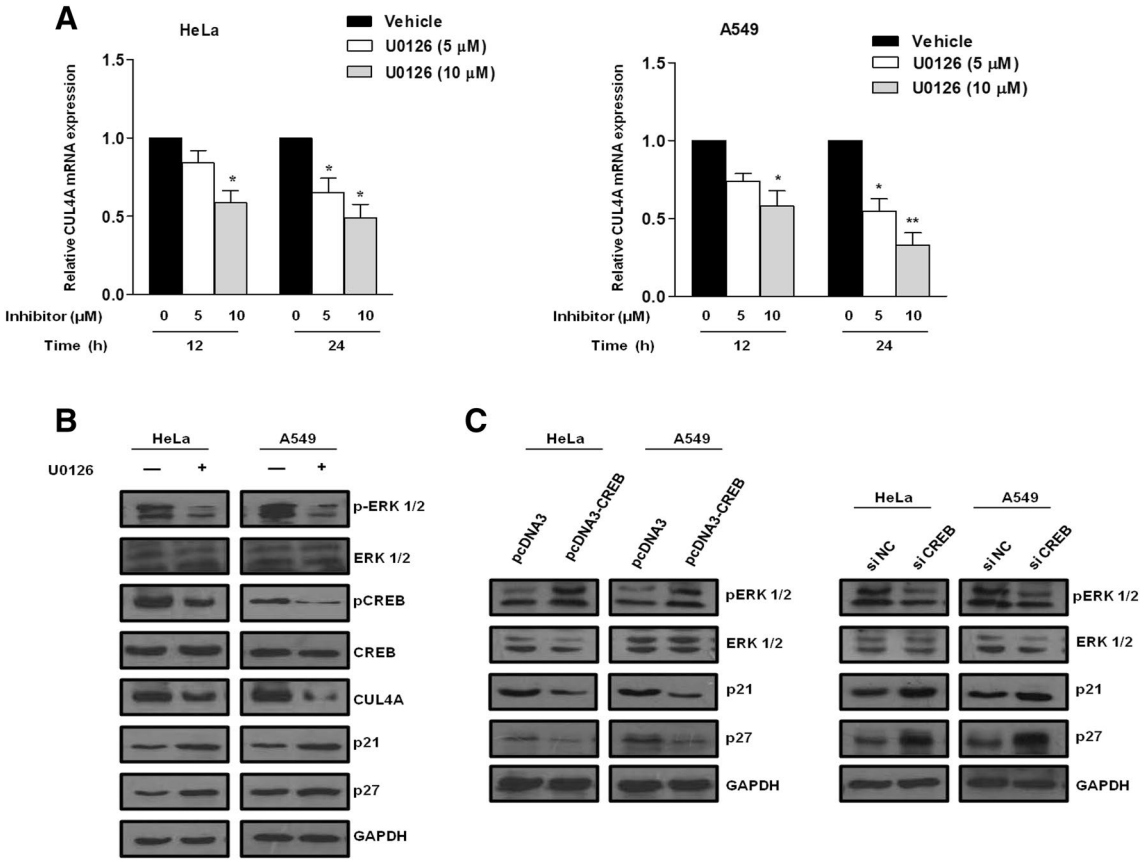

Fig. 3 Effects of MEK inhibitor on CUL4A, CREB, pCREB, ERK, pERK, p21 and p27. HeLa and A549 cells were treated with 5 and 10 μM of U0126, harvested at 12 and 24 h of treatment and the relative CUL4A mRNA was measured by qRT-PCR using specific primers and normalized to GAPDH (**a**). Proteins were isolated from 24 h of 10 μM of U0126 treated and untreated (**b**) and CREB overexpression and silencing condition (**c**; left and right) and analysed by immunoblotting using specific antibodies as indicated in the figure. GAPDH was used as an internal loading control

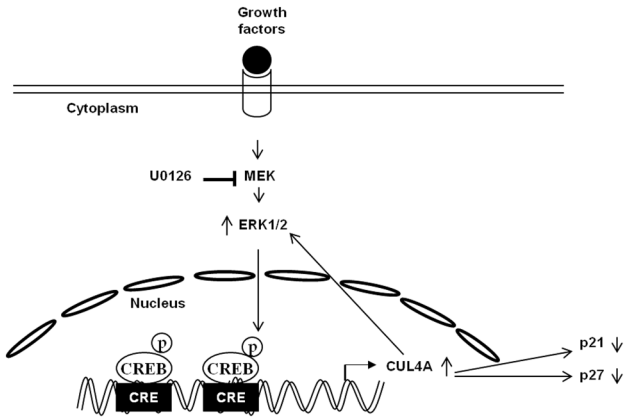

Fig. 4 A model depicting the positive feedback loop of ERK1/2-mediated CREB activation through CUL4A transcriptional regulation

drug-treated cells as expected (Fig. 3b) which supports our earlier results and others. We also analyzed the protein levels of well characterized targets of CUL4A, namely p21 and p27 in CREB upregulation and downregulation (drug induced and siRNA silencing) conditions. As expected, p21 and p27 levels were lower in CREB overexpression and higher in CREB silencing conditions (Fig. 3c). The CREB induced CUL4A and its ERK link connects the CREB signalling activation in cancer cell is shown as schematic in Fig. 4.

Discussion

Appropriate gene expression and proper protein degradation process are critical for normal cell proliferation and any deregulation leads to tumorigenesis. Both CREB and CUL4A are reported to be overexpressed in variety of cancer types showing their possible functional link in terms of cellular proliferation. However, cross talk/molecular link between these proteins have not yet been studied. Though the degradation functions of CUL4A on their targets including cell cycle regulators are well-studied, the transcriptional regulation of CUL4A is not yet studied. Here, in this study, we showed that CREB regulates the transcription of CUL4A by binding to the CREs of the CUL4A promoter.

It is well documented that ERK1/2 signaling activates the CREB by phosphorylation [29, 30]. It is also reported that CUL4A regulates ERK1/2 transcription. Here, we show that the inhibition of MEK/ERK signaling by U0126 not only reduces the CREB activation but also the CUL4A transcription. This suggests the possible feedback mechanism of CREB activation via ERK signaling through CUL4A transcriptional upregulation (Fig. 4). Since the mis-regulation of MEK/ERK signaling is implicated in various cancer types, our findings that CREB regulates CUL4A levels deepens our understanding on MEK/ERK signaling and have a strong relevance in the oncogenic potential of the cells.

Acknowledgements We are grateful to Dr. Pradip Raychaudhuri (UIC, Chicago, USA) for providing CUL4A antibodies. We are also thankful to Dr. Arunkumar Dhayalan for critical reading of the manuscript.

Funding This study was funded by Science and Engineering Research Board (SERB)-INDIA; SB/EMEQ-038/2013 to Prof. Sudhakar Baluchamy. Fellowships from CSIR-UGC to CA, UGC UGC-NFOBC to MS, UGC-BSR to SO, Pondicherry University doctoral research fellowship to LS and DST-SERB-NPDF (2739) to Dr. SP are greatly acknowledged.

Compliance with ethical standards

Conflict of interest No potential conflicts of interest were disclosed.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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