

# CDK5RAP3 Is a Novel Repressor of $p14^{ARF}$ in Hepatocellular Carcinoma Cells

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## Abstract

CDK5 regulatory subunit associated protein 3 (CDK5RAP3) is a novel activator of PAK4 and processes important pro-metastatic function in hepatocarcinogenesis. However, it remains unclear if there are other mechanisms by which CDK5RAP3 promotes HCC metastasis. Here, we showed that in CDK5RAP3 stable knockdown SMMC-7721 HCC cells,  $p14^{ARF}$  tumor suppressor was upregulated at protein and mRNA levels, and ectopic expression of CDK5RAP3 was found to repress the transcription of  $p14^{ARF}$ . Using chromatin immunoprecipitation assay, we demonstrated that CDK5RAP3 bound to  $p14^{ARF}$  promoter *in vivo*. Furthermore, knockdown of  $p14^{ARF}$  in CDK5RAP3 stable knockdown HCC cells reversed the suppression of HCC cell invasiveness mediated by knockdown of CDK5RAP3. Taken together, our findings provide the new evidence that overexpression of CDK5RAP3 promotes HCC metastasis via downregulation of  $p14^{ARF}$ .

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## Introduction

CDK5RAP3 (also called C53/LZAP) was first identified as a binding partner of cyclin-dependent kinase 5 (CDK5) activator,  $p35^{nek5a}$ , in yeast two-hybrid screening [1]. Several studies have investigated the potential roles of CDK5RAP3 in carcinogenesis, but so far its definite roles remain controversial. CDK5RAP3 has been found to promote apoptosis induced by genotoxic stress in HeLa cells by triggering  $G_2/M$  arrest [2]. In addition, CDK5RAP3 has been proposed to be a tumor suppressor because it inhibits the NF- $\kappa$ B cell survival pathway and its protein level is significantly underexpressed in head and neck squamous cell carcinomas [3]. CDK5RAP3 can interact with a well-known tumor suppressor, namely alternate reading frame ( $p14^{ARF}$ ), by which stabilizes and promotes the transcription activity of p53 [4]; yet, the role of this interaction in carcinogenesis has not been explored.

More recently, we demonstrated that *CDK5RAP3* is frequently overexpressed in human HCCs and contributes to HCC metastasis by activating PAK4 [5]. CDK5RAP3 has 2 putative LXXLL motifs, which are the signature motifs for transcriptional co-regulators, mediating the binding on nuclear receptors. In addition, CDK5RAP3 has a leucine zipper domain, which is a structural motif for protein dimerisation and is commonly found in proteins involving in gene expression. Previous study has shown that CDK5RAP3 can associate with a nuclear co-activator, cAMP response element-binding protein (CREB)-binding protein (CBP) [6], suggesting that CDK5RAP3 may also function as a transcriptional co-activator/repressor. Here, we provide evidences that

CDK5RAP3 is a putative transcriptional suppressor of  $p14^{ARF}$  and overexpression of CDK5RAP3 promotes to the metastasis of HCC by downregulating  $p14^{ARF}$ . Thus inhibition of CDK5RAP3 can potentially be used to restore the expression of the important tumor suppressor  $p14^{ARF}$  expression, providing new molecular targets for the therapeutic intervention in HCC and possibly other cancers.

## Materials and Methods

### Antibodies

Anti-CDK5RAP3 antibody was reported previously [5]. Rabbit anti-GFP (FL), mouse anti-Myc (9E10), rabbit anti-Myc (A-14), mouse anti-p53 (DO-1), mouse anti-phospho-p53 (S15) and rabbit anti-MDM2 (C-18) were purchased from Santa Cruz Biotechnology. Rabbit anti- $p14^{ARF}$  was purchased from Abcam. Mouse anti- $\beta$ -actin (AC15) was purchases from Sigma-Aldrich.

### Plasmids

The  $p14^{ARF}$  promoter luciferase reporter construct, pGL3- $p14^{ARF}$ -luc, and the truncation mutants generated from pGL3- $p14^{ARF}$ -luc were generous gifts from Dr. Kiyoshi Ohtani [7]. Plasmid expressing Myc-CDK5RAP3 was reported previously [5] and pEGFP- $p14^{ARF}$  was constructed by subcloning full length of  $p14^{ARF}$  cDNA fragment (clone IMAGE: 6173590) into pEGFP vector (Clontech, Palo Alto, CA). HA-E2F1 expression plasmid was obtained from Addgene (Sellers *et al.*, 1998, Addgene plasmid 10736).

## Cell Culture

Human hepatoma cell line HepG2 and monkey kidney fibroblast cell line COS7 were purchased from ATCC. Human HCC cell line SMMC-7721 was gift from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences [8]. Cells were maintained in DMEM high glucose (Life Technologies, Rockville, MD) supplemented with 1 mM sodium pyruvate and 10% heat-inactivated FBS (JRH Biosciences, Lenexa, KS). Cells were transfected with DNA constructs using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

## siRNA Oligonucleotide Transfection

CDK5RAP3 small interfering RNA (siRNA) duplex was transfected into cells as described [5]. siRNA duplex targeting  $p14^{ARF}$  5'-GCGGAAGGUCCUCAGACAUU-3' (sense strand) and non-targeting siRNA duplex negative control, 5'-UAAGGCUAUGAAGAGAUAC-3' (sense strand) were purchased from Dharmacon, Inc, Lafayette, CO.

## Reverse Transcription-PCR (RT-PCR)

Quantitative real-time PCR (qPCR) was performed as described [9]. The sequence of taqman probes (Applied Biosystems) for *CDK5RAP3* and  $p14^{ARF}$  are 5'-AGGAAAGATGGAGGACCATCAGCAC-3' and 5'-TAGAAGACCAGGTCATGATGATGGG-3' respectively; cellular 18S rRNA was used as an internal control.

## Cell Migration and Invasion Assay

Transwell assay and invasion assay were performed as described [10,11], respectively. The cells were allowed to migrate for 16 hours and invade for 24 hours, respectively. A total of 5 fields were counted for each filter. The experiments were performed three times independently.

## Confocal Microscopy

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X100 [9]. Images were captured by Carl Zeiss LSM510 (HKU Faculty core facility).

## Luciferase Reporter Assay

Cells cultured in 24-well plates were transfected with Myc-CDK5RAP3 in addition to 100 ng of  $p14^{ARF}$ -luc or p53-luc, and 10 ng of pRL-CMV. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI), and light emission was quantified using a microplate luminometer (MicroLumat PLUS, Perkin-Elmer, Shelton, CT).

## Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP Assay Kit (Millipore, Billerica, MA). CDK5RAP3 stable overexpression clone #2 HepG2 cells ( $1 \times 10^7$ ) were used for the assay.

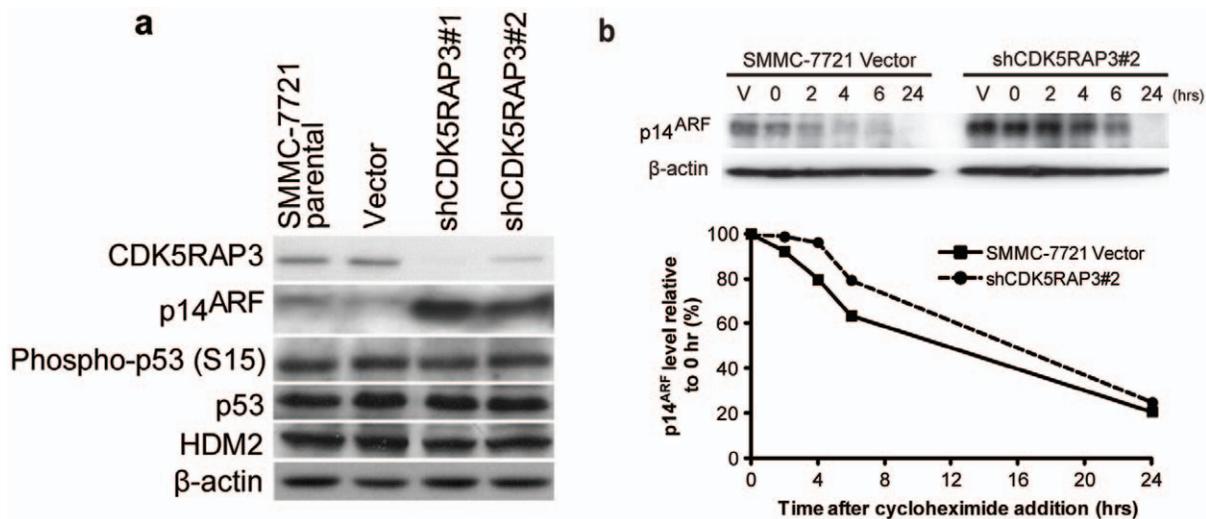
## Statistical Analysis

Student's *t*-test was used for statistical analysis of data. Tests were considered significant with  $P < 0.05$ .

## Results

### Knockdown of CDK5RAP3 Upregulated $p14^{ARF}$

Our previous study demonstrated that overexpression of CDK5RAP3 promoted HCC metastasis by activation of PAK4 [5]. However, as CDK5RAP3 is associated with tumor suppressor,  $p14^{ARF}$  [4], we wonder if  $p14^{ARF}$  also plays a role in CDK5RAP3-mediated HCC formation. To investigate if CDK5RAP3 regulates  $p14^{ARF}$ , Western blotting was performed to detect the protein level of  $p14^{ARF}$  in CDK5RAP3 stable knockdown SMMC-7721 cell lines [5]. Interestingly, the stable knockdown of CDK5RAP3 in SMMC-7721 cells resulted in an upregulation of  $p14^{ARF}$  (Fig. 1a). However, no significant difference was observed for the other components of the  $p14^{ARF}$ /HDM2/p53 pathway, such as HDM2



**Figure 1. Regulation of  $p14^{ARF}$  localization and protein expression by CDK5RAP3.** (a) The  $p14^{ARF}$  and CDK5RAP3 protein levels in stable CDK5RAP3 knockdown SMMC-7721 clones (shCDK5RAP3#1 and #2), vector control and parental cells [5] were compared by Western blotting using indicated antibodies, respectively. (b) The stable CDK5RAP3 knockdown SMMC-7721 (shCDK5RAP3#2) and vector control cells were treated with 100  $\mu$ g/ml cycloheximide or DMSO (vehicle), and harvested at the indicated time points.  $p14^{ARF}$  and  $\beta$ -actin protein levels were determined by Western blotting. V: DMSO treatment. Top: Western blotting; bottom: quantification of  $p14^{ARF}$  protein level. doi:10.1371/journal.pone.0042210.g001

and p53, suggesting that the upregulation of p14<sup>ARF</sup> may be independent of the protein interaction between HDM2 and p14<sup>ARF</sup> (Fig. 1a). Since the protein level of p14<sup>ARF</sup> is largely regulated by protein degradation [12], the possibility that CDK5RAP3 might enhance the turnover rate of p14<sup>ARF</sup> was examined. By treatment with cycloheximide, an inhibitor of protein synthesis, we observed that the protein degradation rate of p14<sup>ARF</sup> in the CDK5RAP3 stable knockdown clone #2 was slower than that of the vector control cells in the first six hours of treatment, but they reached to a similar rate after 24 hours, suggesting that CDK5RAP3 only has a very transient effect on the protein stability of p14<sup>ARF</sup> (Fig. 1b).

### CDK5RAP3 Repressed p14<sup>ARF</sup> Transcription in HCC Cells

To further understand how CDK5RAP3 might enhance the expression of p14<sup>ARF</sup>, and as CDK5RAP3 contains structural motifs suggested to be a transcriptional regulator, we examined if CDK5RAP3 regulates p14<sup>ARF</sup> transcriptionally. p14<sup>ARF</sup> mRNA level was found to be upregulated in the CDK5RAP3 stable knockdown SMMC-7721 clones (Fig. 2a). Consistently, down-regulation of p14<sup>ARF</sup> mRNA expression was also observed in two CDK5RAP3 stable overexpressing HepG2 clones that we established previously [5] (Fig. 2b). To further understand the mechanism by which CDK5RAP3 regulates p14<sup>ARF</sup> transcription, luciferase reporter assay was performed to determine the effect of CDK5RAP3 on p14<sup>ARF</sup> promoter transcription activity. A p14<sup>ARF</sup> promoter luciferase reporter plasmid contained the p14<sup>ARF</sup> promoter region 736 base pair (bp) upstream from the transcription start site was used for the assay [7]. As shown in Fig. 2c, ectopic expression of CDK5RAP3 significantly suppressed the p14<sup>ARF</sup> promoter luciferase activity in a dose-dependent manner in SMMC-7721 HCC cells (Fig. 2c). Similar result was also observed in HepG2 cell line, suggesting that CDK5RAP3 can transcriptionally repress the expression of p14<sup>ARF</sup> (Fig. 3c). To further understand how CDK5RAP3 suppresses p14<sup>ARF</sup> transcription, we used luciferase reporters carrying a series of truncation mutants of the p14<sup>ARF</sup> promoter to map the region responsible for the CDK5RAP3 suppression. As shown in Fig. 2d, CDK5RAP3 suppressed the luciferase activity of the p14<sup>ARF</sup> promoter in the constructs of -736, -327 and -231 to roughly 50%, but not in the construct of -150, suggesting that nucleotides -231 to -151 are important for CDK5RAP3 suppression.

### CDK5RAP3 Bound to Endogenous p14<sup>ARF</sup> promoter

As the ability of CDK5RAP3 to attenuate the p14<sup>ARF</sup> promoter activation and to repress endogenous p14<sup>ARF</sup> expression were demonstrated, we further investigate whether CDK5RAP3 binds to endogenous p14<sup>ARF</sup> promoter by chromatin immunoprecipitation (ChIP) assay using CDK5RAP3 stable overexpressing HepG2 cells [5]. Two sets of primers that has been reported previously were used to amplify p14<sup>ARF</sup> promoter regions (-1888 to -1659 and -48 to +267) [13]. As shown in Fig. 2d, immunoprecipitated with anti-CDK5RAP3 antibody significantly enriched the DNA fragments containing the promoter region of p14<sup>ARF</sup>, as compared with the no antibody control (Fig. 2e), strongly indicating that CDK5RAP3 can directly bind to the p14<sup>ARF</sup> promoter.

### Nuclear Localization of CDK5RAP3 was Important for the p14<sup>ARF</sup> Transcriptional Repressive Activity

To map out whether specific region of CDK5RAP3 is required for the repression of p14<sup>ARF</sup> transcription, a panel of deletion and LXXLL point mutation mutants of CDK5RAP3 was generated. To rule out the possibility that these mutants are unstable within

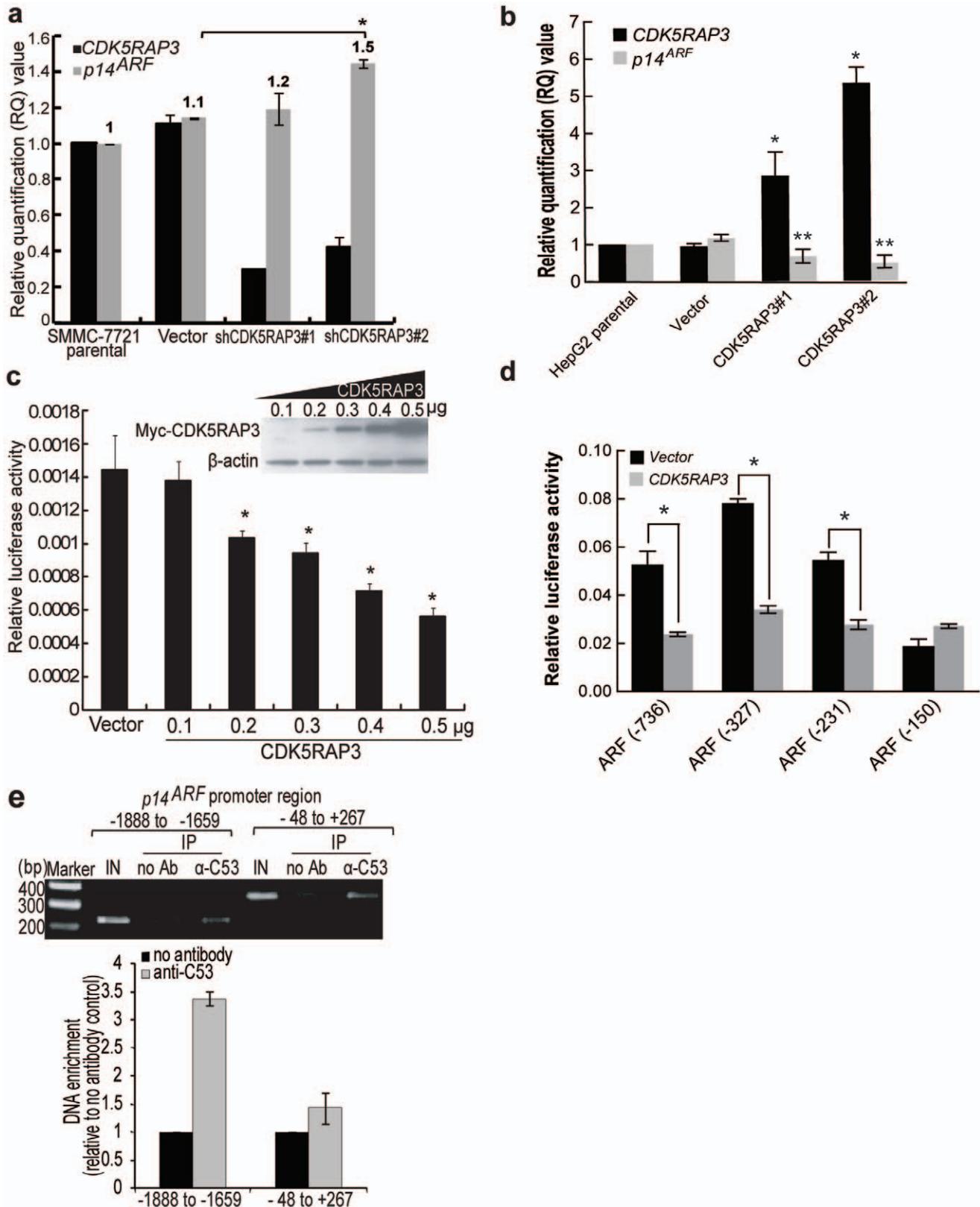
the cells, Western blot analysis was performed to confirm the expression of these mutants. The result showed that all the mutants expressed at similar levels in cells, apart from the 1–217 deletion mutant, which expressed at a lower level (Fig. 3b). To examine the transcriptional suppressive activity of these mutants, luciferase reporter assay was performed. Mutation of both LXXLL motifs on CDK5RAP3 to LXXAA (L117AL118A/L475AL476A) and a 1–362 deletion mutant of CDK5RAP3 was much less potent in repressing the p14<sup>ARF</sup> promoter activity (Fig. 3c), as compared to the full length protein. Similar result of double point mutant was also obtained for single LXXLL/LXXAA mutants (data not shown). However, for the deletion mutants, including 1–256, 255–506 and 434–506, all of them had completely lost their repressive activity on p14<sup>ARF</sup> promoter (Fig. 3c). This result suggests that the overall integrity of CDK5RAP3 protein may be important for the repression activity. Surprisingly, the 1–217 mutant, which was relatively unstable, did not repress, but activates the p14<sup>ARF</sup> promoter activity (Fig. 3c). More interestingly, among the panel of mutants, the a.a. 1–217 mutant was the only mutant that did not localize to the nucleus (Fig. 3d), indicating that the nuclear localization of CDK5RAP3 may be important for its repressive activity on p14<sup>ARF</sup> promoter and protein stability (Fig. 3d). Taken together, our data indicate that CDK5RAP3 repressed p14<sup>ARF</sup> promoter activity in HCC cells.

### CDK5RAP3 had no Effect on E2F1-mediated p14<sup>ARF</sup> Promoter Transactivation

p14<sup>ARF</sup> is the upstream activator for p53 activity as it can abrogate the MDM2-mediated degradation of p53 by inhibiting MDM2 [14,15], we wonder if regulation of p14<sup>ARF</sup> expression by CDK5RAP3 affects p53 transactivation activity. Using p53 luciferase reporter assay, we showed that CDK5RAP3 did not affect the p53 promoter activity (Fig. 4a), indicating that CDK5RAP3 may not have an effect on p14<sup>ARF</sup>-mediated regulation of p53 in HCC cells. To examine if CDK5RAP3 represses the p14<sup>ARF</sup> via regulating the transcriptional activator of p14<sup>ARF</sup>, E2F1, luciferase reporter assays were performed by overexpressing Myc-tagged CDK5RAP3 and HA-tagged E2F1 in HepG2 cells. Although ectopic expression of E2F1 caused a dose-dependent activation of p14<sup>ARF</sup> luciferase reporter (Fig. 4b lane 3 to 5), co-expression of CDK5RAP3 did not affect the E2F1-mediated p14<sup>ARF</sup> transactivation (Fig. 4b lane 6 to 8). Therefore, the mechanism by which CDK5RAP3 downregulates p14<sup>ARF</sup> promoter transcription is most likely independent of E2F1.

### Knockdown of p14<sup>ARF</sup> Reversed the Suppression of Cell Migration and Invasiveness in CDK5RAP3 Knockdown cells

Since evidence has demonstrated that mouse homolog of p14<sup>ARF</sup>, p19<sup>ARF</sup> inhibited HCC cell invasion [16], we speculate that the upregulation of p14<sup>ARF</sup> in CDK5RAP3 stable knockdown HCC clones may cause the decrease in migration of these stable clones [5]. To elucidate whether p14<sup>ARF</sup> plays a role in CDK5RAP3-mediated regulation of invasiveness in HCC cells, we examine the effect of silencing p14<sup>ARF</sup> in CDK5RAP3 stable knockdown clones motility. The specific knockdown of p14<sup>ARF</sup> by two independent p14<sup>ARF</sup> siRNA was confirmed by Western blotting (Fig. 5a). Then we asked if the effect of knockdown of CDK5RAP3 on cell motility and invasiveness could be restored by knockdown of p14<sup>ARF</sup>. As shown in Fig. 5b, knockdown of p14<sup>ARF</sup> in CDK5RAP3 stable knockdown SMMC-7721 cells significantly increased the number of migrated cells as compared to the control siRNA, suggesting that loss of p14<sup>ARF</sup> reversed the suppression of



**Figure 2. Suppression of endogenous expression of  $p14^{ARF}$  by CDK5RAP3.** (a) The  $p14^{ARF}$  and CDK5RAP3 mRNA expression in stable CDK5RAP3 knockdown SMMC-7721 stable clones was determined by Quantitative real-time PCR (qPCR). Data was analyzed by comparative Ct method. Band intensity was analyzed using AlphaEasePC software and normalized with  $\beta$ -actin. Results were mean of three independent experiments. \*,  $P < 0.005$ , Student's  $t$ -test. (b) Similar to (a), the CDK5RAP3 stable expressing HepG2 clones (CDK5RAP3#1 and #2), vector control and parental cells were used for qPCR assay. Results were mean of three independent experiments. \* $P < 0.04$  and \*\* $P < 0.02$  compared with vector control,

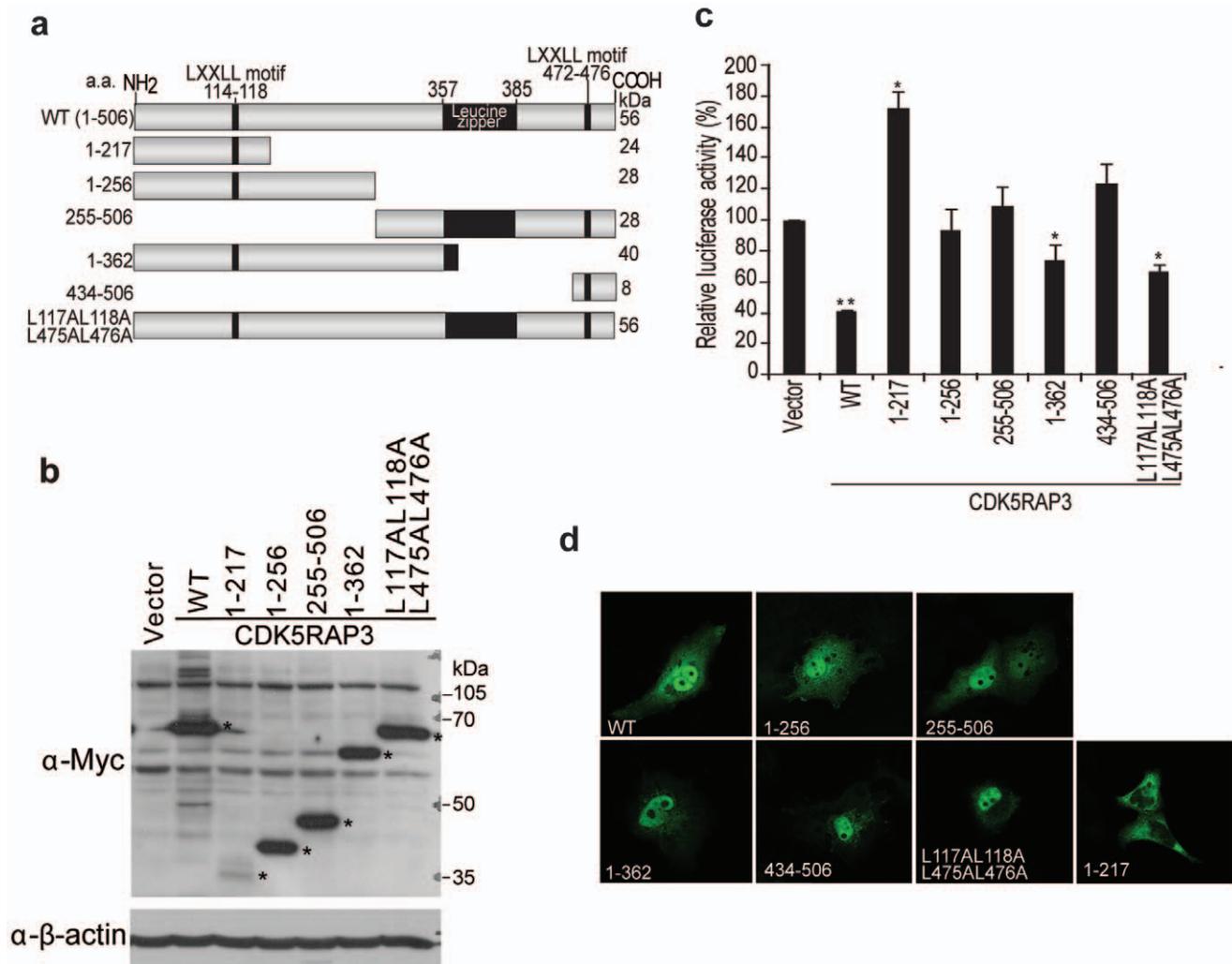
Student's *t*-test. (c) The CDK5RAP3 expression construct and  $p14^{ARF}$  luciferase reporter, pGL3- $p14^{ARF}$  were co-transfected into SMMC-7721 cells for dual-luciferase reporter assay. Results represent mean  $\pm$ SD for triplicate wells. \*,  $P < 0.05$  compared with vector control, Student's *t*-test. (d) Similar to (c), luciferase reporters carrying truncation mutants of the  $p14^{ARF}$  promoter, CDK5RAP3 expression construct (0.3  $\mu$ g) and vector (0.3  $\mu$ g) were used for dual-luciferase reporter assay. Results represent mean  $\pm$ SD for triplicate wells. \*,  $P < 0.02$  compared with vector control, Student's *t*-test. (e) CDK5RAP3 bound  $p14^{ARF}$  promoter by performing chromatin immunoprecipitation (ChIP) analysis on CDK5RAP3 stable overexpression clone #2 HepG2 cells ( $1 \times 10^7$ ). Input (IN) and no antibody control (No Ab) were included. Error bars: mean  $\pm$ SD. doi:10.1371/journal.pone.0042210.g002

cell migration. Consistently, the invasiveness of CDK5RAP3 stable knockdown SMMC-7721 cells was also significantly restored in  $p14^{ARF}$  siRNA knockdown cells (Fig. 5b). Thus these results demonstrated that overexpression of CDK5RAP3 can promote HCC cell metastasis through downregulation of  $p14^{ARF}$ .

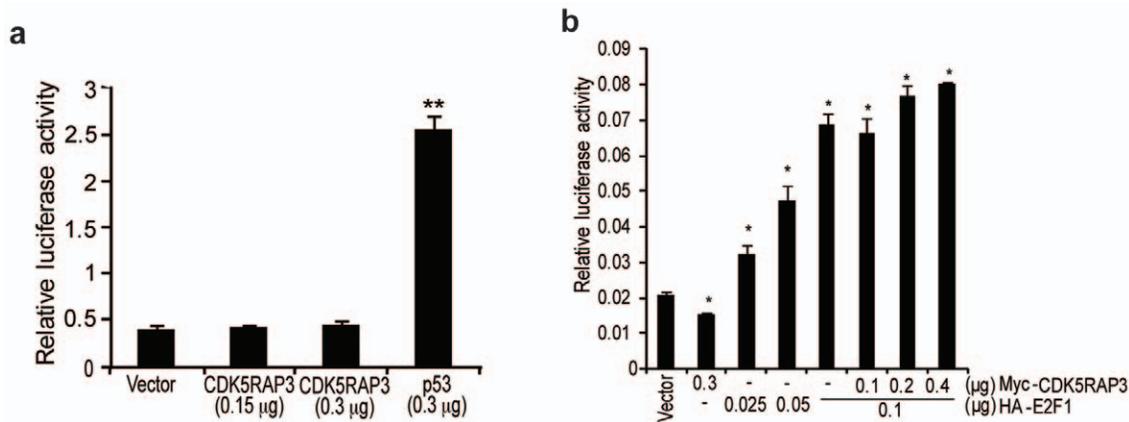
## Discussion

CDK5RAP3 has 2 putative LXXLL motifs, which are the signature motifs for transcriptional co-regulators, mediating the binding on nuclear receptors. Previous study has shown that

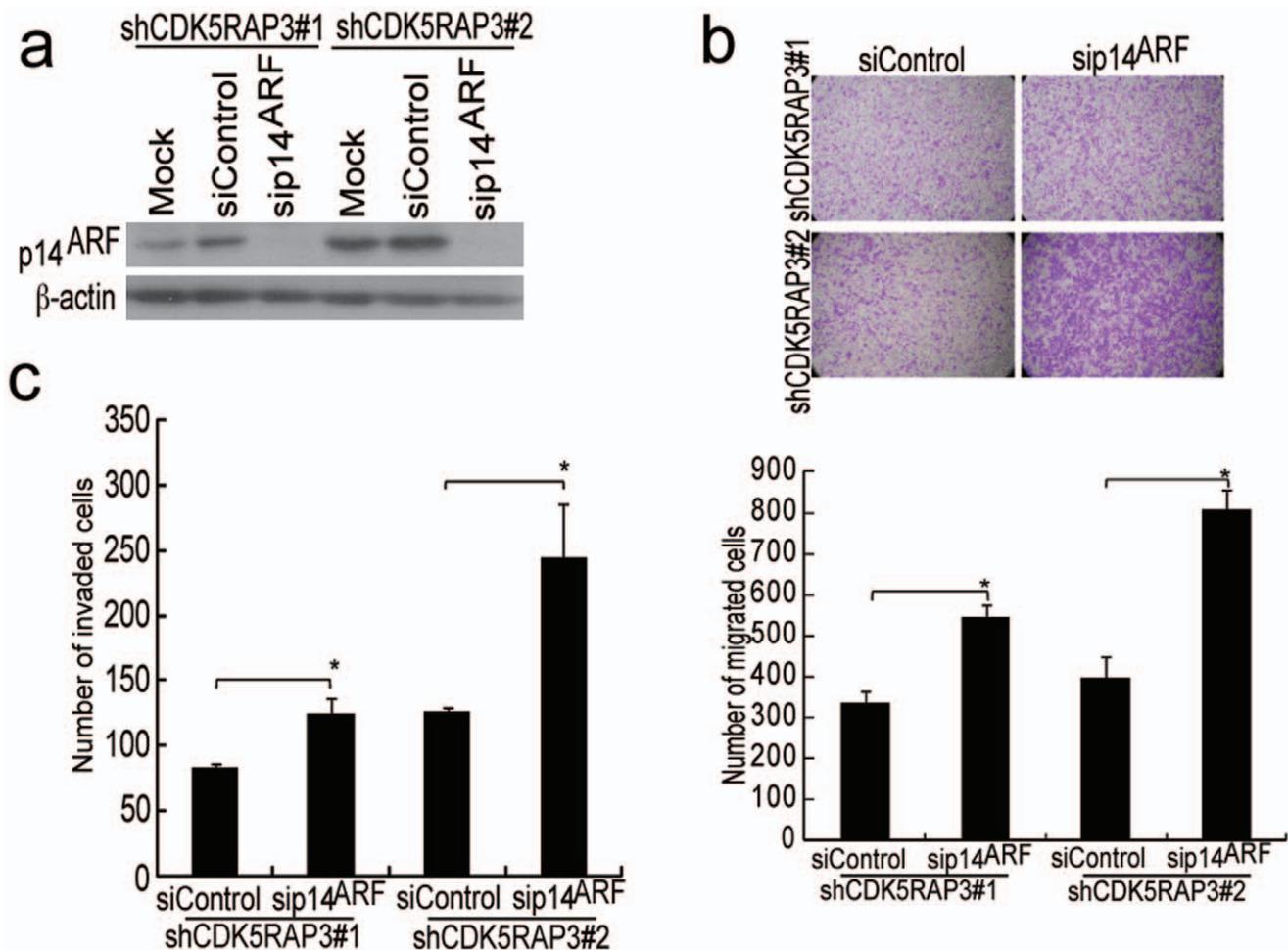
CDK5RAP3 can associate with a nuclear co-activator, cAMP response element-binding protein (CREB)-binding protein (CBP), suggesting that CDK5RAP3 may also function as a transcriptional co-activator/repressor. Recently, *CDK5RAP3* is shown to promote HCC metastasis by activating PAK4 kinase activity [5]. In this study, we provide evidence for a novel mechanism by which CDK5RAP3 promotes HCC metastasis by downregulating tumor suppressor  $p14^{ARF}$  transcriptionally. Our data demonstrated that loss of CDK5RAP3 drastically enhanced the expression of  $p14^{ARF}$  (Fig. 1a and 2a) at both protein and mRNA levels in HCC cells. This notion is supported by three line of evidence. First, we



**Figure 3. Nuclear localization of CDK5RAP3 was important for the suppression of  $p14^{ARF}$  promoter activity.** (a) Schematic diagram of CDK5RAP3 mutants (b) Western blotting showing the expression levels of CDK5RAP3 mutants overexpressed in HepG2. Protein lysates from reporter assay were used for Western blotting probed with anti-Myc antibody. (c) Dual luciferase reporter was performed by co-transfection of CDK5RAP3 mutants with  $p14^{ARF}$ -luc reporter in HepG2. Results were mean of three independent experiments, with promoter activity of vector control set as 100%. \*,  $P < 0.05$  and \*\*,  $P < 0.005$  compared with vector control, Student's *t*-test. (d) Confocal images of wild type (WT) and the indicated deletion mutants of Myc-CDK5RAP3. doi:10.1371/journal.pone.0042210.g003



**Figure 4. CDK5RAP3 transcriptionally regulated p14<sup>ARF</sup> in a E2F1 independent manner.** (a) CDK5RAP3 was co-transfected with p53-responsive element reporter for luciferase assay in HepG2 cells. Results represent mean  $\pm$ SD for triplicate wells. \*,  $P < 0.05$ , \*\*,  $P < 0.005$  compared with vector control, Student's *t*-test. (b) Expression constructs of CDK5RAP3 was co-transfected with HA-E2F1 and p14<sup>ARF</sup>-luc reporter for luciferase assay in HepG2 cells. Results represent mean  $\pm$ SD for triplicate wells. \*,  $P < 0.005$  compared with vector control, Student's *t*-test. doi:10.1371/journal.pone.0042210.g004



**Figure 5. Knockdown of p14<sup>ARF</sup> reversed the suppression of cell migration and invasiveness in CDK5RAP3 knockdown HCC cells.** (a) The CDK5RAP3 stable knockdown SMMC-7721 cells were transfected with p14<sup>ARF</sup> or control siRNA. Top, Western blotting showing p14<sup>ARF</sup> knockdown in cells; bottom, The bar chart showed the quantitation of migrated cells in three independent experiment (\*,  $P = 0.005$ , Student's *t*-test). Representative photomicrographs were shown. (b) Similar to (a), but invasion assay were performed. The bar chart showed the quantitation of the invaded cells in three independent experiment (\*,  $P = 0.05$ , Student's *t*-test). doi:10.1371/journal.pone.0042210.g005

observed that upregulation of p14<sup>ARF</sup> transcripts was observed in CDK5RAP3 stable knockdown HCC clones, whereas downregulation of p14<sup>ARF</sup> transcripts was observed in CDK5RAP3 stable overexpressing HCC clones. Second, using p14<sup>ARF</sup> promoter luciferase reporter assay, we showed that forced expression of CDK5RAP3 repressed the p14<sup>ARF</sup> promoter transcriptional activity in a dose-dependent manner (Fig. 2c). Third, using ChIP analysis, CDK5RAP3 was found to bind directly to p14<sup>ARF</sup> promoter region (Fig. 2e), indicating that CDK5RAP3 may directly regulate the p14<sup>ARF</sup> promoter activity. Indeed, CDK5RAP3 has recently been reported to repress cyclin D1 transcription [17] and NF- $\kappa$ B transcriptional activity [18], further supporting the role of CDK5RAP3 as a transcriptional co-regulator. Taken together, these data indicated that CDK5RAP3 is a putative negative transcriptional regulator of p14<sup>ARF</sup>. As for how CDK5RAP3 can repress the p14<sup>ARF</sup> promoter activity, we find that the overall integrity of the protein may be important. It is because all the truncation of CDK5RAP3 resulted in a completely lost of repressive activity of CDK5RAP3, apart from 1–362 mutant, which retains a moderate repressive activity (Fig. 3b). Interestingly, our data also suggested that the nuclear localization of CDK5RAP3 is important for its repressor activity (Fig. 3d) and this supports our hypothesis that CDK5RAP3 regulates the transcription of p14<sup>ARF</sup> via direct binding to p14<sup>ARF</sup> promoter, as revealed by our ChIP analysis (Fig. 2e).

p14<sup>ARF</sup> is one of the upstream activators for p53 as it can abrogate the MDM2-mediated degradation of p53 by inhibiting MDM2 [14,15]. However, in CDK5RAP3 stable knockdown HCC cells, we found that p53 and phospho-p53 (Ser15) levels remained unchanged (Fig. 1a). Furthermore, overexpression of CDK5RAP3 in HCC cells did not seem to have a significant impact on p53 transactivation activity (Fig. 4a). Thus these results indicate that CDK5RAP3 did not play a significant role in regulating p53 via p14<sup>ARF</sup> in HCC cells. Actually, this observation is in consistent with our previous result showing that the transforming ability of CDK5RAP3 on HCC cells were p53-independent as CDK5RAP3 knockdown reduced proliferation rate and colonies formed in p53-defective PLC/PRF/5 HCC cell line [5].

The mechanism by which overexpression of CDK5RAP3 enhances HCC metastasis is not completely understood. Here, we propose that via the downregulation of p14<sup>ARF</sup>, CDK5RAP3

can enhance the invasiveness of HCC cells. To this end, we used siRNA to specifically knock down p14<sup>ARF</sup> in SMMC-7721 cells with CDK5RAP3 stable knockdown and showed that the loss of p14<sup>ARF</sup> significantly promoted the motility and invasiveness of HCC cells (Fig. 5). Previous studies have shown that mouse homolog of p14<sup>ARF</sup>, p19<sup>ARF</sup> can inhibit the invasion of HCC cells by binding to C-terminal binding protein (CtBP) [16]. Interestingly, while CtBP binds to a.a. residues 42 to 54 of p14<sup>ARF</sup> [16], CDK5RAP3 binds to a.a. residues 1 to 64 of p14<sup>ARF</sup> [4], suggesting that the p14<sup>ARF</sup> binding region of CtBP and CDK5RAP3 may indeed overlap. Thus it is conceivable that CDK5RAP3 may sequester the binding of p14<sup>ARF</sup> to CtBP and release the free CtBPs to promote HCC cell invasion. Further experiment is currently undergoing to test this hypothesis.

Collectively, the underlying mechanism for the transcriptional regulation of p14<sup>ARF</sup> by CDK5RAP3 still not completely clear, and whether CDK5RAP3 regulates generally on *INK4a/p14<sup>ARF</sup>/INK4b* locus or specifically on p14<sup>ARF</sup> requires further investigation. As CDK5RAP3 has been reported to interact with CBP [19], a co-activator for CREB, it remains to be determined whether CDK5RAP3 regulates p14<sup>ARF</sup> promoter by modulating CBP/CREB activity. In HCC, silencing of the p14<sup>ARF</sup> promoter through hypermethylation is frequently observed [20]. However, our data suggest that p14<sup>ARF</sup> transcription can also be repressed by upregulation of CDK5RAP3. In addition, our data provide a novel insight by which overexpression of CDK5RAP3 can enhance HCC metastasis via negatively regulating p14<sup>ARF</sup>. As a result, inhibition of CDK5RAP3 can potentially be used to restore the expression of this important tumor suppressor expression, providing new molecular targets for the therapeutic intervention in HCC.

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## Author Contributions

Conceived and designed the experiments: IOLN YPC. Performed the experiments: GWYM WLL YZ. Analyzed the data: IOLN YPC WLL. Contributed reagents/materials/analysis tools: ML IOLN YPC. Wrote the paper: YPC.

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