



CREB acts as a common transcription factor for major epigenetic repressors; DNMT3B, EZH2, CUL4B and E2F6

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Received: 25 April 2020 / Accepted: 16 July 2020 / Published online: 24 July 2020
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Abstract

CREB signaling is known for several decades, but how it regulates both positive and negative regulators of cell proliferation is not well understood. On the other hand functions of major epigenetic repressors such as DNMT3B, EZH2 and CUL4B for their repressive epigenetic modifications on chromatin have also been well studied. However, there is very limited information available on how these repressors are regulated at their transcriptional level. Here, using computational tools and molecular techniques including site directed mutagenesis, promoter reporter assay, chromatin immunoprecipitation (ChIP), we identified that CREB acts as a common transcription factor for DNMT3B, EZH2, CUL4B and E2F6. ChIP assay revealed that pCREB binds to promoters of these repressors at CREs and induce their transcription. As expected, the expression of these repressors and their associated repressive marks particularly H3K27me3 and H2AK119ub are increased and decreased upon CREB overexpression and knock-down conditions respectively in the cancer cells indicating that CREB regulates the functions of these repressors by activating their transcription. Since CREB and these epigenetic repressors are overexpressed in various cancer types, our findings showed the molecular relationship between them and indicate that CREB is an important therapeutic target for cancer therapy.

Keywords cAMP responsive element · CRE · CREB · DNMT3B · EZH2 · CUL4B · E2F6

Introduction

Transcriptional regulation, both activation and repression, occurs at promoter level and is controlled by the transcription factors (TFs) and functionally different epigenetic regulators. These transcriptional regulators form multi-protein complexes to make specific epigenetic marks on promoters of the target genes to regulate their expression. Activation

of transcription is mediated by various positive TFs like CREB, c-fos, c-jun, etc., and transcriptional co-activators such as CREB binding protein (CBP), E1A binding protein p300, and pCAF, which contains histone acetyltransferase (HAT) activity for relaxing chromatin structure to facilitate transcription [1–4].

Like activation, transcriptional repression is also regulated by various repression-specific epigenetic regulators (hereafter referred as repressors) and transcription factors. There are several repression-specific proteins which form selective repressive complexes depending on the cellular context to regulate target genes expression. The well-studied repressive complexes are Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2). PRC1 contains BMI-RING1B, CBX, and HPH proteins, and PRC2 contains EZH2, EED, SUZ12 and RbAp46 proteins. Histone 3 lysine 27 trimethylation (H3K27me3) is a major repressive mark mediated by EZH2 through its SET domain and maintained by PRC1 complex protein, CBX. These repressive complexes often function in cooperation with other functionally distinct repressors such as DNMT3A/B for promoter CpG

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12032-020-01395-5>) contains supplementary material, which is available to authorized users.

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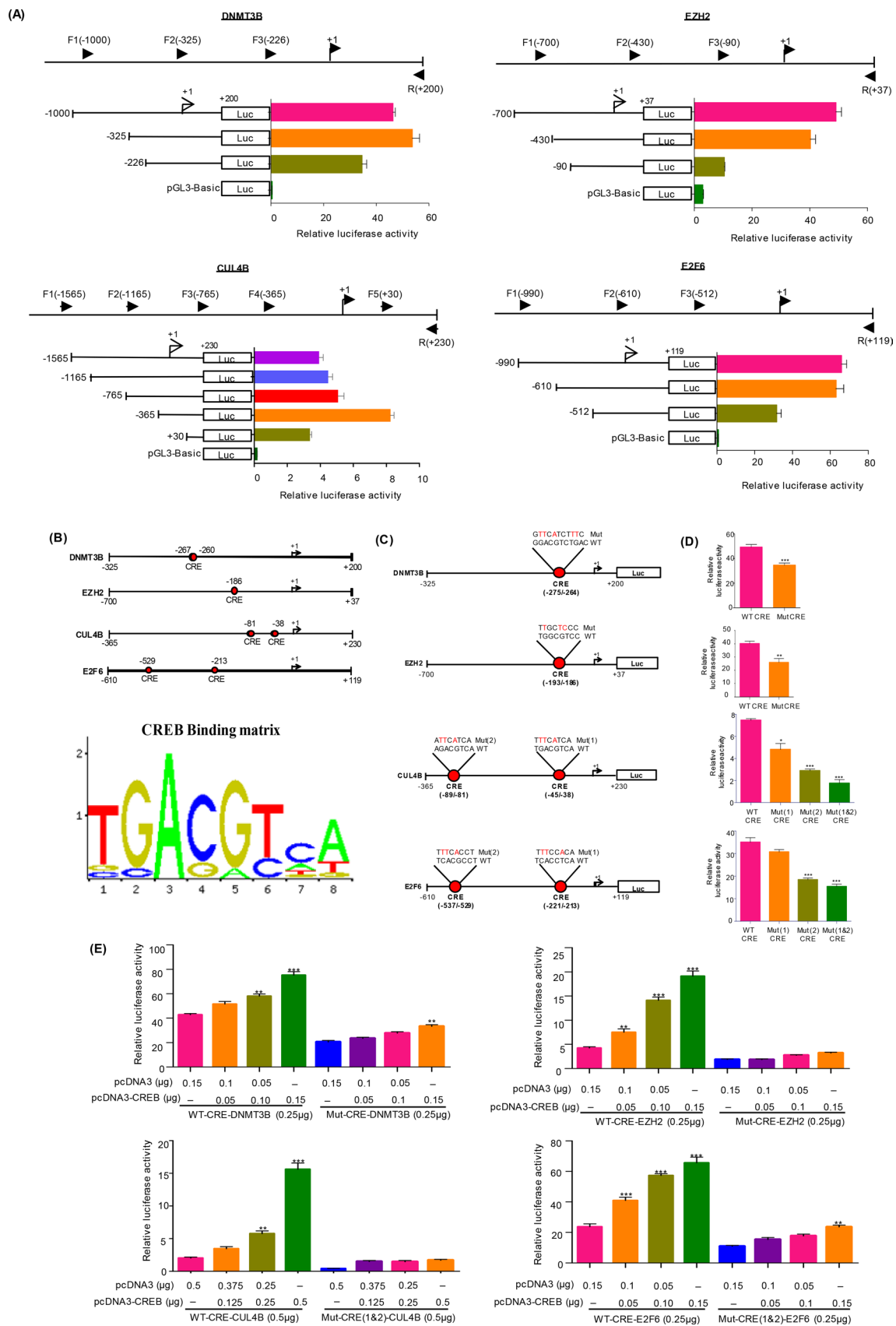


Fig. 1 Identification of CREs in the minimal promoter regions of repressors DNMT3B, EZH2, CUL4B and E2F6 and their transcriptional activation by CREB. **a** Schematic representation of positions of primers used in generating various deletions constructs of CUL4B, EZH2, DNMT3B and E2F6 promoters. HEK293 cells were co-transfected with repressor(s) specific promoter constructs cloned in luciferase vector and Renilla luciferase constructs. After 48 h of transfection, total cell lysates were assayed for luciferase activity and normalized with Renilla luciferase activity. Relative luciferase activities are expressed as mean \pm SE values ($n=3$). **b** Schematic representation of CREs on DNMT3B, EZH2, CUL4B, and E2F6 promoters as predicted by the JASPAR database and the consensus CRE motif sequences are shown below. **c** Schematic representation of CRE WT and mutant sequences and their position in the repressors promoter constructs. Mutated sequences in CREs are highlighted in red. **d** WT or mutant CRE minimal promoter constructs of repressors were co-transfected with Renilla luciferase and the luciferase activity was measured after 48 h of transfection. The relative luciferase values are shown as mean \pm SD ($n=3$). **e** HEK293 cells were co-transfected with WT or mutant CRE 1&2 repressors specific promoter constructs with increasing doses of pcDNA3-CREB plasmid or pcDNA3 as a control and Renilla luciferase constructs. The luciferase activities were measured and the relative luciferase values are shown as mean \pm SE values ($n=3$). ** $p < 0.01$ and *** $p < 0.001$. +1 represents TSS

methylation and E3 ubiquitin ligase CUL4B and RING1B of PRC1 for mono-ubiquitination of H2A at lysine 119 (H2AK119ub) for the efficient silencing of transcription [5, 6]. E2F6 and YY1 are the major TFs which interact with other co-repressors or co-activators at the promoter regions of the target genes to down-regulate or up-regulate their transcription, respectively [7, 8].

Deletion or repression of at least one of the repressor complex protein(s) affects the expression of other repressor(s) [9–11]. For example, CUL4B silencing not only reduces the levels of H2AK119ub but also H3K9 trimethylation, H3K27 trimethylation and DNA methylation [6]. Notably, the Cul4b null embryos phenotype is very similar to Dnmt3b and Ezh2 null embryos showing their functional link during embryogenesis [12, 13]. Similarly, silencing of DNMT3B not only reduces DNA methylation but also histone trimethylation which is mediated by EZH2 [9].

While the functions of major repressors such as DNMT3B, EZH2, CUL4B and E2F6 has been well studied, there is very limited information available on how these repressors are regulated at their transcriptional level. Importantly, these epigenetic repressors (DNMT3B, EZH2, CUL4B and E2F6) are also known to be overexpressed in various cancer types, suggesting an oncogenic function for these repressors [14, 15], but the exact molecular mechanism is still not elucidated. In this study, we report that cyclic AMP response element-binding protein (CREB) up-regulates the transcription of these major repressors.

The CREB is a well-studied signaling molecule shown to be activated in almost all cancer types and under various conditions. Phosphorylation at serine 133 of CREB by

numerous cytoplasmic kinases such as protein kinase A (PKA), protein kinase B (PKB/Akt), mitogen activated protein kinases (MAPKs), p90 ribosome S6 kinase (pp90RSK) etc. activates its transactivation functions [16]. Phosphorylated CREB (pCREB) interacts with major transcriptional co-activators; p300/CBP/PCAF to control the transcriptional initiation and elongation [17]. The pCREB is dephosphorylated by various phosphatases such as protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and phosphatase and tensin homolog (PTEN) [18–20] to inactivate CREB functions. Negative regulators such as RGS13 (Regulator of G protein Signaling 13), DaXX and DREAM are also reported to reduce the CREB-mediated transactivation function [21, 22].

The important CREB-regulated genes include cell cycle regulators (Cyclin D, Cyclin E, etc.), basal transcription factors (TFs) (TFIIB, TFIID, etc.), anti-apoptotic genes (Bcl2, etc.) and metabolic genes (glucose-6-phosphatase, etc.) [23–27]. Moreover, CREB is also shown to interact with other TFs such as MyoD, YY1, Smad3, etc. and tumor suppressors like PTEN, p53, BRCA1, etc. to regulate the gene expression [28–30].

Genome wide analysis predicted that there are nearly four thousand genes whose promoters contain CREB binding motif CRE [31]. Though CREB-mediated transcriptional regulation is known for several decades, how it regulates both positive and negative regulators in normal and cancer cells is still a mystery. Moreover, the mechanism by which CREB regulates these regulators is not well studied. In this investigation, we provide experimental evidences that pCREB binds to the promoters of major epigenetic repressors, DNMT3B, CUL4B, EZH2 and E2F6 through CRE motifs and induce their transcription. These findings suggest that CREB could be exploited as a therapeutic target for cancer therapy.

Results

Identification of CREs in the minimal promoter regions of the repressors; DNMT3B, EZH2, CUL4B and E2F6 and their transcriptional activation by CREB

Though the functional role(s) of the major epigenetic repressor(s) proteins; DNMT3B, EZH2 and CUL4B for their repressive marks on target genes are quite well studied, transcriptional regulation of these repressor is poorly understood. To investigate this, first, we generated several promoter deletion constructs for DNMT3B, EZH2, CUL4B and also for TF, E2F6 with different lengths with respect to their transcription start site (TSS) (+1) in the luciferase reporter vector, pGL3 basic (Fig. 1a). These constructs were

co-transfected with renilla luciferase in HEK293 cells and their promoter strength was measured by quantifying the relative luciferase activity. Figure 1a shows that the region encompassing -325 to $+200$ of DNMT3B, -365 to $+230$ of CUL4B, -700 to $+37$ of EZH2 promoter constructs exhibited maximum luciferase activity as compared to other promoter deletion constructs of the same repressor gene(s). The longer promoter fragments of CUL4B (i.e., -1565 , -1165 and -765) showed relatively less luciferase activity than the smaller construct; -365 to 230 , which showed the maximum luciferase activity (Fig. 1a). This could be due to some unknown repressors occupancy on the regions encompassing -365 to -1565 . For E2F6, the -990 and -610 promoter constructs showed the maximum luciferase activity (Fig. 1a) and the -610 region was used in further experiments. Similarly the promoter constructs of -325 of DNMT3B and -700 of EZH2, which showed the maximal luciferase activities were considered as minimal promoter regions and they were used in further experiments. The schematic of promoter deletion constructs of repressors are shown on top of Fig. 1a.

Next, we predicted the putative and also a common transcription factor binding site(s) within the identified minimal promoter regions of these repressors using two independent computational prediction programs (JASPAR & TF SEARCH) and found the high-affinity binding motifs (CREs) for the transcription factor, CREB (cyclic AMP responsive element-binding protein) in all the four repressors; DNMT3B, EZH2, CUL4B, and E2F6 (Fig. 1b). There are two putative CREs in CUL4B and E2F6 and one CRE in EZH2. Notably, the DNMT3B contains 2 putative CREs adjacent to each other; -260 and -267 positions. The position of CREB binding motifs (CREs) on these repressors promoters and the consensus CRE sequences are shown as schematic in Fig. 1b. The comparison of known CREB target genes CREs motifs with DNMT3B, EZH2, CUL4B and E2F6 CREs is shown in Supplementary Table 1. Hence, we speculated that CREB could be the common transcription factor responsible for the transcription of these repressors. To confirm this, we mutated the putative wild type (WT) CRE sequences by site directed mutagenesis (SDM) (Fig. 1c) and performed the luciferase based promoter reporter assay as mentioned above. As expected, the mutations in the putative CRE regions of the minimal promoters of DNMT3B, EZH2, CUL4B, and E2F6 repressors significantly reduced the luciferase activity as compared to WT-CRE (Fig. 1d). Since CUL4B and E2F6 contain two CREs designated as CRE1 and CRE2 as shown in the schematic (Fig. 1b), we mutated these CREs individually or together by SDM and performed the luciferase assay. We observed a significant reduction of luciferase activity in mutated CREs (Mut 1 and/or Mut 2) of CUL4B. The reduction of luciferase activity of CREs of CUL4B which carry mutations in

both CRE is higher than the CREs which carry individual mutation indicating that both CREs are need for the higher level of transcription of CUL4B (Fig. 1d). In case of E2F6, mutation in CRE1 did not reduce the luciferase activity significantly whereas the mutation in the CRE2 reduced the luciferase activity strongly (Fig. 1d) suggesting that CRE2 of E2F6 is regulated by CREB.

To test whether CREB could induce the transcription of repressors, we performed the co-transfection in HEK293 using CREB expression plasmid and minimal promoter construct of repressors with WT or Mut CRE and measured the luciferase activity. A dose dependent increase of luciferase activity was seen in WT-CRE whereas the Mut CRE did not show any significant increase in the luciferase activity as compared to controls (Fig. 1e). These data clearly indicate that CREB binding on CREs of these repressors is essential for their transcriptional activation.

pCREB binds to CRE motifs of DNMT3B, EZH2, CUL4B and E2F6 promoters and positively regulate their transcription.

To study the promoter occupancy of CREB on repressors promoters, we performed chromatin immunoprecipitation (ChIP) assay from HeLa cells using phospho CREB (pCREB) specific antibody and measured the strength of binding of pCREB to the selected promoter regions of repressors by real-time RT-PCR using primers. Primers were designed for both CRE and non CRE regions for each of these repressors to study the pCREB binding (Fig. 2a). ChIP data reveal that the endogenous pCREB bind on CREs regions at much higher levels of all four repressors promoters as compared to IgG controls (Fig. 2b). However, the binding of pCREB for each of these repressors are different (Fig. 2b). We observed a fold change of 6.2, 3.9, 5.3 and 5.1 for CUL4B, DNMT3B, EZH2 and E2F6 respectively in the promoter DNA amplification of pCREB ChIP DNA as compared to IgG control DNA (Fig. 2b). Notably, in E2F6 promoter, the CRE1 did not show any CREB binding which also support our luciferase data (Fig. 1d). This observation confirms that pCREB physically associates on CRE regions at the promoters of these repressors and regulate their transcription.

Next, we analyzed the expression levels of CREB induced repressors upon the overexpression or knock down of CREB. Different cancer cell lines; HeLa, MCF7, A549 and HEK293 were transfected with CREB overexpression cassette and measured the expression levels of these repressors both at transcripts and proteins by real-time qRT-PCR and immunoblotting, respectively.

As expected, both the mRNA and protein levels of DNMT3B, EZH2, CUL4B and E2F6 repressors are significantly higher in CREB overexpression conditions across all

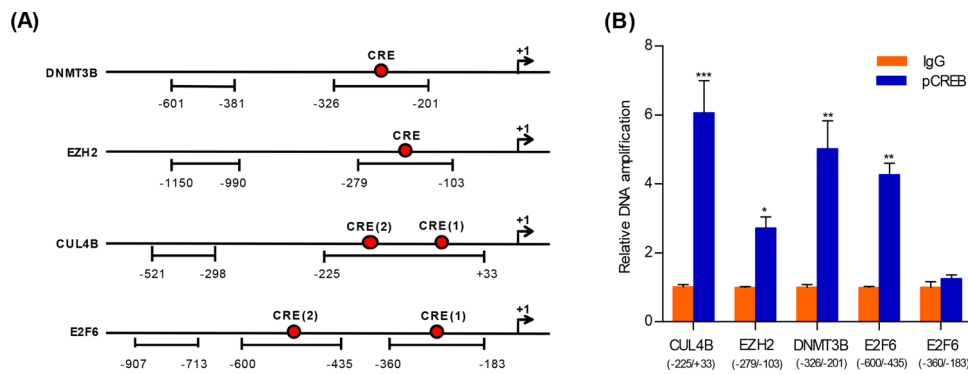


Fig. 2 pCREB binds to CRE motifs of DNMT3B, EZH2, CUL4B and E2F6 promoters and positively regulate their transcription. **a** Schematic representation of CREs on DNMT3B, EZH2, CUL4B, and E2F6 promoters and the primer positions used in ChIP assays. +1 represents TSS. **b** Chromatin were prepared from HeLa cells and sub-

jected to ChIP assay using pCREB antibody followed by quantifying the promoter-bound DNA fragments using specific primers designed at specific regions of repressors promoters as shown above. The fold change of pCREB versus control IgG promoter DNA amplification is shown as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

the tested cancer cell lines (Fig. 3a; left and right). Silencing of CREB expression using CREB specific siRNAs in these cell lines showed the reduction of these repressors levels both at transcript and protein as expected (Fig. 3b; left and right). We previously reported that CREB up-regulates CUL4A both at transcript and protein levels [32]. Here, we identified its paralog CUL4B is also regulated by CREB. The activation of these major epigenetic repressors by CREB was seen in different cancerous cell lines; HeLa, MCF7, A549 and HEK293 indicating that CREB up-regulates these repressors at promoter levels which is not cell line specific. We observed that the levels of pCREB and neddylated active form of CUL4B also increased upon the overexpression of CREB and decreased during the silencing of CREB. In addition, we studied the effect of CREB induced expression of CUL4B and EZH2 repressors on their functional outcomes by studying H2AK119ub and H3K27me3 modifications, respectively. The overexpression of CREB increased and the silencing of the CREB reduced H2AK119ub and H3K27me3 modifications in all the tested cancer cell lines indicating CREB regulates the functions of these repressors by activating their transcription (Fig. 3a and b right).

In addition, we predicted CRE motifs in several other repressors such as DDB1, SUZ12, EED and RbAp46. DDB1 is a part of CUL4A/B complexes and SUZ12, EED and RbAp46 are part of EZH2 containing PRC2 complexes (Fig. 3c; left panel). As observed with major repressors, CREB overexpression also increased the levels of DDB1, SUZ12, EED and RbAp46 transcripts significantly as compared to control (Fig. 3c; right panel) indicating that CREB could regulate several other repressors promoters in a positive manner by binding to the CRE(s).

We also predicted the CRE motifs in other functionally related repressors such as DNMT3A and EZH1 and analyzed their transcripts levels upon CREB overexpression

condition by real-time RT-PCR using specific primers. Though the prediction indicated the presence of a CRE on DNMT3A promoters within -1000, we found no significant change in their transcript levels as compared to controls in CREB overexpressing cells (Fig. 3d) indicating that DNMT3A is not regulated by CREB. Surprisingly, the EZH1; homolog of EZH2 which contains three CREs in its promoter (Fig. 3d left panel) showed the significant reduction in its transcripts levels in CREB overexpression condition instead of activation (Fig. 3d right panel). The down-regulation of EZH1 by CREB indicates that CREB could function as a repressor.

Activation of CREB by K-RAS induces the expression of DNMT3B, EZH2, CUL4B and E2F6.

To further confirm our findings, we overexpressed K-RAS; a well known upstream regulator of CREB activation and studied the expression of these major repressors. For this, we overexpressed WT K-RAS and the mutant K-RAS (G13D, hyper active variant) in HEK293, HeLa, MCF7 and A549 cells and analyzed the protein levels of CREB and pCREB and its induced repressors proteins by immunoblotting using specific antibodies (Fig. 4).

As expected, the pCREB levels were more in K-RAS overexpression conditions as compared to control indicating that CREB is activated by K-RAS (Fig. 4). We observed the up-regulation of the CREB induced repressors proteins DNMT3B, EZH2, CUL4B and E2F6 in K-RAS overexpression conditions (Fig. 4). The mutant K-RAS (hyperactive form) overexpression showed higher levels of pCREB and repressors proteins as compared to WT-K-RAS as expected (Fig. 4). These effects were observed in all the tested cancer cells suggesting that it is not cell line specific.

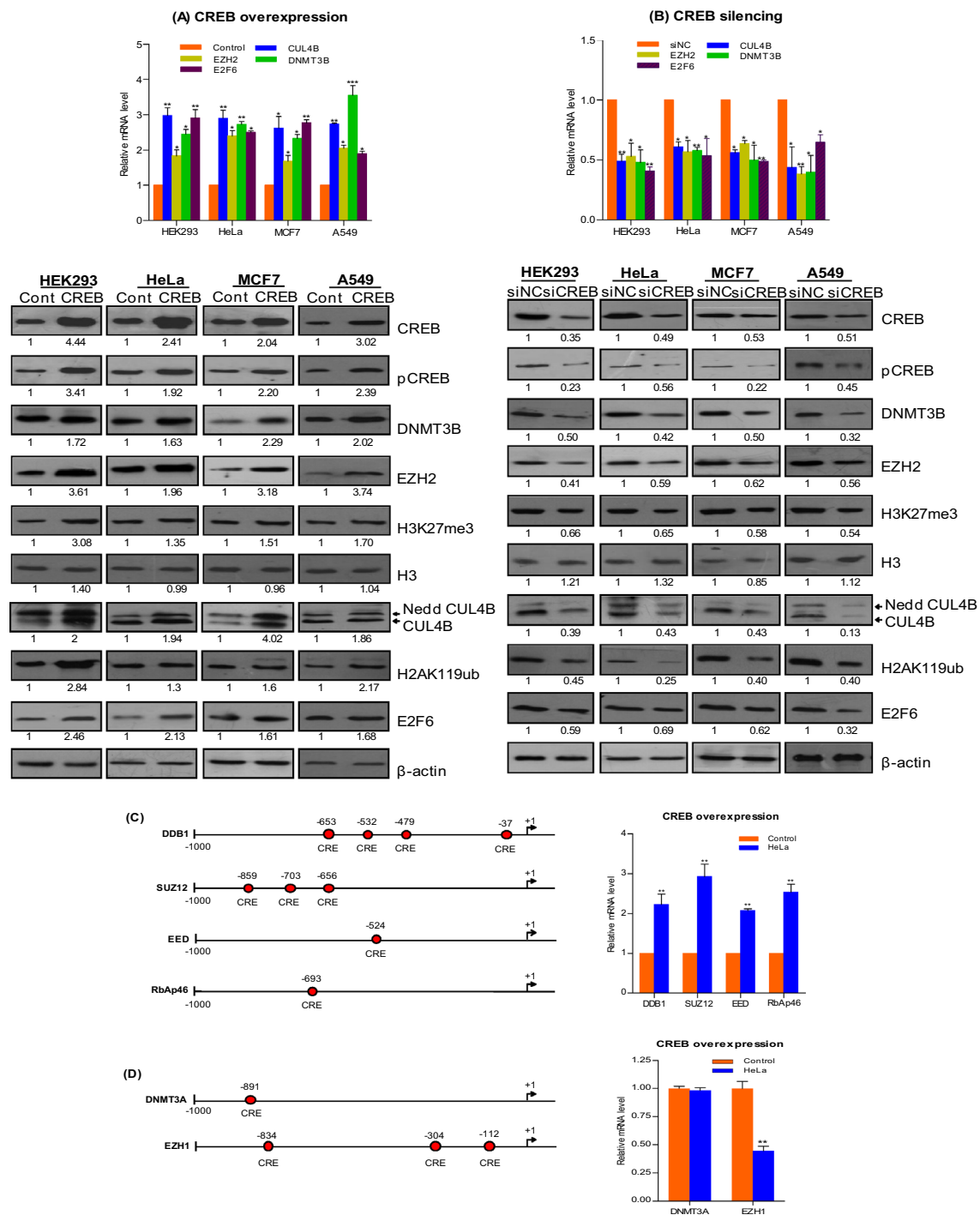
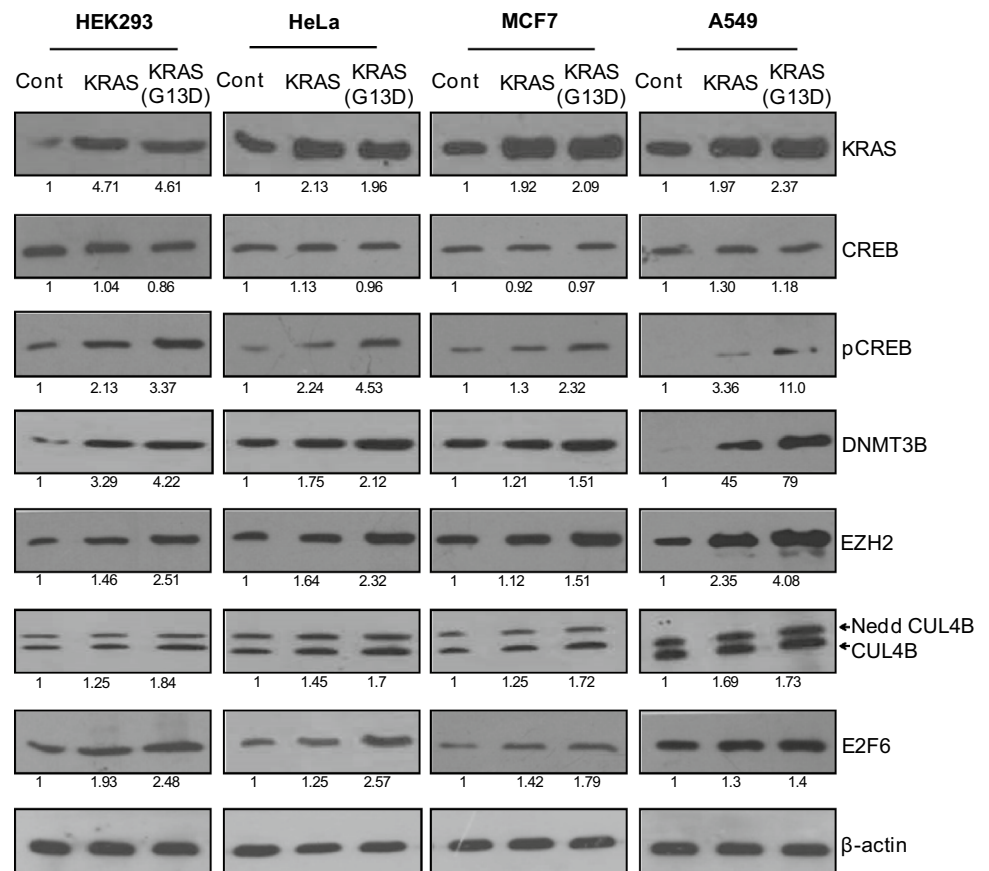


Fig. 3 Effect of CREB on CUL4B, EZH2, DNMT3B, and E2F6 expression. HEK293, HeLa, MCF7 and A549 cells were transfected with CREB overexpression construct or pcDNA3 **a** and CREB specific siRNA or control siRNA **b** and the total RNA and proteins were extracted. qRT-PCR was performed from CREB overexpressed or silenced conditions using specific primers and normalized to GAPDH. Immunoblotting was performed from same conditions using specific antibodies as mentioned in the figure. The β-actin was used as an internal loading control. **c** Schematic representation of CREs on DDB1, EED, SUZ12, and RbAp46 promoter regions as predicted

by the JASPAR database (left). qRT-PCR of DDB1, EED, SUZ12, and RbAp46 transcripts from CREB overexpressed HeLa cells using specific primers (right). **d** Schematic representation of CREs on DNMT3A and EZH1 promoter regions as predicted by the JASPAR database (left). The transcript level of DNMT3A and EZH1 from CREB overexpressed HeLa cells by qRT-PCR using specific primers (right). The expression level of specific gene(s) was normalized with GAPDH and expressed as fold change ± SD (*n* = 3). **p* < 0.05, ***p* < 0.01 and ****p* < 0.001

Fig. 4 Activation of CREB by K-RAS induces the expression of DNMT3B, EZH2, CUL4B and E2F6. HEK293, HeLa, MCF7, and A549 cells were transfected with pcDNA3 or pcDNA3-WT-KRAS or pcDNA3-mut-KRAS (G13D) and the total cell lysates were prepared after 48 h of transfection. Equal amounts of cell lysates were resolved on SDS-PAGE and immunoblotted using specific antibodies as indicated. β -actin was used as an internal loading control



Inhibition of CREB activation by PTEN reduces the expression of DNMT3B, EZH2, CUL4B and E2F6

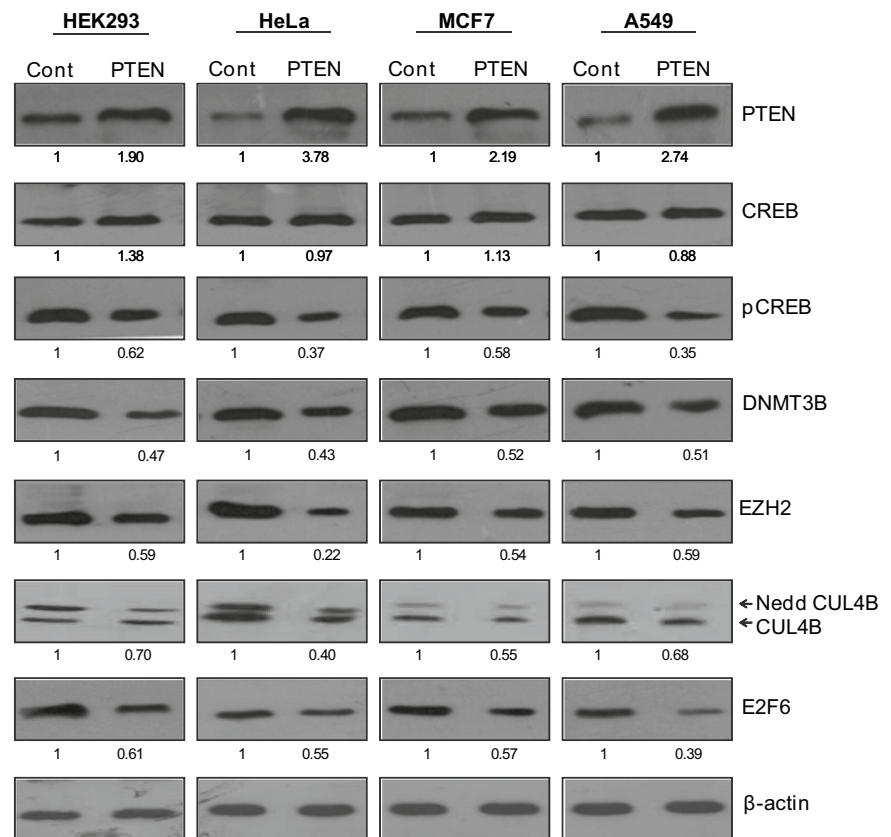
PTEN, a well-studied TSG protein is a negative regulator of CREB through direct physical interaction of CREB, which removes S133 phosphorylation of CREB through its phosphatase activity and inhibits CREB-mediated downstream signaling [18]. To validate our findings that these repressors are indeed trans-activated by CREB, we studied effect of inhibition of CREB activation by PTEN in different cell lines (HeLa, MCF7 A549 and HEK293). To check this, we overexpressed PTEN and analyzed the protein levels of pCREB and CREB induced repressors by immunoblotting using specific antibodies (Fig. 5). As expected, we observed the reduced levels of pCREB and repressors proteins; DNMT3B, EZH2, CUL4B and E2F6 in PTEN overexpression conditions as compared to control (Fig. 5). This further confirms that CREB activation is critical for the transactivation of these repressors and the effect is not cell line specific.

Discussion

CREB and epigenetic repressors are reported to be overexpressed in variety of cancer types showing their possible functional link in terms of cellular proliferation. Understanding the importance of epigenetic regulators which control both activation and repression of genes involved in several cellular processes including proliferation and differentiation is needed and the limited information is available in these aspects. Here, our findings show that CREB acts as a positive and a common transcription factor for major repressors; DNMT3B, EZH2 and CUL4B and its associated TF E2F6.

Though our bioinformatics prediction identified several putative TFs binding sites within the promoter regions of DNMT3B, EZH2, CUL4B, and E2F6, we mainly focused on CREB because it was the only common TF binding site (CRE motif) present in all the four investigated repressors. Our promoter analysis on the repressors using reporter assay revealed that WT-CRE is essential for CREB binding and

Fig. 5 Inhibition of CREB activation by PTEN reduces the expression of DNMT3B, EZH2, CUL4B and E2F6. Cells (HEK293, HeLa, MCF7, and A549) were transfected with pcDNA3 or pcDNA3-PTEN and the total cell lysates were prepared after 48 h of transfection. Equal amounts of cell lysates were resolved on SDS-PAGE and immunoblotted using specific antibodies as indicated. β -actin was used as an internal loading control



for its transcriptional activation. It is reported previously that heterodimers of CREB with ATF family, CREB with CREM and ATF2-c-jun binds to the CREs of the CREB target genes [33, 34]. At present, we do not know whether ATF related TFs; is also involved in the transcriptional regulation of these repressors. To rule out this possibility, future studies are needed. In this study, we focused the roles of CREB exclusively on the expression of repressors.

Our ChIP assay data clearly indicate that pCREB occupy on the CRE regions of all these repressors. Among repressors studied here, only CUL4A (paralog of CUL4B) is regulated by CREB [32], whereas DNMT3A and EZH1 (the homologs of DNMT3B and EZH2, respectively) are not regulated by CREB despite the fact that EZH1 contains 3 CREs on their promoters (at -112 , -304 , and -834) and DNMT3A contains one CRE at -1000 position (Fig. 3d). Our immunoblotting experiments showed an increased levels of H3K27me3 and H2AK119ub repressive marks in CREB overexpressed cells, where pCREB levels are high; and the reverse was observed during CREB silencing/inhibition conditions (Fig. 3). This observation clearly indicates that CREB induced EZH2 and CUL4B are responsible for the increase of H3K27me3 and H2AK119ub, respectively.

DNMT3 family of enzymes are well studied in terms of their CpG methylation particularly in TSGs promoters. DNMT3B is important for X-chromosome inactivation,

development and differentiation, here we show that CREB positively regulate DNMT3B but not DNMT3A indicating that the CREB has strong link with DNA methylation by DNMT3B. Earlier report shows that SP1, SP3 and HOXB3 regulate the DNMT3B transcription [35, 36], here our data show that DNMT3B is also regulated by CREB. Similarly, EZH2 is also well characterized protein for its repressive mark H3K27me3 to target gene silencing. EZH2 transcription is regulated by several transcription factors including ETS, ELK, etc. [37–40]. and here we show that CREB also regulate the EZH2 expression positively. SOX4 is the only TF which is known to regulate the CUL4B transcription [41] and here we show the regulation of CUL4B expression by CREB. E2F1 and NRF-1/ α -PAL are reported to regulate E2F6 transcription [42, 43] and our findings show CREB also regulates the E2F6 expression.

CREB; a major positive transcription factor is predicted to bind on more than four thousands of genes promoters via its binding motifs (CREs) [31]; but so far less than 1% of the targets have been studied in details.

This study provides experimental evidences that how CREB activation regulates the epigenetic repressors positively. Based on this and earlier findings, we speculate that the CREB induced repressors investigated in this study could regulate TSGs negatively at promoter levels to maintain continuous cell proliferation of cancer cells and this part of

investigation is underway. Thus our study strongly suggests that CREB could be a potential drug candidate to inhibit cancer cell proliferation.

Materials and methods

Cell culture

Human cell lines; HEK293 (embryonic kidney cells), HeLa (cervical cancer), MCF7 (Breast cancer), A549 (adenocarcinoma human alveolar basal epithelial cancer) and HCT116 (colon carcinoma) were purchased from National Centre for Cell Sciences (NCCS), Pune, INDIA and cultured in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin at 37 °C under 5% CO₂.

Generation of cDNA clones, promoter deletion constructs and site directed mutagenesis

To generate the full-length cDNA clone(s) of specific gene(s): CREB, WT-KRAS, and PTEN, HEK293 cDNAs were PCR amplified using gene specific primers and cloned in frame into pcDNA3 or pEGFP-C1 vectors between HindIII and BamHI sites. HCT116 cDNAs was used to amplify the mutant-K-RAS (G13D) and cloned at HindIII and BamHI sites of pcDNA3. The clones were confirmed by sequencing using vector or gene specific primers. The primers sequences are listed in Supplementary Table-2.

To generate promoter constructs, human CUL4B (Gene ID: 8450), EZH2 (Gene ID: 2146), DNMT3B (Gene ID: 1789), and E2F6 (Gene ID: 1876) promoters sequences were retrieved from the Eukaryotic Promoter Database (EPD) (<https://epd.vital-it.ch/>). Different lengths of promoters of repressors genes with respect to their putative transcriptional start site (TSS, +1) were PCR amplified from HEK293 genomic DNA using specific primers. The PCR amplified promoter regions of CUL4B and EZH2 were inserted into pGL3 basic vector (Promega) at XhoI and HindIII site and DNMT3B and E2F6 promoters at XhoI and KpnI sites. The primers used in promoter constructs are listed in Supplementary Table-3.

To introduce the point mutations in the CREB binding motifs (CREs) of CUL4B, EZH2, DNMT3B and E2F6 promoters at specific residue as mentioned in the figure, the Quick Change Site-Directed Mutagenesis Kit (Agilent technologies) was used according to manufacturer's instruction. The WT minimal promoters of repressors were used as template to generate CRE mutants (CRE1 or CRE2) and the double mutant (CRE1 and 2) clones were generated using single mutant clone as template. All SDM were confirmed by DNA sequencing. The details of the oligonucleotides are listed in Supplementary Table- 4.

Transfection

Cells (HEK293, HeLa, MCF7, and A549) were freshly cultured for 12 h and transfected with various constructs using TurboFect Transfection Reagent (Thermo Fisher Scientific) and harvested at different time points for RNA or protein isolation. The transfection conditions were optimized to yield of > 80% transfection with > 85% of cell viability. For siRNAs, siCREB or control SiNC were purchased from Santa Cruz Biotechnology and transfected using Lipofectamine RNAiMax (Invitrogen) as per manufacturer's instruction.

Computational analysis

TFSEARCH (<https://diyhpl.us/bryan/irc/protocol-online/protocolcache/TFSEARCH.html>) and JASPAR databases (<https://jaspar.binf.ku.dk/>) were used for the prediction of putative TF binding sites on CUL4B, DDB1, EZH1, EZH2, SUZ12, EED, RbAp46, DNMT3A, DNMT3B, E2F6 promoters.

Luciferase reporter assays

HEK293 cells (0.05×10^6) were seeded in 24-well plates and cultured for 12 h. The cells were co-transfected with different promoter constructs: CUL4B, EZH2, DNMT3B, and E2F6 with pRL-TK Renilla luciferase plasmid (Promega), harvested at 48 h and lysed in passive lysis buffer (Promega). The firefly luciferase activities were measured in a luminometer (Berthold-Centro LB 960) and the values were normalized to Renilla luciferase activities. The relative values are represented as mean \pm SD of triplicates from a representative experiment.

Immunoblotting

Cells were trypsinized, pelleted and washed twice with PBS to remove residual medium. Then, the cells were lysed in RIPA lysis buffer (1% (w/w), NP40, 0.5% (w/v) Sodium deoxycholate, 0.1% (w/v) SDS, 0.5 M NaCl, 5 mM EDTA and 1 mM DTT, and 1X protease inhibitor cocktail) for 30 min and centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant containing proteins was transferred and used immediately or kept at -80 °C for future use. The protein concentration was estimated using Bradford assay. Equal amounts of protein samples were mixed with 6X loading dye, resolved on 10% SDS–Polyacrylamide gel and transferred onto PVDF membrane (GE Healthcare Life Sciences). The blots were blocked in 5% BSA for 1.5 h at RT followed by three washes in 1X TBST. The blot was incubated with specific primary antibodies for 1 to 2 h with gentle agitation and washed thrice with 1X TBST followed by corresponding secondary antibodies conjugated with HRP (Bio-Rad)

incubation for 45 min at RT. Finally, the blots were washed as mentioned above and incubated with ECL reagent (Bio-Rad) and detected using X-ray film. The primary antibodies used were: anti CREB, pCREB, EZH2, PTEN and GAPDH (Cell Signaling Technology); and K-RAS (Santa Cruz); anti CUL4B, and β -actin (Sigma-Aldrich); anti DNMT3B3B and E2F6 (abcam); anti H3K27me3, Trimethyl H3 and H2AK119ub1 (Millipore). The band was quantified using Image J software and normalized to the corresponding beta-actin.

RNA extraction, cDNA preparation and qRT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, USA) and quantified at 260 nm using spectrophotometer NanoDrop™ 2000 (Thermo Scientific). cDNAs were prepared from total RNA (2 μ g) using MMLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, USA). qRT-PCR was performed from cDNAs using gene specific primers and FastStart Essential DNA Green Master (Roche) in *LightCycler 96* Real Time PCR System (Roche Life Science). The reaction cycles were: denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 20 s, and final extension at 72 °C for 7 min. The primers details are provided in Supplementary Table-5. The fold change was calculated with GAPDH values and the relative expression is expressed as mean \pm SD of triplicates.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP assay kit (Millipore, Billerica, MA). Briefly, HeLa cells (1×10^6) were plated on 10 cm dish and cultured. After 24 h, the cells were treated with 1% formaldehyde and incubation continued for 10 min at 37 °C. Then, the cells were washed with ice-cold 1X PBS containing protease inhibitors (1 mM phenylmethylsulfonylfluoride-PMSF, 1 μ g/ml aprotinin and 1 μ g/ml pepstatin A) and centrifuged. Following this, SDS lysis buffer was added to the cells, incubated on ice and sonicated for 4 rounds of 30 s ON/30 s OFF (Diagenode bioruptor sonicator) to shear chromatin to 250–500 base pairs. The chromatin was precleared with 60 μ l of protein G agarose for 1 h followed by specific antibodies or rabbit IgG incubation for overnight at 4 °C. Next day, 60 μ l of protein G agarose beads were added and incubation continued for another 4 h and the immune complexes were eluted from the beads and reverse cross-linked using 5 M NaCl. The chromatin bound DNA was purified using Qiagen PCR cleanup kit and amplified using promoter specific primers by qRT-PCR. The conditions were 95 °C for 5 min; 33 cycles at 95 °C for 15 s, 58 °C for 15 s, and 72 °C

for 20 s, and final extension at 72 °C for 7 min. The details of the primers are listed in Supplementary Table-6.

Statistical analysis

All experiments were carried out three times and results were expressed as mean \pm SD of three independent samples. Statistical significance was calculated by one-way/two-way ANOVA with Bonferroni multiple-comparison post-test. For single comparison between two groups, Student's t test was used. *p* value of <0.05 was considered as significant.

Acknowledgements We thank the members of Prof. Sudhakar Baluchamy laboratory for the technical assistance and helpful discussion. We are grateful to Dr. Arunkumar Dhayalan for valuable suggestions and critical reading of the manuscript. This study was supported by DBT (Department of Biotechnology) INDIA; 6242-P78/RGCB/PMD/DBT/BSKR/2015 and SERB (Science and Engineering Research Board) INDIA; SB/EMEQ-038/2013 to Prof. Sudhakar Baluchamy. Fellowships from CSIR-UGC to CA, UGC-NFOBC to MS, DST-SERB-NPDF (2739) to Dr. SP are greatly acknowledged.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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