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A novel crosstalk between calcium/calmodulin kinases II and IV regulates cell proliferation in myeloid leukemia cells

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ABSTRACT

CaMKs link transient increases in intracellular Ca^{2+} with biological processes. In myeloid leukemia cells, CaMKII, activated by the *bcr-abl* oncogene, promotes cell proliferation. Inhibition of CaMKII activity restricts cell proliferation, and correlates with growth arrest and differentiation. The mechanism by which the inhibition of CaMKII results in growth arrest and differentiation in myeloid leukemia cells is still unknown. We report that inhibition of CaMKII activity results in an upregulation of CaMKIV mRNA and protein in leukemia cell lines. Conversely, expression of CaMKIV inhibits autophosphorylation and activation of CaMKII, and elicits G_0/G_1cell cycle arrest, impairing cell proliferation. Furthermore, U937 cells expressing CaMKIV show elevated levels of Cdk inhibitors $p27^{kip1}$ and $p16^{ink4a}$ and reduced levels of cyclins A, B_1 and D_1.
These findings were also confirmed in the K562 leukemic cell line.
The relationship between CaMKII and CaMKIV is also observed in primary acute myeloid leukemia (AML) cells, and it correlates with their immunophenotypic profile. Indeed, immature MO/M1 AML showed increased CaMKIV expression and decreased pCaMKII, whereas highly differentiated M4/M5 AML showed decreased CaMKIV expression and increased pCaMKII levels.
Our data reveal a novel cross-talk between CaMKII and CaMKIV and suggest that CaMKII suppresses the expression of CaMKIV to promote leukemia cell proliferation.

1. Introduction

Multifunctional CaMKs (CaMKI, CaMKII and CaMKIV) are important mediators of intracellular Ca^{2+} signalling that play pleiotropic roles in cell physiology. These serine–threonine (Ser/Thr) protein kinases are activated upon Ca^{2+}/CaM binding. The upstream CaMK kinases (CaMKKs) phosphorylate a critical Thr 200 in the activation-loop to activate CaMKIV, whereas CaMKII is fully activated by autophosphorylation of its own Thr 286 [1,2]. Ca^{2+}/CaM signaling is necessary for cell cycle progression, and CaM-dependent pathways influence cyclin-Cdk activation at different phases of the cell cycle [3]. Several studies suggest the involvement of CaMKs at G_1/S and G_2/M transitions of the cell cycle [4–8]. Furthermore, treatment of several cell types with KN93, a pharmacological inhibitor of CaMKs, elicits a reversible G_1 arrest that correlates with enhanced association of $p27^{kip1}$ with Cdk2 and reduced Cdk2/4 activity [9].

CaMKIV is a predominantly nuclear, monomeric kinase with tissue-restricted expression [1]. It plays a role in mediating Ca^{2+}-regulated transcription through phosphorylation and/or activation of different transcription factors, including cyclic adenosine mono phosphate

Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}-calmodulin dependent kinase; RAR, retinoic acid receptor; CDKi, cyclin dependent kinase inhibitor; AML, acute myeloid leukemia; Rb, retinoblastoma.

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(cAMP) response element binding protein (CREB), activating transcription factor (ATF), serum response factor (SRF), and transcriptional co-factors including CREB binding protein (CBP) [10]. Embryonic expression of CaMKIV correlates with periods of differentiation and terminal mitoses, supporting for this kinase a pivotal role in regulation of cell differentiation [11]. Indeed, in hematopoietic stem cells (HSCs), CaMKIV/CREB/CBP pathway restricts cell proliferation by expression of Bcl-2, a pro-survival protein with additional roles in cell quiescence [12]. In cancer cells, CaMKs appear to play a role in aggressiveness: CaMKK2 signaling through adenosine monophosphate dependent protein kinase (AMPK) [13] and/or CaMKI [3] modulates cell migration, invasiveness and malignancy in prostate cancer and medulloblastoma [14,15]. Notably, CaMKIV is not expressed in these two cancer cell types, a feature that the authors attributed to reduced expression of genes required for differentiation [14].

The ubiquitously expressed, multimeric CaMKII is predominantly cytoplasmic, although 3 alternatively spliced variants: α_B,γ_A, and δ_B are targeted to the nucleus [16,17]. CaMKII exerts a broad range of biological functions, and is an important regulator of cell proliferation [18,19]. It modulates Raf1 activity leading to extra-cellular receptor kinase (ERK) activation and induction of cell proliferation. The interplay between CaMKII and ERK has been observed in several cell types including thyroid [20,21], fibroblasts [22], skeletal muscle [23] and vascular smooth muscle [24], suggesting that it is a general mechanism in the control of cell proliferation. CaMKII is also a target of RET-PTC oncogene, raising the possibility that it plays a central role in the abnormal growth of tumor cells [25].

Many studies suggest the involvement of CaMKII in the homeostasis of tumor cells. Indeed, Si and Collins reported that myeloid leukemia cell lines as well as primary acute myeloid leukemia (AML) patient samples express elevated levels of activated CaMKII. CaMKII promotes G_1/S cell cycle progression [26] and its inhibition impairs myeloid leukemia cell proliferation. Leukemia cells undergoing growth arrest and/or terminal differentiation present markedly reduced levels of CaMKII [26], suggesting that downregulation of CaMKII may be a pre-requisite for inducing terminal differentiation in myeloid leukemia cells.

Because of the reciprocal aspects of CaMKII and CaMKIV on proliferation [12] and terminal differentiation [11,27,28] in hematopoietic progenitors, we hypothesized that CaMKIV may be repressed in a CaMKII dependent manner in myeloid leukemia cells. In the present study, we investigated a potential cross-talk between CaMKII and CaMKIV in human myeloid leukemia U937 cells [29], which express very low CaMKIV and high CaMKII levels. We found that CaMKII represses *CaMK4* transcription and promotes proliferation in human myeloid leukemia cells. On the other hand, transgenic enhanced expression of CaMKIV negatively modulates CaMKII activity and induces G_0/G_1 cell cycle arrest in these malignant cells. We propose that CaMKII and CaMKIV counterregulation is not restricted to leukemia cells and can be exploited in the development of targeted therapies against myeloid leukemia and other myeloid disorders.

2. Materials and methods

2.1. Cell culture and reagents

U937 and K562 (CRL1593.2 and CCl243, respectively, American Type Culture Collection (ATCC), Manassas, VA) were cultured in RPMI-1640 Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Human embryonic kidney HEK 293A cells (R70507, Invitrogen) were grown in DMEM supplemented with 10% FBS (Invitrogen). Cells were stimulated with 2 μM ionomycin (Sigma, St. Louis, MO) for 15 min or with 10% FBS following 12 h serum starvation. CaMKII pharmacological inhibition was obtained with 10 μM of the CaMKs inhibitor KN93 (Biomol Plymouth Meeting, PA); or with 5 μM of the CaMKII selective inhibitory peptide, AntCaNtide [30].

2.2. RNA interference

Four CaMKIIγ-specific shRNA (SureSilencing shRNA plasmids) and control shRNA vector were purchased from SuperArray. The targeted sequences are shRNA1: ACCTGCTGCTGGCGAGTAAAT; shRNA2;GAACGTGA GGCTCGGATATGT; shRNA3: GAGGCCTACACGAAGATTTGT; shRNA-4: GAGTGTTTGCGCAAGTTCAAT. CaMKIIγ-shRNA design was based on the GenBank accession number NM_001222. CaMKIIγ- shRNA transfections were performed using Attractene Transfection Reagent.

These four plasmids were, first, transiently transfected in U937 cells according to the manufacturer's instructions, and then selected in G418 (800 μg/mL) for 10 d. We first indentified one shRNA that caused >80% reduction in the endogenous CaMKIIγ levels.

2.3. Lentiviral infection

Lentivirus-green fluorescent protein (GFP) constructs were generated and characterized as mentioned before [31]. Briefly, full-length WT CaMKIVcDNA (a gift from Dr. Anthony R. Means, Duke University Medical Center) was cloned into a Lenti-green fluorescent protein (GFP) vector [32]. Empty Lenti-GFP-control ("Mock"), Lenti-GFP-CaMKIV ("CaMKIV") and Lenti-GFP-CaMKIV-K71M viruses were generated as previously described [3,4] using second generation helper plasmids in 293T cells (a gift from Dr. Anthony R. Means, Duke University Medical Center and described in Kitsos et al. [12]). Virus titers were calculated using 293T cells and CaMKIV expression confirmed by immunoblotting. Approximately 2×10^6 of U937 cells were infected with control (Mock) or Lenti-GFP-CaMKIV (CaMKIV) viruses at a multiplicity of infection of 10:1.

2.4. Cell viability and [^{3}H] thymidine (T) incorporation assays

50,000 cells/well were counted daily following trypan blue staining (Invitrogen). 5000 cells were serum-starved for 12 h, and DMEM-10% FBS with 0.5 μCi [^{3}H] thymidine (T) and CaMKII inhibitors were added. After 24 h, [^{3}H] T uptake was evaluated (BD Biosciences, San Jose, CA).

2.5. Cell cycle distribution assay

Cells were fixed in 70% ethanol, incubated with 0.02 mg/ml propidium iodide (PI) and 0.25 mg/ml ribonuclease A (Sigma), and analyzed for DNA content (FACScan, BD). Acquisition and analyses were performed using CellQuest (BD) software.

2.6. Annexin V apoptosis assay

3×10^6cells were incubated with AnnexinV-PE and PI (BDPharmingen, USA) and analyzed by flow cytometry. Samples were acquired with a CYAN flow cytometer (DAKO Corporation, San Jose, CA) and analyzed using SUMMIT® software.

2.7. Ki-67 staining

Cells were incubated with Ki-67-PE antibody and 7-amino actinomycin D (7AAD, BD) and analyzed (FACSAria, BD).

2.8. Bromo deoxy uridine (BrdU) labeling

Cells were pulse-labeled with 10 μM BrdU (Sigma). Samples were processed and incubated with BrdU-PE antibody and 7AAD (BD), and analyzed on a FACSAria.

2.9. Transient transfection and dual luciferase assay

HEK 293A cells were transiently transfected with 0.1 μg of renilla luciferase pRLTK vector and 1 μg each of fire fly luciferase reporter construct containing a *CaMK4* promoter harboring RARβ response elements (RRE) [33], expression constructs encoding RAR-β, chicken ovalbumin upstream promoter transcription factor (COUP-TF) and CaMKII. Two days later, transfected cells were treated with 2 μM retinoic acid (Sigma) for 2 h and assayed using a dual Luciferase Assay Reporter System Kit (Promega Co., Madison, WI).

2.10. CaMKII activity assays

Endogenous CaMKII was immunoprecipitated with 5 μL of anti-CaMKII antibody and 25 μL of protein G plus/protein A agarose beads/1 mg total cell extract (Santa Cruz, CA). CaMKII was incubated for 30 min at 30 °C with 1 mM CaCl2 and 1 μM CaM in 50 μl of reaction mixture (50 mM HEPES pH 7.5, 10 mM MgCl2, 0.5 mM dithiothreitol (DTT), 100 nM microcystin, 0.1 mM cold ATP). In a second reaction step, an aliquot from the first reaction was incubated with the CaMKII substrate Autocamtide and [32P]-γATP (GE-Amersham, Piscataway, NJ); EGTA was added to quantify CaMKII autonomous activity.

2.11. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted with a RNA 4-PCR kit (Applied Biosystems, Foster City, CA) and reverse transcribed using a High Capacity Reverse Transcriptase Kit (Applied Biosystems). QRT-PCR was performed by Bio-Rad IC5 thermo-cycler (Bio-Rad Laboratories, Hercules, CA). Primers sequences used for hCaMKIV are: 5′-GGC ACA GGC TGA GCT GAT G-3′ forward and 5′-CTA GTT CCA GGT CAG CCA CCT TT-3′ reverse primers. Cycle threshold (Ct) values from 3 independent experiments were normalized to the internal β-actin control. The ratio of fold change was calculated using the Pfaffl method [34].

2.12. Patient specimen collection

AML specimens were obtained from peripheral blood or bone marrow of patients following informed consent at the time of diagnostic biopsy. Weekly calibration using beads (Beckman Coulter Inc. Brea, CA) was performed. Whole BM samples in EDTA were washed 3 times in phosphate-buffered saline (PBS) and resuspended with 1% bovine serum albumin in PBS. 100 μL were incubated with each of the following titered monoclonal antibodies (MoAbs) for 15 min: 5 μL HLA-DR FITC, 5 μL Myeloperoxidase FITC, 5 μL CD2-PeCy7, 5 μL CD3 PE, 5 μL CD4 PE, 5 μL CD5-PeCy7, 5 μL CD8-PeCy5, 5 μL CD7 FITC, 5 μL CD10 PE, 5 μL CD11a FITC, 5 μL CD11b-PeCy5, 5 μL CD11c PE, 5 μL CD13-PeCy5, 5 μL CD14 FITC, 5 μL CD15 FITC, 5 μL CD19 PeCy5, 5 μL CD20 PE, 5 μL CD24 PE, 5 μL CD25 FITC, 5 μL CD33 PeCy5, 5 μL CD34 PeCy7, 5 μL CD45RO PE, 5 μL CD45RA PE (Beckman Coulter Inc. Brea, CA). Erythrocytes were lysed by BD FACS lysing solution. Cells were pelleted (416 g for 5 min), washed and resuspended in 0.5 mL of 1% of paraformaldehyde. 160,000 to 1,000,000 total BM cells were acquired/sample. Based on FAB/WHO immunologic criteria, 4 cases with the immunophenotype MPO +/−, CD13 +/, CD33 +/−, CD34 +/−, CD117 +, HLA-DR +/− were classified as M0/M1 AML, 2 cases MPO +, CD13 +, CD15 +, CD19 +, CD33 +, CD34 +, CD56 +, CD117 +/−, HLA-DR + as M2 AML, 1 case MPO +, CD13 +, CD14 +/−, CD15−, CD33 +, CD34 −, CD56 −, CD64 +, CD117 −/+, HLA-DR- as AML M3, 6 cases MPO +, CD2 +, CD13 +, CD14 +/−, CD15 +/−, CD33 +, CD34−/+, CD64 +, CD117 +, HLA-DR + as M4 AML, 7 cases MPO −/+, CD13 +, CD14 +/−, CD33 +, CD34−, CD64 +, CD117 +/−, HLA-DR + as AML M5, 1 case MP0-, CD13 +/−, CD14 +/−, CD33 +/−, CD34−, CD61 +, CD117 −, HLA-DR +/− as M7 AML.

Small samples of normal bone marrow were collected from the sack left following transplant.

2.13. Flow cytometry on primary human cells

Bone marrow aspirate specimens were tested on a dual laser system Beckman Coulter, Cytomics FC500 (Beckman Coulter Inc. Brea, CA) using CXP software (see SM).

2.14. Statistical analysis

Student's *t*-test was used for statistical significance; a p-value < 0.05 was deemed significant.

3. Results

3.1. Inhibition of CaMKII activity elevates CaMKIV expression

Pharmacological inhibition of CaMKII activity induces cell cycle arrest in several cell types including myeloid leukemia cells [5,7,26]. To identify the underlying mechanism of growth inhibition, U937 cells were treated with 10 μM KN93. Whereas the levels of total CaMKII did not change, autophosphorylation of CaMKII became significantly reduced within 6 h of KN93 treatment, and completely repressed after 24 h (p-CaMKII, Fig. 1A). In contrast to CaMKII, which is abundantly expressed in U937 myeloid leukemia cells, expression of CaMKIV is very low (Fig. 1A and Supplementary Fig. S1). However, levels of CaMKIV increased as a function of time in U937 cells following exposure to KN93 (Fig. 1A). This result was reproduced also in K562 cells, confirming that this mechanism is not restricted to U937 cells (Fig. 1F). To understand whether CaMKIV increase occurs at the transcriptional level, we analyzed *CaMK4* mRNA in U937 cells treated with KN93. As indicated in Fig. 1B, *CaMK4* mRNA levels increase by 24 h and become maximally up-regulated by 72 h after KN93 treatment, indicating that CaMKIV increase following CaMKII inhibition involves enhanced *Camk4* transcription. To further confirm the effect of CaMKII inhibition on CaMKIV expression, we analyzed *CaMK4* mRNA and protein levels in U937 cells following treatment with the CaMKII-specific inhibitory peptide, AntCaNtide [21–23,30]. Accordingly, antCaNtide treatment resulted in a time-dependent increase of CaMKIV protein levels (Fig. 1C). Inhibition of CaMKII activity using AntCaNtide also resulted in a significant induction of *CaMK4* mRNA within 6 h (Fig. 1D). Altogether, these data suggest that inhibition of CaMKII promotes *CaMK4* mRNA synthesis. Since CaMKIIγ is the predominantly expressed isoform of CaMKII in both myeloid cells and U937 [35,36], we also determined the effect of silencing CaMKIIγ by shRNA on CaMKIV expression in this latter cell line. As shown in Fig. 1E, CaMKIIγ shRNA in U937 cells increased CaMKIV protein expression in a similar way of KN-93 and AntCaNtide treatments. This result is consistent with CaMKIV upregulation after pharmacological inhibition of CaMKII.

It is known that CaMKII directly binds to and phosphorylates the nuclear hormone receptor transcription factor RAR [35]. In myeloid cells, this phosphorylation results in enhanced binding of RAR to transcriptional co-repressors, inhibiting RAR-mediated transcription from target genes. The 5′ flanking region of human *CaMK4* promoter contains RAR response elements (RRE) and treatment with retinoic acid (RA) results in transactivation of the CaMK4 promoter through the RRE [33]. In fact, RAR acts in concert with chicken ovalbumin upstream promoter transcription factor (COUP-TF) at the RRE elements on *CaMK4* promoter. Indeed, in embryonic stem (ES) cells differentiated into neurons, endogenous CaMKIV is regulated by T3, and a single complex element in the rat CaMKIV 5′-flanking region confers this regulation. The isolated element is also stimulated by RA. COUP-TF1, which suppresses the T3 response and augments the RA response in vitro, binds to an overlapping sequence in the same site [33]. We surmised that in proliferating leukemia cells, CaMKII could mediate repression of RAR-mediated

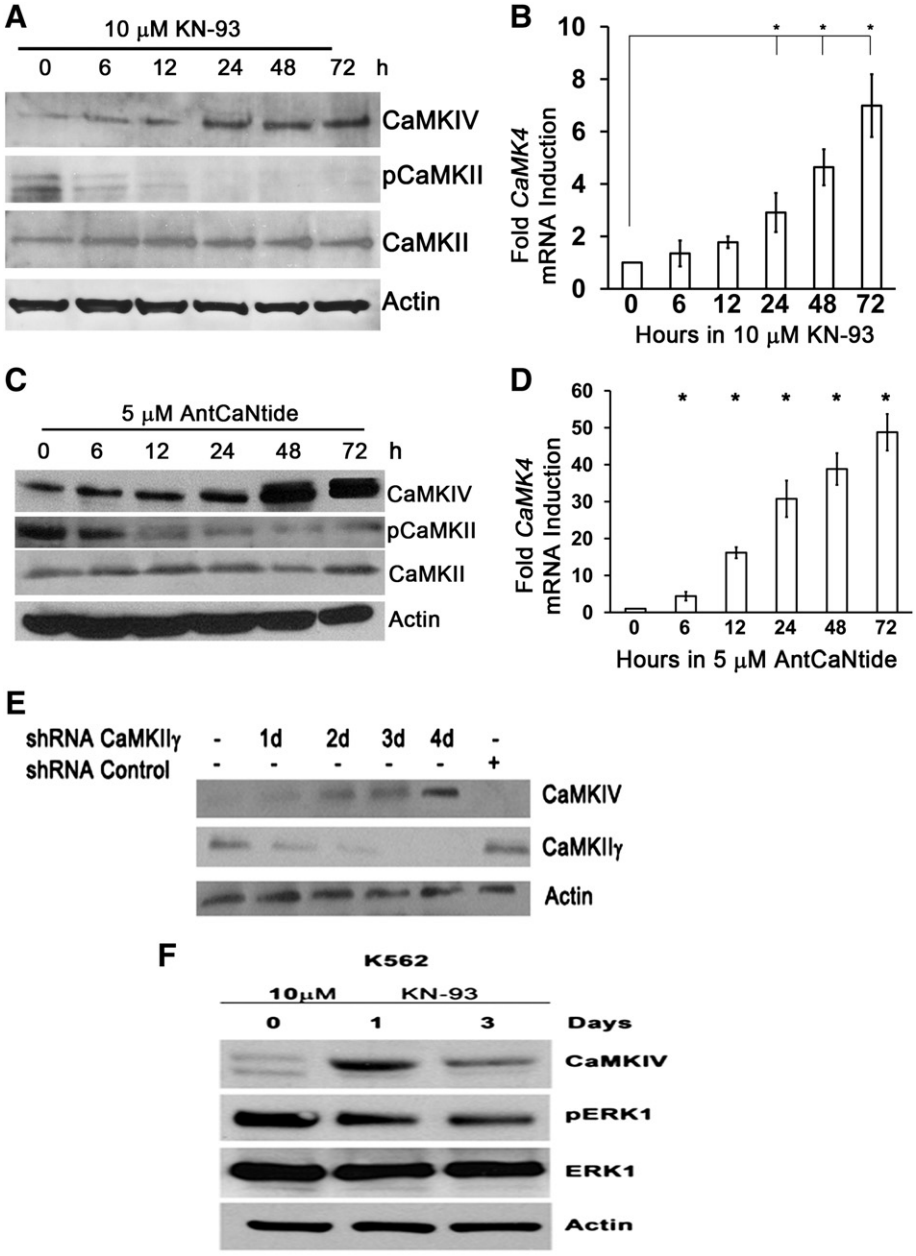

Fig. 1. Pharmacological inhibition of CaMKII induces the expression of CaMKIV. A) KN93-mediated inhibition of CaMKII activity results in the elevation of CaMKIV protein. Representative immunoblots showing levels of CaMKIV, pT286CaMKII (p-CaMKII), total CaMKII and Actin (loading control) in U937 cells treated with 10 μM KN93 for indicated time points (n = 3). B) Inhibition of CaMKII by KN93 results in the upregulation of *CaMK4* mRNA. Average *CaMK4* mRNA levels, normalized to internal control β-Actin, in U937 cells at 0, 6, 12, 24, 48 and 72 h after treatment with 10 μM KN93 from three experiments are shown. Data are indicated as fold induction over untreated (0 h time point); error bars represent ± standard deviation (SD); **p*-value < 0.05. C) Inhibition of CaMKII activity using the highly specific inhibitory peptide AntCaNtide results in highly increased CaMKIV protein level. Immunoblots depicting CaMKIV and Actin in U937 cells treated with 5 μM AntCaNtide for the indicated time points (n = 3). D) Treatment of U937 cells with 5 μM AntCaNtide results in a significant upregulation of *CaMK4* mRNA from 6 h to 72 h. Average ± SD *CaMK4* mRNA levels (normalized to β-Actin), measured by qRT-PCR assays and indicated as fold induction over control (0 h), are shown; n = 3; *p*-value < 0.001. E) U937 stable transfected with shRNA CaMKIIγ were cultured 4 days and show an increase of CaMKIV protein expression (n = 3). F) KN93-mediated inhibition of CaMKII activity results in the elevation of CaMKIV protein in K562 cells . Representative immunoblots showing levels of CaMKIV, p ERK, ERK and Actin (loading control) after treatment with 10 μM KN93 for indicated time points; (n = 3).

transactivation of the *CaMK4* promoter. To investigate this, we performed transient transactivation assays with a *CaMK4* promoter construct containing RRE, upstream of a fire-fly luciferase reporter gene [33] and found that RAR-β and COUP-TF conferred an 11-fold transactivation of the *CaMK4* transgene, in the presence of RA (Fig. 2). However, co-transfection of full-length CaMKII resulted in a significant, 2-fold repression of RA/RAR-β/COUP-TF/RA-mediated transactivation of *CaMK4* promoter (Fig. 2). We concluded that CaMKII inhibits *CaMK4* gene expression by repressing its transcription.

3.2. Overexpression of CaMKIV in U937 cells inhibits CaMKII activation

CaMKIV is only slightly expressed in U937. To determine if CaMKIV expression could affect CaMKII expression or activation, we expressed the full-length CaMKIV using a Lenti-GFP virus system (CaMKIV) [37], or a Lenti-GFP virus as a control (Mock). Treatment of U937 cells with the Ca^{2+} ionophore ionomycin induces the autophosphorylation of CaMKII (p-CaMKII). This elevation in p-CaMKII levels was blocked in cells expressing CaMKIV (Fig. 3A). These data suggest that CaMKIV

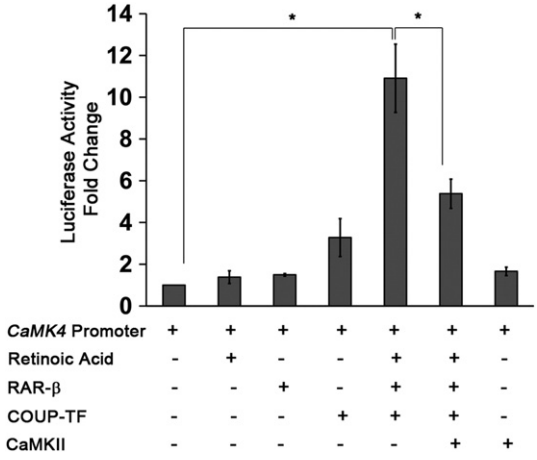

Fig. 2. CaMKII represses *CaMK4* transactivation. CaMKII inhibits RA-dependent transactivation from RRE of *CaMK4* promoter by RAR-β/COUP-TF complexes. Luciferase activity representing transactivation from *CaMK4* promoter, following 48 h of treatment with 2 μM RA, in the presence of RAR-b, COUP-TF and/or full-length CaMKII is shown. Data indicated as average (±SD) fold induction over promoter-only constructs from n = 6 experiments; * *p*-value <0.0008.

suppresses CaMKII phosphorylation in U937 cells. Also ionomycin-induced elevation of ERK phosphorylation was repressed in CaMKIV-U937 cells. Suppression of CaMKII activation by CaMKIV was further confirmed by *in vitro* kinase assays using CaMKII immunoprecipitates from Mock and CaMKIV-U937 cells stimulated with ionomycin (Fig. 3B). Ionomycin induced activity of CaMKII was significantly inhibited in U937 cells over-expressing CaMKIV. Altogether, these data indicate that CaMKIV suppresses the autonomous activity of CaMKII in U937 leukemia cells.

3.3. Increased expression of CaMKIV in U937 cells restricts cell proliferation

CaMKII is a potent stimulator of growth factor-induced cell proliferation in several normal and cancer cells including U937 leukemia cells [23,26]. As over-expression of CaMKIV suppresses CaMKII activity, we hypothesized that CaMKIV would inhibit CaMKII-dependent proliferation of U937 cells. We measured proliferation of CaMKIV-U937 and Mock-U937 cells in regular growth medium for three days, and found that the overexpression of CaMKIV in U937 cells does indeed result in a significant retardation of cell growth (Fig. 4A). To understand whether the retardation of cell growth induced by CaMKIV was dependent on its kinase activity, we expressed a kinase-inactive mutant of CaMKIV [38] in U937 cells and found that this mutant does not affect proliferation (Supplementary Fig. S2A). This observation suggests that the effect of the overexpression of CaMKIV on cell proliferation requires its kinase activity and is not due to an indirect, non-specific effect. To determine if the lack of increase in cell number is due to apoptosis in we tested Annexin V/propidium iodide (PI) reactivity. As shown in Fig. 4B, we did not observe any significant difference in apoptosis between CaMKIV- and Mock- U937, either in the presence or the absence of serum (FBS), indicating that CaMKIV-induced inhibition of proliferation was not secondary to a cell survival defect. On the contrary the growth retardation observed in CaMKIV-U937 was due to reduced cell proliferation, as shown by [^{3}H] thymidine (T) uptake assay, in Fig. 4C. A similar 2-fold inhibition of [^{3}H] T uptake was observed in Mock-U937 cells treated with either KN-93 or AntCaNtide, whereas these inhibitors had no effect on [^{3}H] T uptake by CaMKIV-U937 cells. Taken together, these data support the hypothesis that CaMKIV expression negatively regulates cell proliferation.

3.4. CaMKIV induces G_0/G_1 cell cycle arrest in U937 leukemia cells

We analyzed the cell cycle profile of U937 cells over-expressing CaMKIV to determine at which stage of the cell cycle CaMKIV played its inhibitory role. Flow-cytometric analysis of DNA content showed a significant 3.5-fold increase in the percentage of cells arrested in the G_0/G_1 phase in CaMKIV-U937cells. Conversely, the fraction of CaMKIV-U937 cells in S and G_2/M phase was significantly decreased (Fig. 5A), suggesting that over-expression of CaMKIV in myeloid leukemia cells results in G_0/G_1 arrest, a feature that is consistent with cell quiescence. We therefore probed these cells with an antibody against the nuclear protein Ki-67, which is present in all cycling cells but uniquely absent in quiescent (G_0) cells [39,40]. By combining Ki-67 reactivity with DNA content analysis (7AAD), we assessed G_0, G_1 and S-G_2-M populations (Fig. 5B) and found that expression of CaMKIV increases the number of Ki-67 and 7AAD double negative cells (G_0) (Fig. 5Bi and Bii). This accumulation of CaMKIV-U937 cells in the G_0/G_1 phase of the cell cycle requires its kinase activity as expression of a CaMKIV-K71M mutant did not elicit a G_0/G_1 arrest in U937 cells (Supplementary Fig. S2). CaMKIV-mediated cell cycle arrest was further confirmed through BrdU labeling combined with 7AAD profile analysis. Thus, a significantly lower population of CaMKIV-U937 cells entered S-phase of the cell cycle compared to Mock U937 (Fig. 5Ci and Cii), further indicating that expression of CaMKIV in U937 cells results in the induction of G_0/G_1 cell cycle arrest.

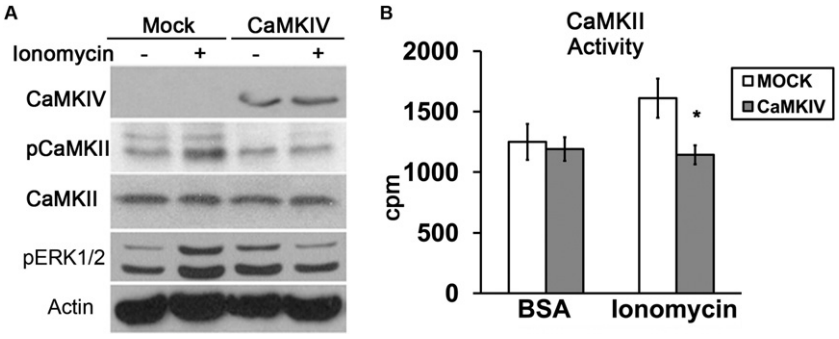

Fig. 3. CaMKIV inhibits CaMKII activity in U937 leukemia cells. A) Expression of CaMKIV in U937 cells results in the inhibition of CaMKII activation. U937 cells were infected with Lenti-GFP-virus (Mock) or Lenti-GFP-CaMKIV virus (CaMKIV) and stimulated with ionomycin (2 μM, 15 min). Levels of CaMKIV, p-CaMKII, total CaMKII, p-ERK and Actin were visualized by immunoblot analyses (n = 3). B) CaMKIV effect on CaMKII activation was determined by in vitro kinase assays. Endogenous CaMKII was immunoprecipitated from Mock or CaMKIV-U937 cells stimulated for 15 min with 2 μM ionomycin. The activity of immunoprecipitated CaMKII was measured by in vitro kinase assays using its specific peptide substrate, Autocamtide. Results are presented as total incorporated counts per minute (cpm). Average ± SD values from n = 4 experiments are shown. *p-value ≤ 0.05.

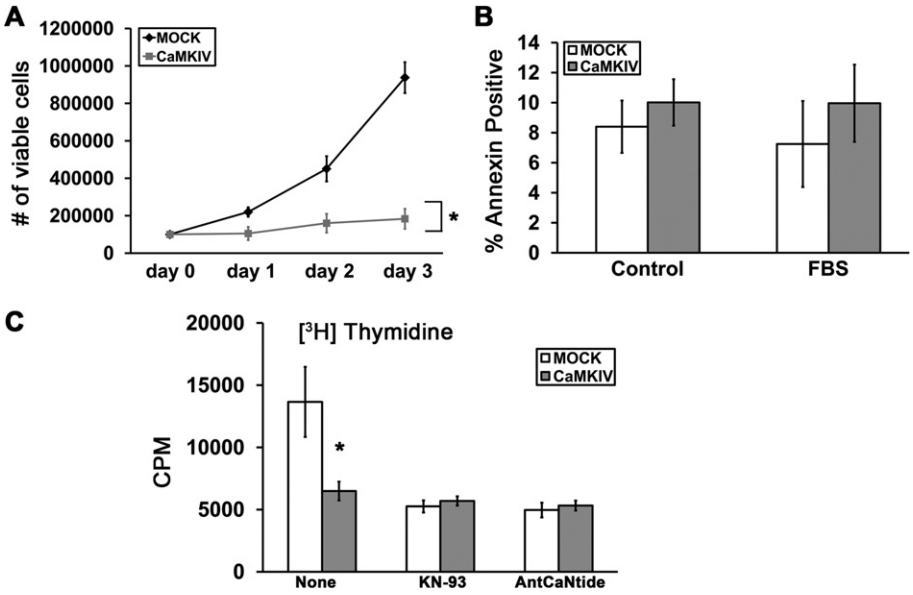

Fig. 4. Over-expression of CaMKIV in U937 inhibits cell proliferation. A) Expression of CaMKIV significantly restricts U937 cell proliferation. Mock or CaMKIV U937 cells were seeded in triplicate in 6-well dishes and the total number of viable cells was assessed daily over a period of three days. Average ± SD numbers of viable cells from n = 6 experiments are shown; *p*-value < 0.001. B) Expression of CaMKIV in U937 cells does not induce apoptosis. AnnexinV reactivity in Mock and CaMKIV U937 cells was measured during homeostasis and/or following stimulation with 5% FBS. Data are reported as average ± SD of n = 3 experiments. C) CaMKIV restricts [^{3}H] thymidine incorporation into U937 cells. Mock and CaMKIV U937 cells were serum starved overnight and treated with vehicle (none), 10 μM KN93 and/or 5 μM AntCaNtide for 30 min. FBS and [^{3}H] thymidine were then added and radioactivity measured after 24 h by liquid scintillation counting. Average cpm incorporation in cells from n = 3 experiments is shown; *p*-value < 0.02.

3.5. CaMKIV expression in U937 induces upregulation of Cdk inhibitors $p27^{kip1}$ and $p16^{ink4a}$ and downregulation of cyclins A, B1 and D

Progression of cells through the G_1-S phase of the cell cycle requires hyper-phosphorylation of the retinoblastoma protein (Rb; p-Rb) by G_1 cyclin-Cdk complexes and subsequent activation of the E_2F family of transcription factors [41]. To probe the mechanism(s) by which CaMKIV orchestrates G_0/G1 arrest in U937 cells, we analyzed the expression levels of G_1-CDKIs. Mock-U937 cells displayed a typical expression profile of actively proliferating tumor cells, with low levels of $p27^{kip1}$ and barely detectable $p16^{ink4a}$ (Fig. 6A, densitometry showed in Supplementary Fig. S3A). In addition, they possessed markedly elevated p-Rb levels, both in basal conditions and following FBS stimulation. In contrast, in CaMKIV-U937 we observed significant downregulation of p-Rb in CaMKIV-U937 cells and an upregulation of $p27^{kip1}$ and $p16^{ink4a}$, consistent with a G_0/G1 arrest (Fig. 6A and Supplementary Fig. S3A). These data suggest that modulation of G_1-CDKIs expression is a key mechanism by which CaMKIV may suppress U937 cell proliferation.

We further examined the modification of cyclin expression in U937 cells. CaMKIV-U937 expressed significantly lower levels of cyclin D1 under basal conditions and following stimulation with FBS, which explains the reduced proliferation and G_0/G_1 arrest that we observed in U937 cells over-expressing CaMKIV.

We also evaluated whether over-expression of CaMKIV modulates the expression of cyclins A and B1, which regulate entry of cells into G2/M phases of the cell cycle in a CaMKII dependent manner [6,7]. In comparison to control, CaMKIV-U937 showed lower levels of cyclin A in both basal and stimulatory (FBS) media (Fig. 6B and Supplementary Fig. S3B), suggesting the reduced ability of these cells to progress through the S-G2 phases of the cell cycle. Interestingly, a product of degradation of Cyclin A was detectable only in CaMKIV-U937 cells, which is consistent with enhanced turnover of cyclin A, possibly due to elevated $p27^{kip1}$ [42]. Furthermore, the levels of cyclin B1 after serum stimulation were also decreased in CaMKIV-U937. (Fig. 6B and Supplementary Fig. S3B), indicating that CaMKIV could also impair G2-M phases entry (Fig. 6B and Supplementary Fig. S3B). Our data suggest that the expression of CaMKIV represses cell proliferation by forcing the cells into a G_0/G_1 arrest via upregulation of CDKIs $p27^{kip1}$ and $p16^{ink4a}$.

3.6. CaMKII and CaMKIV expression in primary AML cells and correlation with the FAB/WHO phenothype

We also studied CaMKIV and CaMKII crosstalk in primary leukemic BM cells by Western blotting analysis. As shown in Fig. 7A, 4 AML patients showed increased expression of CaMKIV associated with simultaneous decreased activity of CaMKII (CaMKIV+/pCaMKII− patients: 10, 14, 9, 17), whereas 10 AML patients showed decreased expression of CaMKIV associated with simultaneous increase of CaMKII activity (CaMKIV−/pCaMKII+ patients: 6, 3, 12, 13, 11, 8, 18, 19, 20, 21); we could not find an inverse correlation between CaMKIV expression and CaMKII activation only in the remaining 7 AML patients (n: 1, 2, 4, 5, 7, 15, 16).

In addition, we documented that all AML patients with the characteristic immature immunophenotype MO/M1 according to WHO classification showed increased expression of CaMKIV and decreased pCaMKII. By contrast, 10/13 AML patients with high differentiation immunophenotype M4/M5 showed decreased expression of CaMKIV and increased pCaMKII (CaMKIV−/pCaMKII+). We also performed a Western blot analysis for pCaMKII and CaMKIV, normalized for CaMKII, on normal bone marrow cells as a control (Fig. 7B). Neither pCaMKII nor CaMKIV were expressed in normal BM samples.

3.7. Inhibition of CaMKII activity elevates CaMKIV expression in primary monocytes from LMA patients

Since we observed CaMKIV and CaMKII crosstalk in leukemic BM cells, we also analyzed CAMKIV expression in primary monocytes of LMA patients following KN93 exposure. Monocytes were isolated from peripheral blood obtained from LMA-patients. Monocytes were serum-starved for 1 h and treated with 10 μM of KN93 for 3, 6 and 24 h. CaMKIV and CaMKII expression were analyzed by Western blot.

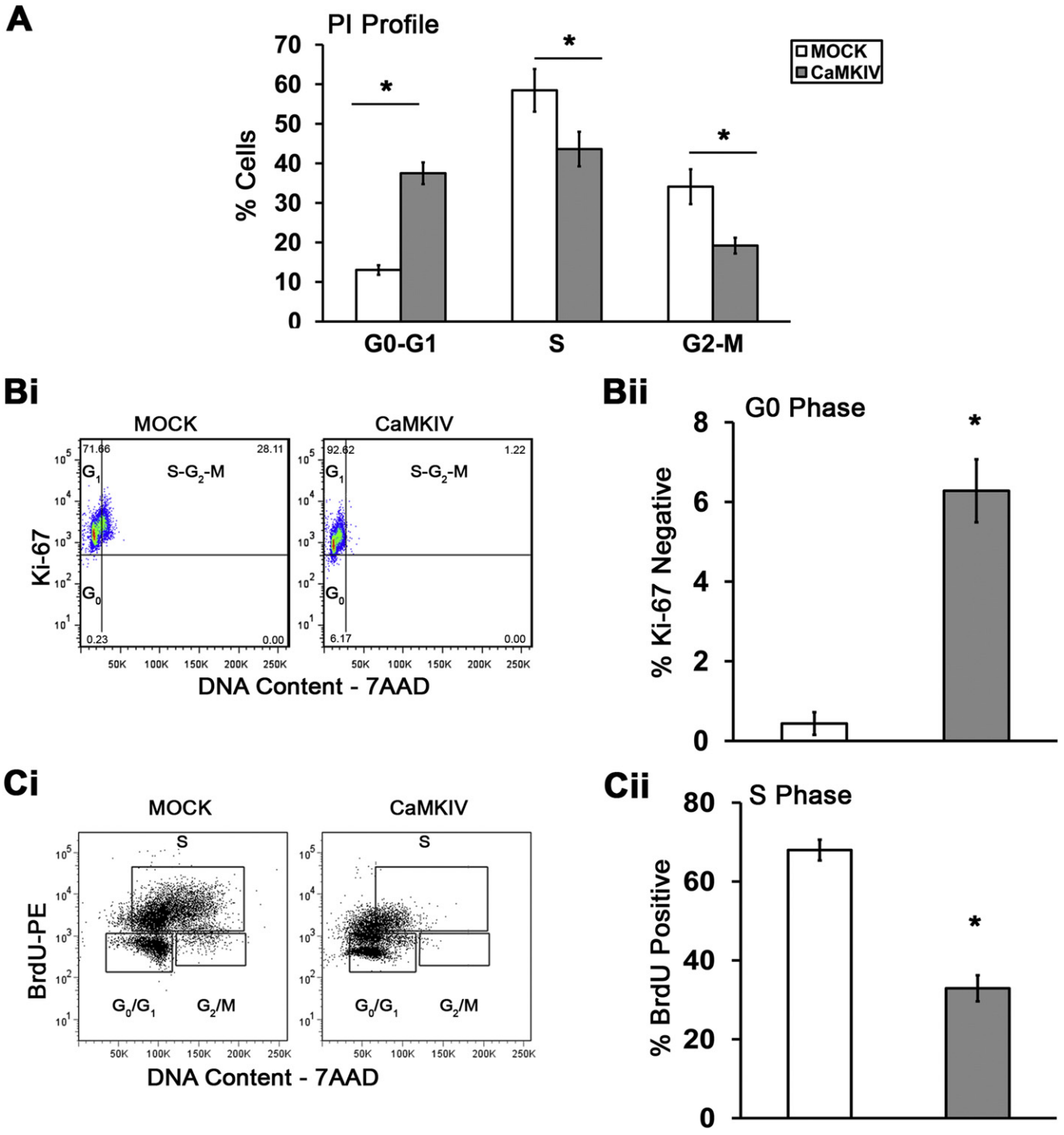

Fig. 5. CaMKIV induces G_0/G_1 cell cycle arrest in U937 cells. A) CaMKIV expression results in the accumulation of U937 cells in the G_0/G_1 phase of the cell cycle. Propidium iodide (PI) profile of Mock and CaMKIV U937 cells showing the average (n = 4) percentage of cells in each phase of cell cycle; error bars represent ± SD; *p-value < 0.01. Bi) CaMKIV expression triggers quiescence in U937 cells. Representative (n = 3) dot plots depicting Ki-67 reactivity versus DNA content (7AAD) analyses in Mock and CaMKIV U937 cells. Bii) Average (±SD) Ki-67/7AAD double negative cell populations, representing G_0 phase of the cell cycle, were calculated from three independent experiments; p-value < 0.001. Ci) CaMKIV restricts entry of U937 cells into the S phase of cell cycle. Representative (n = 3) histograms depicting BrdU incorporation versus DNA content (7AAD) in Mock and CaMKIV U937 cells. Cii) Boxed gates representing G_0/G_1, S and G_2/M phases of cell cycle were used to calculate the percentage of cells in the S-phase of the cell cycle in the indicated genotypes. The histograms represent the % of cells in S phase. Data are reported as average ± SD of n = 3 experiments; p-value < 0.001.

As shown in Fig. 8 whereas the levels of total CaMKII did not change, autophosphorylation of CaMKII (p-CaMKII) was significantly reduced following 3 and 6 h of KN93 treatment, and completely inhibited at 24 h. Of note, low constitutive expression of CaMKIV in primary monocytes increased as a function of time following exposure to KN93 (Fig. 8).

4. Discussion

The present study is the first demonstration of the reciprocal role of CaMKII and CaMKIV on cell proliferation in cancer cells. We demonstrated that CaMKII activation in leukemia blasts inhibits CaMKIV expression. A role for CaMKII in the regulation of proliferation and

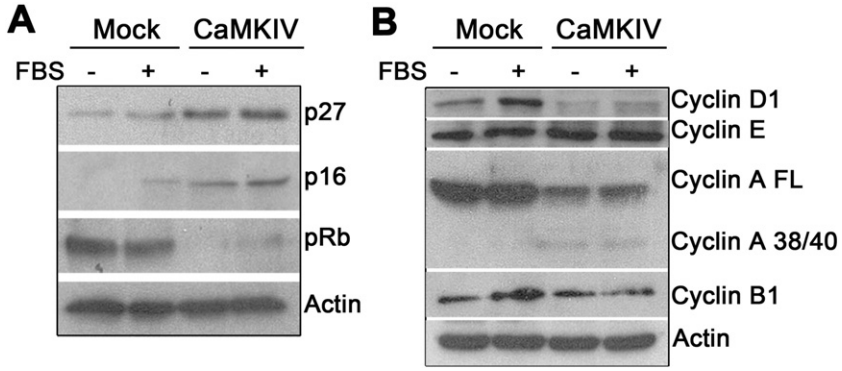

Fig. 6. CaMKIV over-expression triggers the upregulation of CDKIs $p16^{ink4a}$ and $p27^{kip1}$ and a concomitant downregulation of cyclins D1, A and B1 in U937 cells. A) CaMKIV expression in U937 cells leads to significantly enhanced $p16^{ink4a}$ and $p27^{kip1}$levels and a concurrent inhibition of Rb phosphorylation. Immunoblots depicting $p16^{ink4a}$, $p27^{kip1}$, p-Rb and Actin in Mock and CaMKIV U937 cells, during homeostasis and following FBS stimulation are shown. B) Forced expression of CaMKIV results in decreased levels of cyclins D1, A and B1; and in the proteolytic cleavage of cyclin A. Cell lysates from Mock and CaMKIV U937 cells were subjected to immunoblot analyses with antibodies against cyclins D1, E, A and B_1. Positions of full-length (60 kDa) and cleavage intermediates (38 kDa and 40 kDa) of cyclin A are indicated.

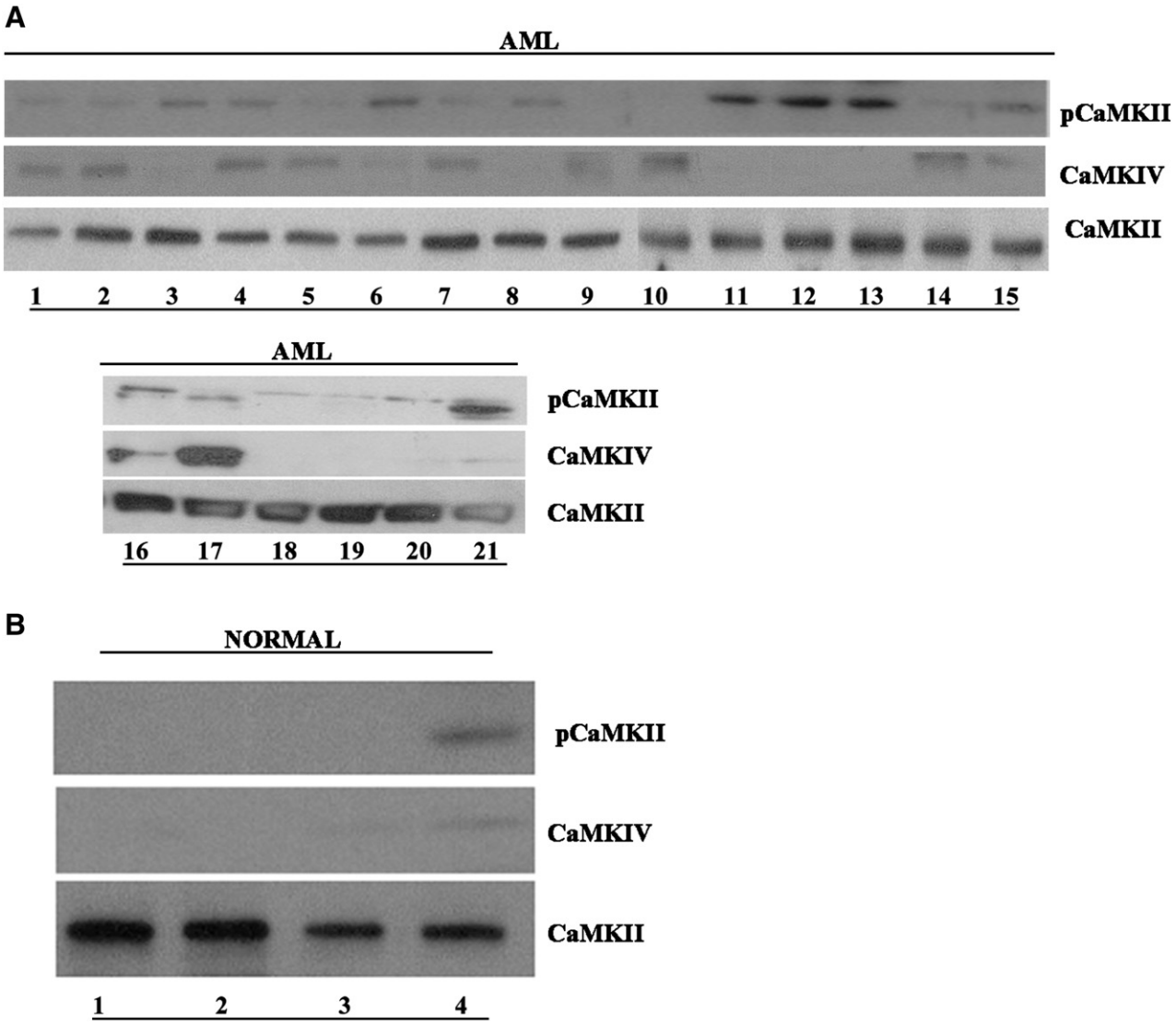

Fig. 7. Cross-regulatory relationship between CaMKII and CaMKIV in primary acute myeloid leukemia (AML) cells. A) Bone marrow samples were obtained, after informed consent, during diagnostic procedures from 21 patients with AML. Collected speciemens were analyzed by Western blotting to evaluate CaMKII activation and CaMKIV expression. 4 AML patients showed increased expression of CaMKIV associated with simultaneous decreased activity of CaMKII, whereas 10 AML patients showed decreased expression of CaMKIV associated with simultaneous increase of CaMKII. B) Samples of normal bone marrow were analysed by Western blot for pCaMKII, CaMKII and CaMKIV.

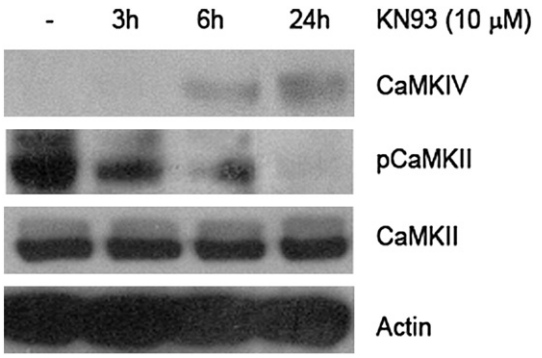

Fig. 8. Pharmacological inhibition of CaMKII induces the expression of CaMKIV in primary monocytes. KN93-mediated inhibition of CaMKII activity results in the increase of CaMKIV protein. Representative immunoblots showing levels of CaMKIV, pT286CaMKII (p-CaMKII), total CaMKII and Actin (loading control) in primary monocytes isolated from peripheral blood of LMA-patients, treated with 10 μM KN93 for indicated time points (n = 3).

cell cycle has been demonstrated both in normal and tumor cells [3,7,22,25]. One of the mechanisms by which CaMKII stimulates growth factor-induced cell proliferation is the activation of the ERK pathway [20,43]. It has been reported that activation of CaMKII is essential for cell proliferation by facilitating G_1-S, G_2-M and metaphase to anaphase transitions [3,6,44,45]. In contrast, CaMKIV can restrict aberrant proliferation and promote survival of hematopoietic cells by activating the CREB/Bcl2 pathway [12,46]. However, a cross-talk between these two multifunctional CaMKs in the modulation of cell physiology has never been reported. In the present study we demonstrate a novel interplay between CaMKII and CaMKIV, which could have implications in the control of leukemia cell proliferation.

Under resting conditions, the human leukemic monocyte lymphoma U937 cells express high levels of CaMKII and very low, barely detectable, levels of CaMKIV. We provide evidences that CaMKII inhibits CaMKIV gene expression by repressing RAR-induced activation of the *CaMK4* promoter. Pharmacological inhibition of CaMKII de-represses the *CaMK4* promoter to enhance CaMKIV mRNA and protein expression in two different cell lines: U937 and K562. We also demonstrated that the pharmacological inhibition of CaMKII by KN93 in primary monocytes, increases the levels of CaMKIV as a function of time (Fig. 8). It has been demonstrated that in several hematopoietic cell lines the expression of CaMKIIγ negatively correlates with the expression of CaMKIV. Whether this counterregulation is casual or mechanistic has been never investigated. We show that forced expression of CaMKIV in U937 cells suppresses CaMKII activity and its pro-proliferation function. Specifically, CaMKIV overexpression induces G_0/G_1 cell cycle arrest in U937 cells, through upregulation of CDKIs $p27^{kip1}$ and $p16^{ink4a}$ and downregulation of G_1 and G_2/M cyclins. These findings suggest that, in U937 leukemia cells, CaMKII is a positive regulator of cell proliferation, whereas CaMKIV plays an opposite role, inhibiting cell cycle progression.

Cell cycle arrest subsequent to pharmacological inhibition of CaMKII by KN93 has been reported in several myeloid leukemia cell lines, including U937, and in primary AML patient samples [26,47]. Moreover, CaMKII levels become markedly reduced in leukemia cells undergoing growth arrest and/or terminal differentiation [47]. Results from our study are consistent with these previous findings. Further, they provide additional evidence that the block in cell cycle progression in U937 cells following pharmacological inhibition of CaMKII is, at least in part, due to the re-expression of CaMKIV, which promotes a cell cycle arrest in G_0/G_1.

CaMKIV induced the expression of $p16^{ink4a}$, which plays a pivotal role in cell cycle progression at the G_1-S checkpoint. Rb and $p16^{ink4a}$ acts in a common pathway, where hyper-phosphorylation of Rb results in the activation of E_2F, leading to the expression of cyclin D1, cdc2 and cyclin A. Rb is a tumor suppressor [48] that is associated with the elevation of $p16^{ink4a}$ in different cell models [49–51], which is compromised in senescent fibroblasts and several cancers [51,52]. CaMKIV-mediated enhancement of $p16^{ink4a}$ and subsequent downregulation of cyclin D1 results in G_1 cell cycle arrest. Further, CaMKIV expression elevates $p27^{kip1}$ in U937 cells. Previous reports have demonstrated that CaMKII

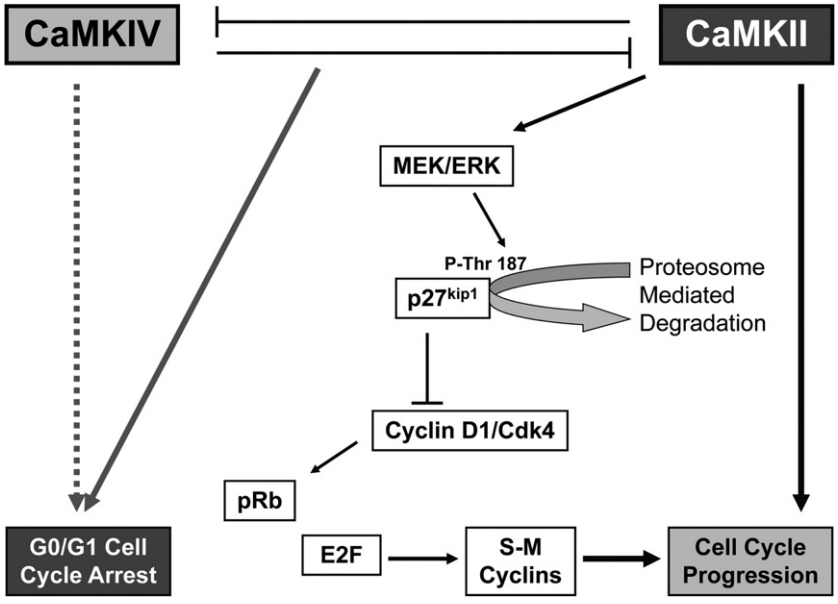

Fig. 9. Cross regulatory relationship between CaMKII and CaMKIV in the regulation of leukemia cell proliferation. In U937 cells CaMKII is expressed at very high levels while CaMKIV is barely detectable. CaMKII suppresses CaMKIV transcription whereas CaMKIV over-expression promotes the inhibition of CaMKII autonomous activity and cell cycle arrest. Previous studies have shown that CaMKII regulates the half-life of $p27^{kip1}$ by mediating its degradation via the Mek/Erk pathway [43]. Therefore, we hypothesize that by suppressing CaMKII activity, CaMKIV inhibits the proteasome-mediated degradation of $p27^{kip1}$ via the CAMKII-Erk pathway. Stabilized $p27^{kip1}$ will then inhibit Cdk4/cyclinD1 complexes, and therefore Rb phosphorylation and E2F activation. As a consequence, cell cycle progression is blocked. In this case, CaMKIV promotes cell cycle arrest (solid gray arrow). Further, expression of CaMKIV in U937 cells elevates $p16^{ink4a}$ and represses cyclin A levels through unknown mechanisms and elicits cell cycle arrest (denoted by the dashed gray arrow). We conclude that CaMKIV and CaMKII oppose each other in the regulation of myeloid leukemia cell proliferation, with CaMKII promoting proliferation and CaMKIV promoting quiescence.

is a negative regulator of $p27^{kip1}$, mainly through its activation of the ERK pathway and subsequent proteolytic degradation of $p27^{kip143}$. Thus CaMKIV may cause the upregulation of $p27^{kip1}$ in U937 leukemia cells, indirectly, through its suppression of CaMKII activity (Fig. 6).

Levels of cyclins A, B and D1 were considerably lower in CaMKIV-U937 cells and cyclin A appeared to be proteolytically cleaved. Previous reports suggest that $p27^{kip1}$ elicits the proteolytic cleavage of cyclin A within its N-terminal domain to form products that lack mitotic activity [42]. In dividing myeloid progenitor cells, this cleavage of cyclin A is important for the onset of differentiation [53]. The correlation between CaMKIV expression and the appearance of the A38/40 cyclin A fragment is therefore not surprising, as CaMKIV modulates the differentiation of a number of cell types, including neurons, osteoclasts, and dendritic cells [12,27,28,46,54].

Of note, we found a similar regulatory cross-talk between CaMKII and CaMKIV in primary AML cells. Indeed, CaMKII activation was associated with CaMKIV down-regulation and, conversely, CaMKIV over-expression with increased CaMKII activation. Moreover, pCaMKII activation mediating concurrent CaMKIV downregulation was associated with differentiated M4/M5 AML phenotype, whereas pCaMKII inhibition mediated by CaMKIV upregulation was associated with immature M0/M1 AML phenotype.

This inverse correlation between CaMKIV and CaMKII activation, although assessed in a limited number of samples, suggests a cross regulatory relationship between CaMKII and CaMKIV in the regulation of leukemia cell proliferation and/or differentiation. In these cells, CaMKII represses the expression of CaMKIV. In contrast, overexpression of CaMKIV exerts a negative regulation on CaMKII activation that results in inhibition of the MAPK pathway and cell cycle arrest (Fig. 9).

Although larger studies are needed to identify the mechanisms of the crosstalk between CaMKII and CaMKIV in primary AML cells, and demonstrate the existence of a correlation between pCaMKII/CaMKIV ratio and FAB, our findings suggest that CaMKII and CaMKIV might have opposite roles in transformed leukemia cells and that the balance of their expression and activities can regulate not only cell proliferation, but also drive their differentiation.

The focus of our paper is the reciprocal regulation of CaMKII and CaMKIV as a general feature for cellular proliferation and survival. Indeed, the cellular process we describe in the manuscript is a widely diffuse feature, present in, but not restricted to, AML. Indeed, we analyzed two leukemia cell lines (U937 and K562) and a number of primary AMLs, and in these models we confirm the existence of such regulatory mechanism, even despite different patterns of oncogene expression. In particular, K562 cells are bcr-abl positive, while U937 are not. We also have some experimental data (*paper in submission*) that this regulation between CaMKII and CaMKIV occurs also in solid tumors and in particular in cancer cell lines such as KAT-4 and HT-29. Thyrosine kinase inhibitors, targeting proteins such as BCR-ABL, VEGFR, and Raf are currently used in the therapy of several tumors including leukemia [55,56], The interplay between CaMKII and CaMKIV could allow the identification of novel therapeutic targets in the same pathways to exploit in the treatment of myeloproliferative disorders.

Conflict of interest

The authors declare that there aren't any competing financial interests in relation to the work described.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2014.11.007.

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