

Original Paper

Down-Regulation of CK2 α Leads to Up-Regulation of the Cyclin-Dependent Kinase Inhibitor p27^{KIP1} in Conditions Unfavorable for the Growth of Myoblast Cells

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Key Words

Ck2 α • Cell cycle progression • p27^{KIP1} • Dyrk1B • AMPK

Abstract

Background/Aims: Compelling evidence indicates that CK2 α , which is one of the two catalytic isoforms of protein kinase CK2, is required for cell viability and plays an important role in cell proliferation and differentiation. While much is known on CK2 in the context of disease states, particularly cancer, its critical role in non-cancerous cell growth has not been extensively investigated. **Methods:** In the present study, we have employed a cell line derived from rat heart with inducible down-regulation of CK2 α and CK2 α -knockout mouse tissue to identify CK2-mediated molecular mechanisms regulating cell growth. For this, we have performed Incucyte[®] live-cell analysis and applied flow cytometry, western blot, immunoprecipitation, immunohistochemistry, RT-qPCR and luciferase-based methods. **Results:** Here, we show that lack of CK2 α results in significantly delayed cell cycle progression through G1, inhibition of cyclin E-CDK2 complex, decreased phosphorylation of Rb protein at S795, and inactivation of E2F transcription factor. These events are accompanied by nuclear accumulation and up-regulation of the cyclin-dependent kinase inhibitor p27^{KIP1} in cells and CK2 α -knockout mouse tissues. We found that increased levels of p27^{KIP1} are mainly attributable to post-translational modifications, namely phosphorylation at S10 and T197 amino acid residues catalyzed by Dyrk1B and AMPK, respectively, as silencing of FoxO3A transcription factor, which activates *CDKN1B* the gene coding for p27^{KIP1}, does not result in markedly decreased expression levels of the corresponding protein. Interestingly, simultaneous silencing of CK2 α and p27^{KIP1} significantly impairs cell cycle progression without increasing cell death. **Conclusion:** Taken together, our study sheds light on the molecular mechanisms controlling cell cycle

progression through G1 phase when myoblasts proliferation potential is impaired by CK2 α depletion. Our results suggest that elevated levels of p27^{KIP1}, which follows CK2 α depletion, contribute to delay the G1-to-S phase transition. Effects seen when p27^{KIP1} is down-regulated are independent of CK2 α and reflect the protective role exerted by p27^{KIP1} under unfavorable cell growth conditions.

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Introduction

Cell cycle progression is tightly controlled by the cyclin-dependent kinases (CDKs). These enzymes are regulated at a post-translational level and by their association to specific activating cyclins, and inactivating cyclin-dependent kinase inhibitors (CKIs) [1]. In mammals, the decision to pass through the “restriction point” in late G1 coincides with the D cyclins pairing with CDK4 or -6 while the interaction between cyclin E and CDK2 is active at the G1/S boundary, and the complex between cyclin A and CDK2 modulates cell cycle transition through the S phase [2]. p27^{KIP1} belongs to the CIP/KIP (CDK interacting protein kinase inhibitory protein) family of CKIs and it regulates the G1-S cell cycle transition by specifically inhibiting the cyclin E/CDK2 and cyclin A/CDK2 complexes, respectively [3]. The levels and localization of p27^{KIP1} in cells are controlled by transcriptional mechanisms, post-translational modifications and signaling pathways regulating the ubiquitin-proteasome system [4, 5]. Although one of the major functions of p27^{KIP1} is the inhibition of the G1 cyclin/CDK complexes in response to unfavorable conditions (e.g. lack of mitogenic signals), it is known that this protein may significantly contribute to cell viability while having only a limited effect on the regulation of the cell cycle [6, 7].

Another enzyme that has been linked to the control of various intracellular processes including the regulation of the cell cycle, is protein kinase CK2 (reviewed in [8-11]). CK2 is typically described as a constitutively active heterotetrameric enzyme composed of two catalytic subunits α and/or α' and two regulatory β -subunits. However, mounting evidence has suggested that these three subunits may be functionally specialized, and exist outside the tetrameric complex as individual isoforms (reviewed in [12]). They can display different subcellular localization, expression levels and have independent interaction partners [12-15]. The notion that the three CK2 isoforms have distinct cellular functions is also supported by experiments based on gene targeting with homologous recombination showing that knock-out of CK2 α or CK2 β leads to early embryonic lethality while homozygous deletion of CK2 α' in mice results in a viable offspring, though, the males are infertile and affected by oligozoospermia [16-18].

CK2 has been implicated in every stage of cell cycle progression in eukaryotic cells by forming a complex and/or phosphorylating cell cycle regulatory proteins including PLK1, p53, p21^{WAF1} as well as p27^{KIP1} (reviewed in [9, 12, 19]). The role of CK2 in cell survival has been extensively studied in cancer cell lines and solid human tumors where it has been shown that its increased activity and expression correlate with the degree of malignancy of the tumor and resistance towards induction of cell death (reviewed in [11]). In contrast to the large number of studies focusing on the role of CK2 in human diseases, particularly cancer, the function of CK2 in non-cancerous cells has been partially addressed.

We have recently established a myoblast cell line (hereafter referred to as H9c2-CK2 α -44) derived from rat heart tissue with the ability to induce down-regulation of CK2 α by a doxycycline-inducible shRNA [20]. An important observation that emerged from the study is that lowered expression levels of CK2 α resulted in decreased cell proliferation and a significant slightly increased G1 population over the course of several days. However, unlike cancer cells, reduced cell proliferation was not accompanied by increased cell death either *in vivo* or *in vitro* [20].

The aim of the present study was to shed light on the underlying molecular mechanisms responsible for the observed cell cycle delay and impaired cell proliferation observed in myoblasts upon depletion of CK2 α . Our data show that lack of CK2 α resulted in a strong

inhibition of CDK2 activity and promoted increased levels of p27^{KIP1} both in cells and *in vivo*. Stabilization of p27^{KIP1} was mainly attributable to post-translational modifications implicating AMPK and Dyrk1B as the kinases responsible, at least in part, for this effect. Subsequent siRNA-mediated experiments aiming at decreasing the expression of p27^{KIP1} indicated that lack of proliferation potential induced by the silencing of CK2 α triggered a p27^{KIP1}-dependent protective mechanism in conditions critical for cell growth.

Materials and Methods

Cell culture and treatments

The cell line H9c2-CK2 α -44 was derived from the parental myoblast H9c-2 cell line originated from rat heart (American Type Culture Collection, ATCC, Rockville, MD, USA) and cultivated at 37°C under a 5% CO₂ atmosphere in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Taastrup, Denmark) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany). Cells were added 1 μ g/ml doxycycline (Sigma-Aldrich, Brøndby, Denmark) as indicated in the figure legends. Cell synchronization at G0/G1 was achieved by incubating cells in the presence of 0.1% fetal bovine serum for 48 hours. The cell cycle was resumed by adding complete growth medium and cells were harvested at various time points as indicated in the figure legends.

Down-regulation of protein expression was carried out by RNA interference as previously described [21]. Sets of four small interfering RNA duplexes (ON-TARGET plus SMART pools, Thermo Scientific, Rockford, IL, USA) directed against the mRNA coding for proteins indicated in the figures were used. Wortmannin, aphidicolin, propidium iodide and rat recombinant IGF-I were purchased from Sigma-Aldrich, Mirk/Dyrk1B inhibitor (Compound A) was obtained from Calbiochem (Darmstadt, Germany) while Dorsomorphine was obtained from Tocris Bioscience (Abingdon, United Kingdom).

IncuCyte S3 image capture and analysis

Cells were seeded in 96-well plates or 6-well plates and imaged within 30 minutes from plating or according to experimental conditions shown in the figures, using phase contrast channel in the IncuCyte S3 platform (Sartorius, Göttingen, Germany). Four sets of phase contrast images from distinct regions within each well were taken at intervals indicated in the figures using a 10X objective. IncuCyte S3 image analysis software was set to detect the edges of the cells and to determine their confluence in percentage. Graphs were generated with the IncuCyte image analysis software graph/export functions and Microsoft Excel software.

Preparation of cell lysate, western blot, antibodies, immunoprecipitation and kinase assays

Harvested cells were further processed for SDS-PAGE and subsequent western blot analysis as described in [22]. Proteins were detected by incubating western blot membranes with the following antibodies: polyclonal anti-CK2 α obtained by immunizing rabbits against the human full-length protein sequence; polyclonal anti-CK2 α' obtained by immunizing rabbits with a specific peptide sequence of human CK2 α' (i.e. SQPCADNAVLSSGTAAR); mouse monoclonal anti-CK2 β (KinaseDetect Aps, Odense, Denmark); rabbit polyclonal anti-phospho-Rb (S795), rabbit monoclonal anti-cyclin E1, rabbit monoclonal anti-p27^{KIP1}, rabbit polyclonal anti-AKT, rabbit polyclonal anti-phospho-AKT (T308), rabbit polyclonal anti-acetyl CoA carboxylase (ACC), rabbit polyclonal anti-phospho-ACC (S79), rabbit polyclonal anti-AMPK α , rabbit monoclonal anti-FoxO1, rabbit monoclonal anti-FoxO3A, rabbit monoclonal anti-phospho-FoxO3A (S253), rabbit monoclonal anti-MCM4 and rabbit monoclonal anti-MCM7 (all from Cell Signaling Technology, MA, USA); mouse monoclonal anti-Rb, rabbit polyclonal anti-cyclin A, rabbit polyclonal anti-cyclin D1, rabbit polyclonal anti-E2F, goat polyclonal anti-MCM3, goat polyclonal anti-MCM6, mouse monoclonal anti-p21^{WAF1} and mouse monoclonal anti-FoxO3A (all from Santa Cruz Biotechnology, Heidelberg, Germany); rabbit monoclonal anti-phospho-p27^{KIP1} (S10, Abcam, Cambridge, United Kingdom); mouse monoclonal anti- β -actin (Sigma-Aldrich); rabbit polyclonal anti-phospho-p27^{KIP1} (T197) (R&D Systems, Abingdon, UK); rabbit polyclonal anti-phospho-AKT (S129) (Abgent, San Diego, CA, USA).

Whole cell lysate (500 μ g) was employed for immunoprecipitation of CDK2 using mouse monoclonal anti-CDK2 antibody (Santa Cruz Biotechnology) essentially as previously described [23]. Immunoprecipitated

CDK2 was employed as kinase source in enzyme assay performed for 15 minutes at 30°C in a 25 μ l reaction mixture containing kinase buffer (50 mM Tris/HCl pH 7.4, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 10 mM β -glycerophosphate, 50 μ M ATP, 10 μ Ci [γ -³²P] ATP) and 1 μ g histone H1 (Sigma-Aldrich). Reactions were stopped by adding SDS-PAGE sample buffer and further processed as previously described [22].

Cell cycle analysis and quantification of cell death

Cells were collected by trypsinization after treatment as indicated in the figure legends, washed with PBS and fixed overnight in 70% ethanol at -20°C. Cell cycle analysis was carried out employing a FACSCalibur flow cytometer (BD Biosciences, Franklin Lake, New Jersey, USA) following staining with 20 μ g/ml propidium iodide in the presence of 40 μ g/ml RNase (Sigma-Aldrich). Compensation was applied to eliminate overlap between propidium iodide and GFP emissions due to inducible expression of the protein in the presence of doxycycline. Data acquisition was carried out with Cell Quest Pro Analysis software (BD Biosciences). Determination of the percentage of cells with reduced/fragmented DNA content (i.e. sub-G1 cells) indicates activation of cell death.

Luciferase reporter assay

The transcription activity of E2F-1 was determined by transfecting cells with a luciferase reporter vector (pGL2-AN) carrying the E2F-1 promoter sequence (Addgene plasmid #20950). Control experiments were performed transfecting cells with the empty vector. Transfection of cells was carried out with Lipofectamine 3000 (Invitrogen). After 48 hours from transfection, whole cell extracts were employed for measuring the activity of luciferase applying the Luciferase Reporter Assay System kit according to the manufacturer's guidelines (Promega, Stockholm, Sweden). Luminescence was measured using a Perkin Elmer Victor Light 1420 luminescence counter at 560 nm. Three independent experiments, each prepared in triplicate, were performed with reproducible results.

Immunostaining

Immunostaining was carried out essentially as described in [24] employing rabbit monoclonal anti-p27^{KIP1}, anti-FoxO3A and anti-FoxO1 antibodies (Cell Signaling Technology), respectively. Incubation with the primary antibodies was followed by incubation with biotinylated swine anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) and subsequently streptavidin-conjugated Alexa Fluor 555 (Thermo Fisher Scientific). Cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and analyzed on a Leica DMRBE microscope equipped with a DFC 420C camera and Leica Application Suite V 3.3.0 software (Leica Microsystem, Wetzlar, Germany) at 40x magnification.

Immunological analysis of mouse tissue sections was performed essentially as described in [20]. Detection of p27^{KIP1} was carried out staining three somite-paired *WT* and *CK2 α -/-* embryos (*KO*) with rabbit polyclonal anti-p27^{KIP1} (Thermo Fisher Scientific). Nuclear staining of the cells was carried out by incubation with DAPI (Invitrogen). As negative controls, to one section of each slide no primary antibody was added, and to another neither primary nor secondary antibodies were added. Photographs were taken with a Nikon Eclipse TE-2000E/Photometrics CoolSnapHQ2 camera with NIS-elements software at the same magnification and exposure time, and analyzed using ImageJ (NIH, USA).

qPCR analysis

Total RNA from cultured cells was isolated using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and phenol-chloroform extraction. cDNA synthesis was performed using SuperScript® VIL0™ cDNA Synthesis Kit (Invitrogen, CA, USA). 2.5 ng of cDNA was used in triplicate for the qPCR reaction with the SYBR-green premix [FastStart Essential DNA Green Master (Roche Diagnostics, CH)]. The qPCR reaction was run on a Roche LightCycler 480. The absolute quantification method was used to determine the concentration of each sample in relation to a standard curve of 7 (5 -fold) serial dilutions of known concentration, made with a cDNA mix of all the samples, and a no-template control. PCR efficiency values (= 10^(-1/slope) - 1) were between 1.80 and 2.18, and r² = 0.96 or higher. The specificity of each amplification was analyzed by melting curve analysis.

The primers (LGC Biosearch Technologies, Risskov, DK), were designed to span exon-exon junctions of the *Rattus norvegicus* genes and were as follows: *CDKN1B* (TTCGCAAAACAAAAGGGCCA and AGGAAT-TATTCTTAATTCGGAGCTG), and *HPRT1* (CTTCTCTCAGACCGTTT and CATCACTAATCACGACGCTGG).

Statistical analysis

Where indicated, the results were subjected to statistical analysis employing the Student's *t* test. The level of significance is indicated in the figure legends.

Results

Down-regulation of CK2 α causes delayed cell cycle progression through G1 and the G1/S border, molecular mechanism dynamics

Earlier work in our laboratory showed that myoblasts with down-regulation of CK2 α proliferate more slowly and display an increase in the fraction of cells in G1 as compared to the parental cells [20]. To further investigate this, we used the IncuCyte S3 live-cell imaging system to measure cell proliferation employing a real-time kinetic platform. Results shown in Fig. 1 demonstrated that lack of CK2 α induces significant inhibition of cell growth and delayed transition through G1 and/or to S phase. This is consistent with our previous findings [20]. Furthermore, doxycycline did not show any significant effect on the parental cell line (i.e. H9c-2 cells, Supplementary Fig. S1).

To better evaluate the dynamic of S phase entry in cells expressing or depleted of CK2 α we measured the cells' DNA content by flow cytometry following synchronization in G0/G1 induced by serum withdrawal. Synchronized control cells entered the S phase within 10 h after addition of serum, whereas, entry into S phase was delayed to after 12 h in cells depleted of CK2 α (Fig. 2A, B). In order to examine the changes in the expression levels of key signaling molecules controlling progression through G1 phase and beyond, we performed western blot analysis of whole lysate from cells with down-regulated CK2 α . This showed decreased cyclin D1 levels and delayed expression of cyclin E and cyclin A while the phosphorylation of the retinoblastoma protein (pRb) at S795 was similar at 10 h and appeared reduced at later time points as compared to control cells (Fig. 2C). In line with previous observations

Fig. 1. Down-regulation of protein kinase CK2 α leads to decreased proliferation rate and delayed G1/S cell cycle transition. (A) H9c2-CK2 α -44 cells were seeded in 96-well plates (1000 cells/well) in the absence (Control) or presence of 1 μ g/ml doxycycline (+ Dox) in order to induce down-regulation of CK2 α in the cells. Cell growth was analyzed with the Incucyte S3 live-cell analysis system. Data represent mean values +/- standard deviation (STDEV) of n = 8 experiments and expressed in percentage, *P<0.0001. Insert: western blot analysis of whole cell lysate from control cells or cells treated with doxycycline for the indicated times. Western blot membranes were incubated with antibodies against CK2 α and β -actin, respectively. Detection of β -actin served as loading control. (B) The cell cycle profile of cells expressing or lacking CK2 α induced in the presence of doxycycline for the indicated times was determined by flow cytometry analysis following propidium iodide staining. The experiments were repeated three times. Average values are expressed in percentage +/- STDEV.

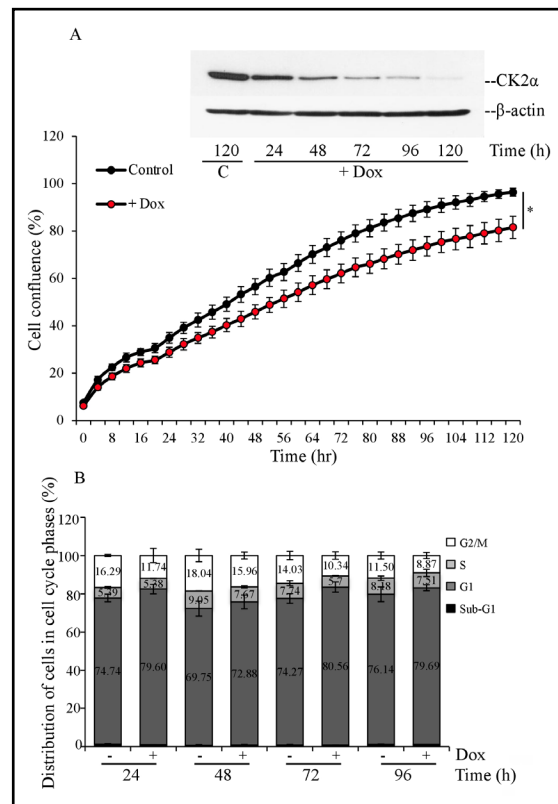
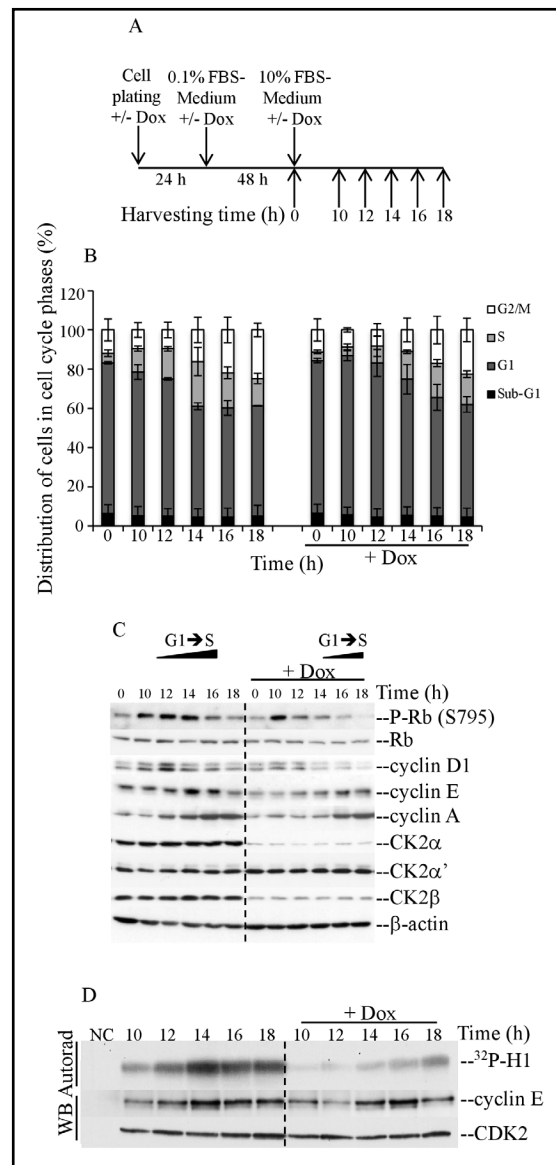


Fig. 2. Silencing of protein kinase CK2 α results in marked delay in cell progression through G1 and inhibition of cyclin E-CDK2 activity. (A, B) 24 h after plating, cells were starved in growth medium containing 0.1% fetal bovine serum for 48 hours in the presence or absence of doxycycline as indicated in (A). Cells were collected at the indicated time points after adding full growth medium and subsequently analyzed by FACS as shown in (B). (C) Cells were treated as indicated in (A) and then harvested for protein expression analysis. Whole cell lysates were examined by western blot employing antibodies against the indicated proteins. (D) Whole lysate from cells treated as described in (A) was subjected to immunoprecipitation in the presence of either control serum (NC) or mouse monoclonal anti-CDK2 antibody. Immunoprecipitates were subjected to phosphorylation assay employing histone H1 as substrate target. Phosphorylation of histone H1 was revealed by autoradiography (Autorad). Immunoprecipitates were analyzed by western blot using the indicated antibodies (WB). Experiments were repeated at least three times obtaining similar results.

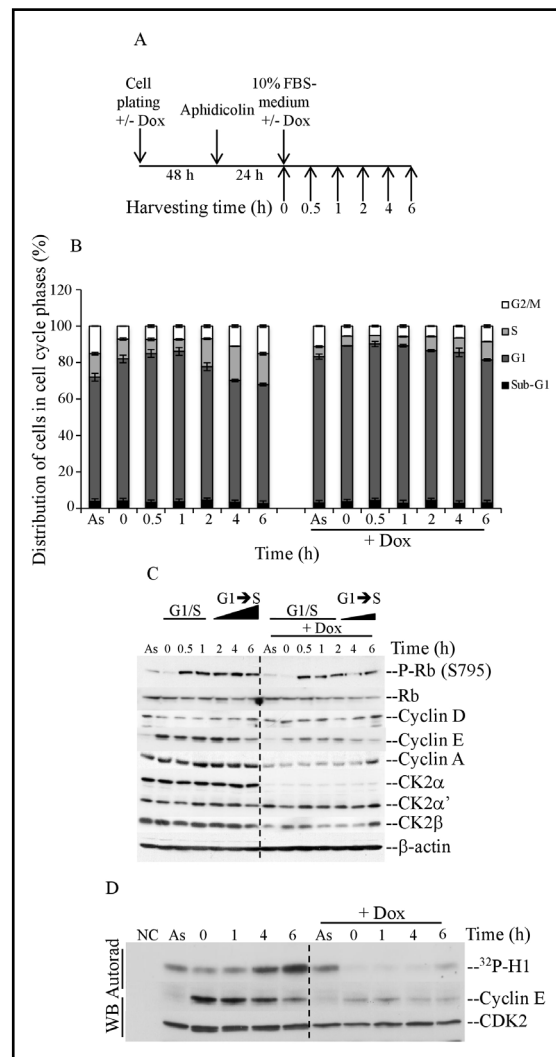


([20] and reviewed in [12]), down-regulation of CK2 α was accompanied by lower expression levels of CK2 β (Fig. 2C) supporting the notion that the stability of CK2 β is dependent on the expression of CK2 α and possibly regulated by an intermolecular phosphorylation event (reviewed in [19]).

Because phosphorylation of pRb at S795 is catalyzed by cyclin D/CDK4 and/or CDK2 in complex with cyclin E and cyclin A, respectively [25], we analyzed the kinase activity of CDK2 which plays a critical role in the G1 phase while mostly bound to cyclin E [2]. Whole lysate from cells harvested at different time points was used in immunoprecipitation assays with antibodies directed against CDK2. The kinase activity in immune complexes was determined by *in vitro* kinase assay using histone H1 as substrate. As shown in Fig. 2D, down-regulation of CK2 α resulted in significantly reduced CDK2 kinase activity. Except at 16 h, this was accompanied by a decreased signal derived from the interacting cyclin E. It should be taken into account, though, that the kinase activity of CDK2 is additionally regulated by the associated cyclin A (results not shown) which contributes to the level of phosphorylation of histone H1 and, thus, of pRb at S795 in cells.

Next, we examined the ability of cells to progress to S phase following incubation with aphidicolin, which is a potent inhibitor of DNA polymerase α , δ and ϵ that reversibly arrests cells at the G1/S boundary ([26] and reviewed in [27]). Control cells arrested at the G1/S border, resumed proliferation and entered S phase within 2 hours after release from the aphidicolin block. In contrast, cells with down-regulated CK2 α resumed the cell cycle and entered S phase after approximately 6 hours after the removal of aphidicolin (Fig. 3A, B). Western blot analysis of whole lysates from control cells showed that the levels of cyclin E decreased gradually as the cells entered S phase and this was accompanied by sustained expression of cyclin A. Conversely, in cells lacking CK2 α , cyclin E expression was weakly detectable while cyclin A signal started to increase at approximately 6 hours after adding full growth medium coinciding with cells actively entering S phase (Fig. 3C). The measurement of CDK2 activity in whole lysates from control cells treated as outlined in Fig. 3A showed an increase in kinase activity for up to 6 hours after release from the aphidicolin block (Fig. 3D). As we did not detect a concomitant increase in the binding to cyclin E, we suspect that CDK2 activity mainly derived from its association with cyclin A. Conversely, we found that CDK2 activity in whole lysates from cells depleted of CK2 α was considerably lower as compared to control experiments. Overall, these results suggest that decreased phosphorylation of pRb at S795 in cells lacking CK2 α (Figs. 2C and 3C) could be attributable to reduced CDK2 kinase activity.

Fig. 3. Release from aphidicolin block causes delay G1/S cell cycle transition in cells depleted of CK2 α . (A, B) Cells were synchronized in the presence of 3 μ M aphidicolin for 24 hours as indicated in (A). After release from aphidicolin block, cells were harvested at the indicated time points and analyzed by flow cytometry (B). (C) Whole cell lysate from cells treated as reported in (A) was examined essentially as described in Fig. 2C. (D) Whole cell lysate was also employed for immunoprecipitation experiments with antibody against CDK2 or in the presence of control serum (NC). Cyclin E-CDK2 activity was measured by H1-based kinase assay. CDK2 and cyclin E protein levels were measured by western blot analysis using antibodies reacting against the indicated proteins. As: asynchronous cells.



Sequential phosphorylation of pRb by cyclin-CDK complexes (i.e. cyclin D-CDK4/6, cyclin E-CDK2, and cyclin A-CDK2) results in the release of the transcription factor E2F from pRb [28]. As E2F is essential for the transcription of genes necessary for entry into S phase (e.g. cyclin E) and for S phase progression (e.g. cyclin A) [29], we further investigated whether lowered expression levels of cyclin E and cyclin A observed in cells lacking CK2 α correlated with changes in the transcription activity of E2F and/or its expression levels. To test this, a reporter assay was carried out employing plasmids containing a 3XE2F promoter fused to the luciferase reporter gene (E2F-1-Luc, [30, 31]). Cells were brought to quiescence by serum withdrawal and then stimulated with growth medium essentially as indicated in Fig. 2A. The activity of endogenous E2F was tested at the indicated time intervals (Fig. 4A). The promoter activity was relatively low in quiescent and early G1 control cells but increased approximately threefold as cells approached the S phase in response to serum stimulation. Conversely, the increase in E2F activity was lower in cells lacking CK2 α under the same experimental conditions. The expression of endogenous E2F was verified by immunoblot and results showed that the levels in control cells were similar to those in doxycycline-treated cells (Fig. 4B). As an independent measure of E2F activity, we analyzed expression of minichromosome maintenance (MCM) proteins that are known E2F gene targets. The MCM complex consists of six closely related proteins (MCM2-7) required for the initiation of S phase by coordinating initiation of DNA replication, when associated to chromatin, as DNA unwinding enzymes [32]. Transcription of the MCM genes is up-regulated in G1 phase by E2F [33-35]. We previously showed that the transcripts coding for MCM proteins were among the most significantly down-regulated in cells depleted of CK2 α [20]. Thus, since *MCM* genes are transcriptional targets of E2F, we also examined the expression levels of the corresponding proteins following stimulation of quiescence cells with growth medium (Fig. 4C). The analysis of the time course experiment showed that serum stimulation induced an

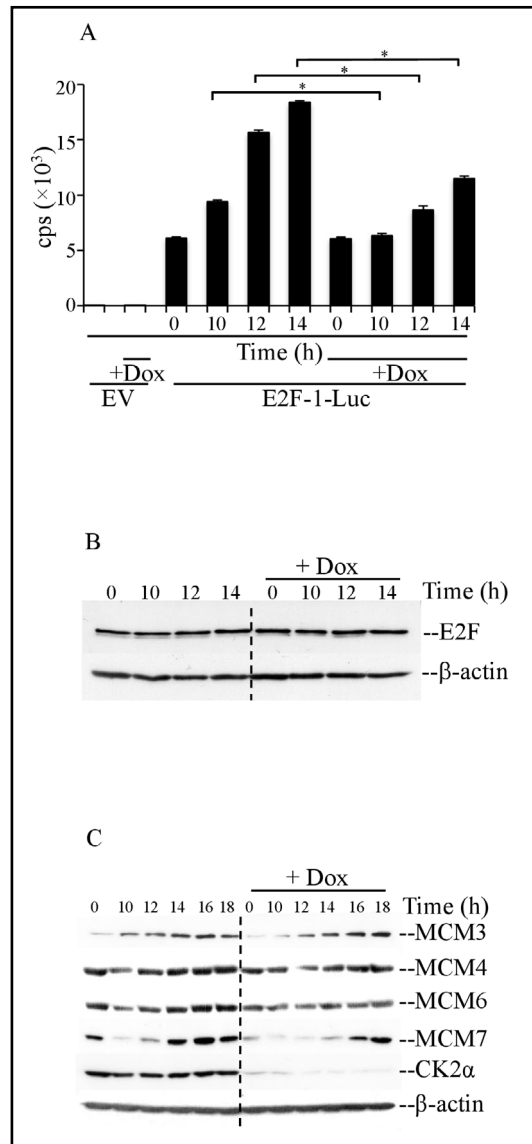


Fig. 4. Cells with reduced expression levels of CK2 α display decreased activity of E2F-1 transcription factor. (A) Cells were transfected with empty vector (EV) or a vector expressing firefly luciferase under the control of the E2F-1 promoter (E2F-1-Luc). Cells were synchronized by serum starvation essentially as described in Fig. 2A and, subsequently, harvested at the indicated time points. E2F-1 transcription activity was measured by a luciferase reporter assay employing 20 μ g whole cell lysate. E2F-1 transcription activity is expressed in counts/s (cps). Mean values \pm SDEV, *P<0.005. (B, C) Expression of E2F and proteins indicated in (C) was analyzed by western blot employing whole lysate from synchronized cells as described in Fig. 2A. Experiments were performed three times obtaining similar results. One representative experiment is shown.

increase in the expression of the MCM proteins as cells entered S phase and it was more pronounced in the control cells than in cells with lowered expression of CK2 α . Interestingly, MCM4 and MCM6 were expressed in quiescent cells and their total expression levels only marginally increased after serum stimulation. Similarly, Arata *et al.* [36] analyzed the expression of MCM4 and MCM7 using a different cell line and demonstrated the persistent abundance of these helicases along the cell cycle, although their association with chromatin was significantly low in quiescent cells.

Overall, these data suggest that the lack of activation of E2F is responsible, at least in part, for the delayed cell cycle progression in cells with reduced levels of CK2 α possibly by affecting the expression of genes coding for proteins (e.g. cyclin E and cyclin A) that play an essential role in S phase entry and progression.

Down-regulation of CK2 α results in up-regulation of p27^{KIP1} in myoblasts and in vivo

The activity of CDK2 is regulated positively and negatively by post-translational modifications and negatively by the association to proteins of the CIP/KIP family (reviewed in [37]). Evidence suggests that p21^{WAF1} and p27^{KIP1} may induce similar effects, however, numerous observations indicate that these CKIs are not biologically equivalent as they have non-overlapping functions and different patterns of expression [38]. In resting cells and early G1 phase, p27^{KIP1} exerts a more prominent role while p21^{WAF1} levels are low and increase during G1 phase progression in response to mitogenic signals [39]. In fibroblasts, the expression of p27^{KIP1} is elevated in G0 and G1 and associated to the cyclin E-CDK2 complex while it is rapidly degraded when cells enter S phase [40, 41]. Given the significant inhibition of CDK2 activity observed in cells lacking CK2 α , we analyzed the expression of p27^{KIP1} in cultured cells synchronized by serum starvation (Fig. 5A). We examined the expression of p27^{KIP1} transcripts from cells treated as shown in Fig. 5A by quantitative PCR. The analysis revealed a significantly higher p27^{KIP1} mRNA levels in quiescent cells with low expression of CK2 α as compared to control cells. However, no further differences were observed between cells expressing or lacking CK2 α (Fig. 5B). Next, immunoblot analysis revealed that the levels of p27^{KIP1} in quiescent cells lacking CK2 α were higher than in control cells and the protein appeared to be present for a longer period of time after serum stimulation (Fig. 5C). In contrast, appreciable differences in the amounts of p21^{WAF1} between control and doxycycline-treated cells were not detected during the entire time-course period.

Distinct post-translational modifications regulate the stability of p27^{KIP1} during G0/G1 to S phase [41]. Specifically, phosphorylation of p27^{KIP1} at S10 correlates with increased stability of the protein and its nuclear accumulation in quiescent cells [41-44]. Phosphorylation of p27^{KIP1} at T197 (T198 in humans) also appears to stabilize the protein in G0 cells (reviewed in [41, 44]). Thus, we examined the levels of phosphorylation of p27^{KIP1} at S10 and T197, respectively, by using phospho-specific antibodies. Immunoblot analysis revealed significantly higher levels of phosphorylation of p27^{KIP1} at S10 in quiescent cells depleted of CK2 α as compared to control cells. In response to serum stimulation, phosphorylation of p27^{KIP1} at S10 appeared to decrease more rapidly in control cells (Fig. 5C). In line with findings reported by Sun *et al.*, [45], phosphorylation of p27^{KIP1} at T197 was not promptly detected in cells arrested in G0, however, the signal raised within 2 hours after release from quiescence and appeared to be stronger in cells lacking CK2 α where it remained detectable for up to 6 hours (Fig. 5C). Phosphorylation of p27^{KIP1} at S10 has been found to regulate this CKI's stability and subcellular localization [42, 46]. Hence, we analyzed the distribution of p27^{KIP1} in myoblasts under experimental conditions described in Fig. 5A and *in vivo*. Cells expressing CK2 α displayed a predominant nuclear pool of p27^{KIP1} and a weak cytoplasmic expression similar to findings reported in mouse embryonic fibroblasts [43] (Fig. 5D and Supplementary Fig. S2A). The localization of p27^{KIP1} in cell lacking CK2 α showed a similar pattern; however, the overall signal was significantly stronger than in control cells supporting the results obtained by western blot analysis (Fig. 5C). Next, immunohistological analysis of p27^{KIP1} expression was performed in tissues of wild-type (+/+) and CK2 α -knockout (-/-) embryos at E10.5 (Fig. 5E, F). This revealed that p27^{KIP1} was mainly localized in the

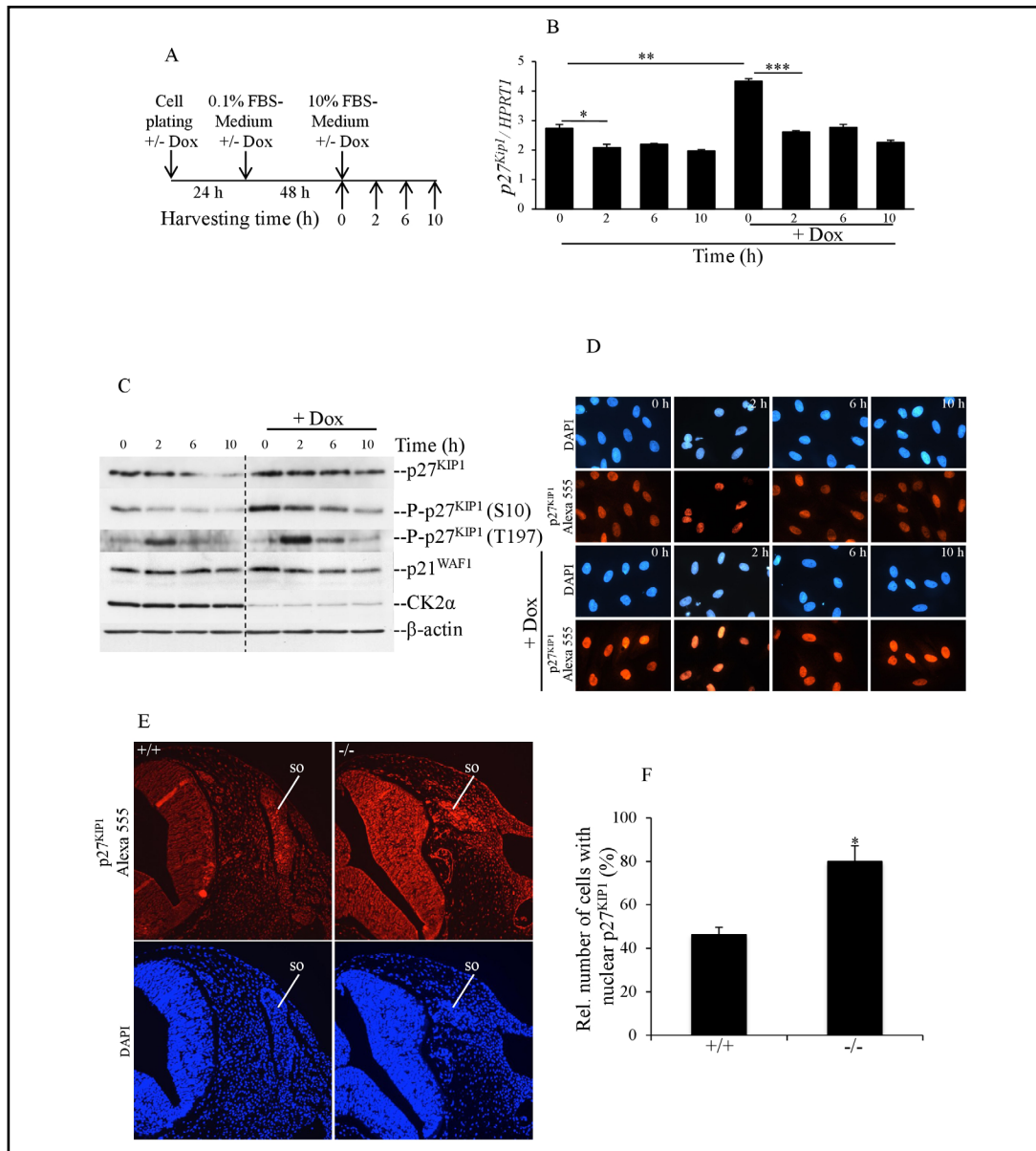


Fig. 5. Delayed G1/S cell cycle transition is accompanied by up-regulation of p27^{KIP1} in myoblasts depleted of CK2 α and in CK2 α -KO mouse embryos. (A) Cells were synchronized by serum starvation in the presence or absence of doxycycline for 48 hours and harvested at the indicated time points after adding full growth medium. (B) Total RNA was isolated and used for reverse transcription quantitative PCR (RT-qPCR). Graph shows the ratio p27^{KIP1}/HPRT1 mRNA. Data are the average of three independent experiments +/- standard error of the mean (SEM). *P = 0.02, **P = 0.0031, ***P = 0.004. (C) Whole lysate from cells treated as described in (A) were analyzed by western blot employing antibodies against the indicated proteins. (D) Immunostaining of cells with rabbit polyclonal anti-p27^{KIP1} and subsequently, a biotin-conjugated secondary anti-rabbit IgG antibody. Expression and localization of p27^{KIP1} was revealed by cell staining with Alexa Fluor 555-conjugated streptavidin. Cell nuclei were visualized by DAPI staining. Cell pictures were taken at 40x magnification. (E) p27^{KIP1} staining of tissue sections from wild-type (+/+) and knockout (-/-) mouse embryos at E10.5, respectively. Photographs were taken at 20x magnification. Fluorescence pictures were pseudo-colored and show p27^{KIP1} nuclear staining (red). Cell nuclei were visualized as in (D). (F) Bar-graph showing in percentage the ratio of p27^{KIP1}-positive cells/total number of cells in the embryos. Three to five sections each from three pairs of wild-type and knockout embryos were analyzed. Values are mean +/- STDEV, *P<0.0005. SO: somite.

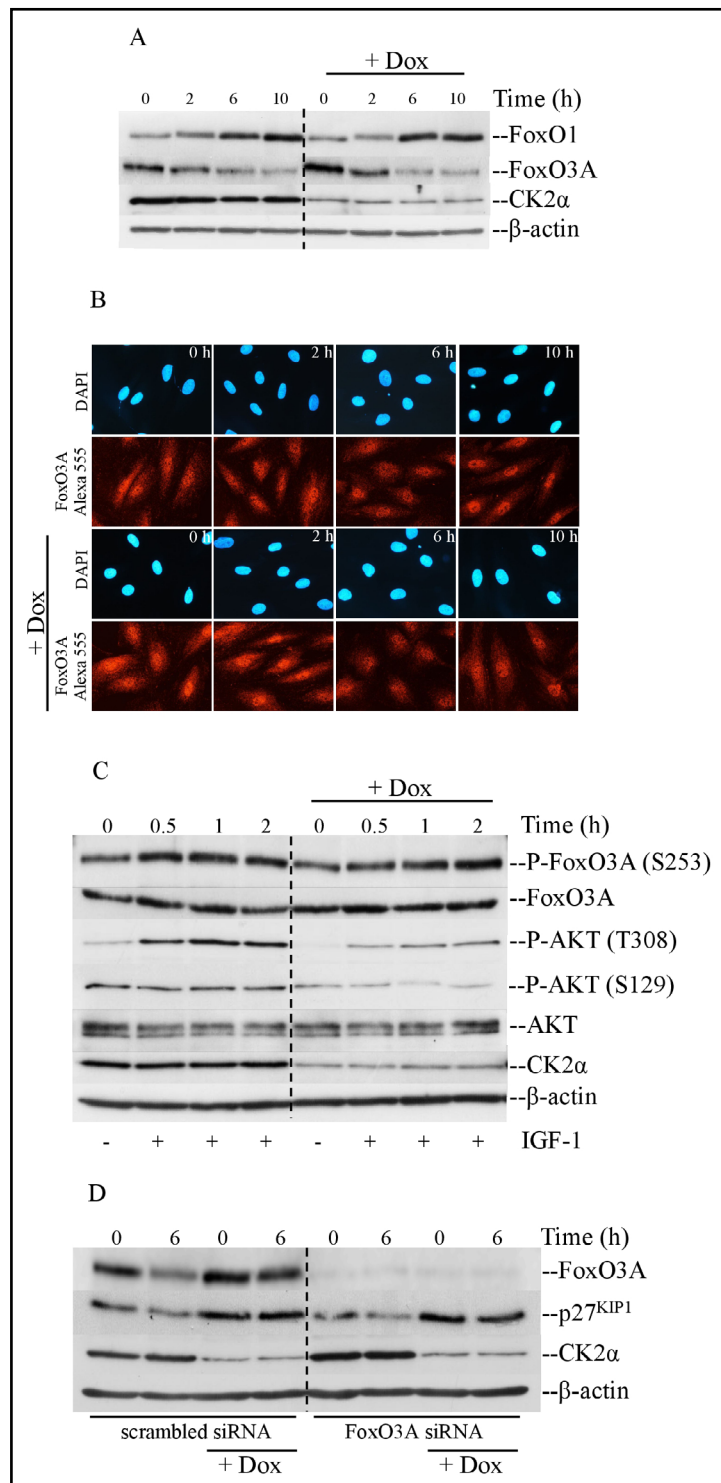
nuclei and the signal was reproducibly stronger than the background signal detected in the negative control (Supplementary Fig. S2B). Interestingly, significant differences in signal intensity could be detected in wild-type and knockout tissue sections, respectively (Fig. 5F), indicating that loss of CK2 α significantly enhances the levels of p27^{KIP1} *in vivo* during early mouse development.

The FoxO transcription factors do not affect the expression of p27^{KIP1} in cells depleted of CK2 α

The mammalian FoxO proteins comprise, among others, FoxO1 (FKHR) and FoxO3A (FKHRL1). These are transcription factors mainly expressed in the nucleus to direct the transcription of specific gene targets controlling cell survival, proliferation, metabolism, DNA repair, and differentiation [reviewed in [47, 48]]. Overexpression of FoxO proteins induces arrest in G1 phase of the cell cycle attributable to the induction of cell cycle regulatory proteins, including the retinoblastoma-related protein p130 and p27^{KIP1} [reviewed in [41, 49]]. Within this family, FoxO3A is reported to be the major regulator of p27^{KIP1} expression [50, 51]. To test whether FoxO proteins contribute to the up-regulation of p27^{KIP1} in cells with down-regulated CK2 α , we analyzed the expression of FoxO1 and FoxO3A in cells synchronized by serum withdrawal (Fig. 6A). The expression of endogenous FoxO1 appeared significantly elevated 6 hours after serum stimulation and remained still well detectable 10 hours afterward; however, no significant differences in FoxO1 levels between control and CK2 α -depleted cells could be detected (Fig. 6A). Conversely, FoxO3A expression was mainly elevated in quiescent cells and up to 2 hours after serum stimulation and it was slightly higher in cells lacking CK2 α than in cells expressing endogenous CK2 α . Serum deprivation induces nuclear localization of FoxO proteins to stimulate gene transcription and post-translational modifications regulate their nuclear/cytoplasmic shuttling [reviewed in [52]]. Based on these findings, we investigated the cellular localization of FoxO3A by staining the cells with a FoxO3A antibody. Immunofluorescence analysis revealed that FoxO3A was more abundantly localized in the nucleus and marginally in the cytoplasm under both serum starvation and stimulation conditions (Fig. 6B and Supplementary Fig. S2C). Moreover, the intensity of fluorescence signal followed the pattern seen by the western blot analysis (Fig. 6A). Analysis of FoxO1 localization indicated that the protein was mainly localized in the nucleus in quiescent cells and in the perinucleus or cytoplasm following serum stimulation. Cells expressing reduced levels of CK2 α showed a stronger signal around the nucleus as compared to control cells (Supplementary Fig. S3A, B).

FoxO proteins are negatively regulated by the PI3K/AKT signaling pathway in response to stimulation with cytokines or growth factors [52]. Activated AKT preferentially phosphorylates an amino acid residue located within the nuclear localization signal, which corresponds to S253 in FoxO3A [52]. This event is followed by FoxO3A nuclear export and degradation [49]. To shed light on the role of AKT in the regulation of FoxO3A phosphorylation, serum-starved cells were stimulated by IGF-1 for increasing amounts of time (Fig. 6C). Analysis of the time-course experiment showed detectable levels of phosphorylation of FoxO3A in quiescent cells. Control cells promptly responded to IGF-1 stimulation within 30 minutes and over the entire time course of the experiment. This was accompanied by a concomitant stimulation of AKT kinase activity as indicated by its phosphorylation at the activating T308 and S129 catalyzed by PDK1 and CK2, respectively [53, 54]. Conversely, activation of AKT was significantly lower in cells depleted of CK2 α and the phosphorylation status of FoxO3A slightly increased above basal levels approximately between 1 and 2 hours after IGF-1 stimulation. These findings suggest that AKT-mediated phosphorylation at S253 might be crucial for the regulation of FoxO3A function and localization in myoblasts. Finally, synchronized cells treated with scrambled siRNA or siRNA against FoxO3A (Fig. 6D) or FoxO1 (Supplementary Fig. S3B) transcripts did not result in a significant change in the expression levels of p27^{KIP1} under the indicated experimental conditions although down-regulation of FoxO3A could induce a partial decrease of p27^{KIP1} mRNA levels (Supplementary Fig. S3C). Overall, these results suggest that the persistently high levels of p27^{KIP1} in cells depleted of CK2 α are mainly a consequence of regulation by post-translational modifications.

Fig. 6. Up-regulation of p27^{KIP1} correlates with slightly higher levels of FoxO3A in cells with lowered expression of CK2 α . However, FoxO3A silencing does not affect the expression of p27^{KIP1}. (A) Cells were serum-starved for 48 hours in the presence or absence of doxycycline and harvested at the indicated time points. Whole cell lysate was analyzed by western blot employing antibodies against FoxO1 and FoxO3A, respectively. Detection of β -actin confirmed equal protein loading. (B) Cells treated as indicated in (A) were labeled with rabbit polyclonal anti-FoxO3A. Proteins were visualized by staining with Alexa Fluor 555-conjugated streptavidin (red fluorescence) after incubation with a secondary anti-rabbit IgG antibody conjugated with biotin. Cellular DNA was detected by staining with DAPI reagent (blue fluorescence). Cell pictures were taken at 40x magnification. (C) Cells were rendered quiescent by serum withdrawal. Subsequently, they were stimulated by adding 100 ng/ml IGF-I and harvested at the indicated time points as shown in the Fig. Whole cell extract was analyzed by western blot employing antibodies against the indicated proteins. (D) Down-regulation of FoxO3A was obtained by transfecting cells with siRNA targeting FoxO3A-mRNA. Control cells were transfected with scrambled siRNA. Cells were synchronized by serum starvation and harvested after 0 hours (i.e. corresponding to quiescent cells) and 6 hours after stimulation. Experiments were repeated at least three times obtaining similar results. One representative experiment is shown.



Dyrk1B and AMPK contribute to stabilize p27^{KIP1} in CK2 α -depleted cells in quiescent cells and in early G1 phase

Although still a matter of debate, it has been reported that several protein kinases are able to phosphorylate and stabilize p27^{KIP1} in G0 and early G1 cells. The high expression levels/stability is due to enhanced translation and phosphorylation levels at S10 and T197 ([43, 55, 56] and reviewed in [41, 44]). Known kinases responsible for the modifications of these sites include AKT which targets p27^{KIP1} at S10 and possibly T197 (reviewed in [44, 57]), Dyrk1B which targets p27^{KIP1} at S10 ([55] and reviewed in [41, 44]) and AMPK which catalyzes the phosphorylation of p27^{KIP1} at T197 ([56] and reviewed in [41]). To determine whether these protein kinases contribute to the stability of p27^{KIP1} in our model system, quiescent cells were stimulated in the presence of full growth medium after pre-treatment with inhibitors specific for the aforementioned protein kinases (Fig. 7A). Incubation of cells with Wortmannin, a potent inhibitor of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling cascade, significantly compromised the kinase activity of AKT as seen by the complete absence of the activating phosphorylation at T308. However, this was not accompanied by changes in the phosphorylation of p27^{KIP1} at S10 or in the expression levels of endogenous p27^{KIP1} (Fig. 7B). Conversely, treatment of cells with Compound A, a selective inhibitor of Dyrk1B [58], resulted in partially decreased levels of total p27^{KIP1} and phosphorylation at S10 confirming previous findings of a role of Dyrk1B kinase in the phosphorylation and stability of p27^{KIP1} mainly in quiescent cells (Fig. 7C, [44, 55]). Similarly, pre-treatment of cells with Dorsomorphin effectively inhibited AMPK [59], as indicated by decreased phosphorylation of its substrate targets acetyl-CoA carboxylase [P-ACC (S79)] and p27^{KIP1} [P-p27^{KIP1} (T197)] and resulted in lower expression levels of endogenous p27^{KIP1} (Fig. 7D). This is consistent with the notion that AMPK might cooperate with other protein kinases in the stabilization of p27^{KIP1} in early G1 phase of the cell cycle in myoblasts [56, 60, 61].

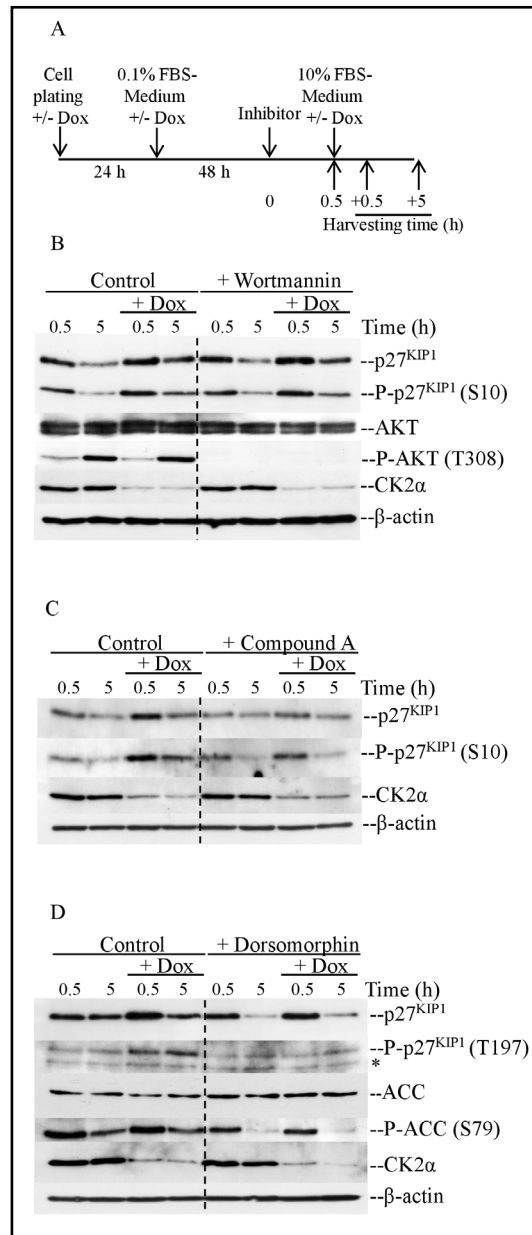


Fig. 7. Dyrk1B and AMPK contribute to the stabilization of p27^{KIP1} by increasing its phosphorylation at S10 and T197, respectively, in cells lacking CK2 α . (A-D) Cells were serum-starved for 48 hours in the absence or presence of doxycycline as indicated in the Fig.. Thirty minutes before adding full growth medium, cells were added 0.1 μ M Wortmannin (A), 10 μ M Compound A (C), 10 μ M Dorsomorphin (D) or an equivalent volume of DMSO (Control). Cells were harvested 30 minutes after release from starvation (+0.5) and after 5 hours (+5), respectively. Whole cell lysate was analyzed by western blot employing antibodies directed against the indicated proteins. Detection of β -actin was used as a loading proteins. * Denotes unspecific signal not corresponding to phosphorylated p27^{KIP1}.

Down-regulation of CK2 α creates unfavorable conditions for cell proliferation signaled by the up-regulation of p27^{KIP1}

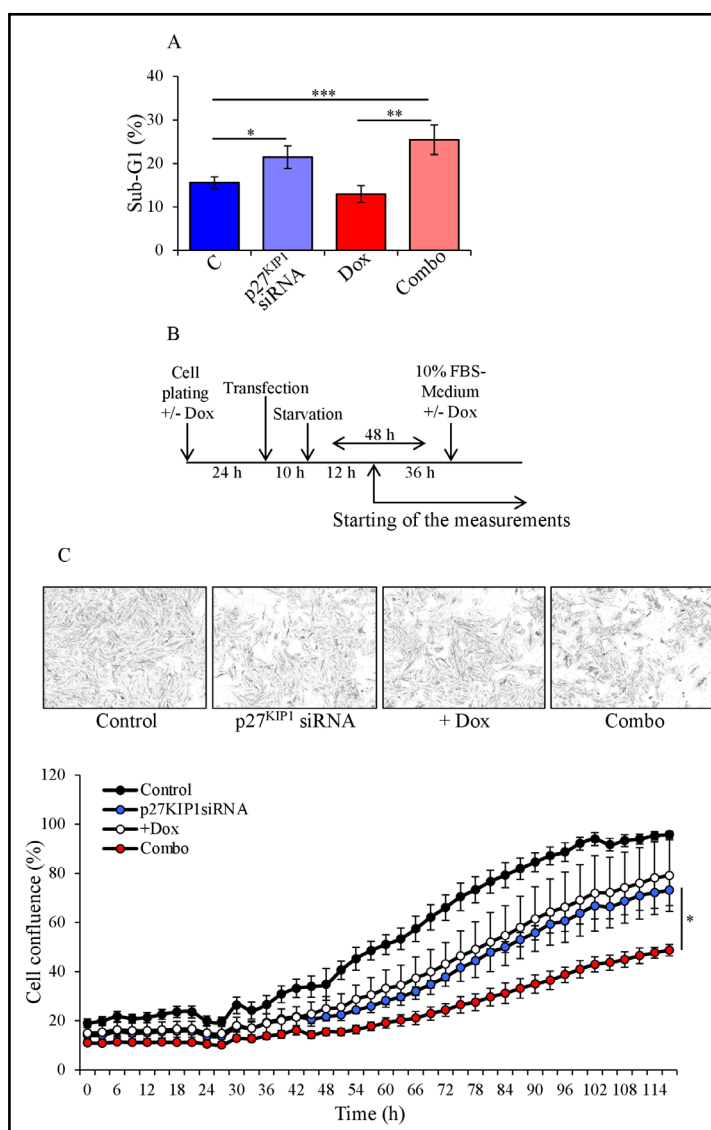
p27^{KIP1} levels increased when exponentially growing cells ceased proliferation in response to serum withdrawal. However, we showed that in cells depleted of CK2 α the expression of p27^{KIP1} was further up-regulated and remained detectable for a longer period of time upon mitogenic stimulation as compared to control cells. The percentage of cell death was negligible under the indicated culture conditions (Fig. 1B, [20]). We hypothesized that increased levels of p27^{KIP1} could be responsible for the delayed cell cycle progression observed in cells with silenced CK2 α or, alternatively, that p27^{KIP1} could protect myoblasts from cell death under unfavorable growth conditions triggered by the absence of growth factors and proliferation signal caused by CK2 α depletion. This is plausible, as it has been shown that the role of p27^{KIP1} goes beyond stimulation of cell cycle arrest [6, 62]. To test this, cells were transfected with scrambled siRNA (i.e. Control and Doxycycline-treated cells) or siRNA directed against p27^{KIP1} transcripts alone or in combination with CK2 α silencing (i.e. Combo), synchronized by serum withdrawal and, subsequently, stimulated by adding complete growth medium. As shown in Fig. 8A, silencing of p27^{KIP1} resulted in significantly increased cell death as compared to control cells. Down-regulation of both CK2 α and p27^{KIP1} (i.e. Combo) resulted in a slightly higher percentage of cell death with respect to p27^{KIP1}-siRNA treated cells which was, however, not significant. These data altogether suggest that increased expression of p27^{KIP1} following silencing of CK2 α does not promote further protection from apoptosis. We noticed, however, a significant difference in cell density when both proteins were simultaneously down-regulated (Fig. 8C and data not shown). Hence, in order to evaluate the role of p27^{KIP1}-knock-down on myoblasts proliferation, we subjected the cells to experimental conditions indicated in Fig. 8B and analyzed proliferation by using the Incucyte S3 live-cell analysis system. As shown in Fig. 8C, down-regulation of p27^{KIP1} and CK2 α , respectively, resulted in markedly slower proliferation rate of the cells as compared to control experiment. Remarkably, combined silencing of both proteins resulted in significantly impaired proliferation of the cells. In support of these results, we additionally carried out experiments similar to those shown in Fig. 8C following siRNA-mediated down-regulation of AMPK α . We anticipated that silencing of AMPK α would lead to decreased proliferation of the cells by destabilizing p27^{KIP1} expression as seen in cells treated with Dorsomorphin. Data reported in Supplementary Fig. S4 confirmed our prediction and revealed a pattern similar to that shown on Fig. 8C. However, altogether, these results raise the possibility that p27^{KIP1} exerts a protective role not linked to CK2 α in cells lacking proliferation potential.

Discussion

Ample evidence indicates that CK2 α plays a crucial role in the regulation of cell cycle progression in cancer cells. However, its importance in non-cancerous cells has been partially addressed. To elucidate the molecular mechanisms by which CK2 α regulates cell replication, we investigated the expression and phosphorylation levels of proteins that regulate the G1 to S cell cycle transition. Here, we propose a model explaining how CK2 α may contribute to cell cycle progression at G1 phase in myoblasts (Fig. 9). Low expression of p27^{KIP1}, which plays a major role in the control of G0/G1 cell cycle progression, potentiates cyclin E-CDK2 complex activation. G1 to S progression is subsequently promoted by the phosphorylation of Rb allowing activation of E2F transcription factor and the expression of a number of cell cycle-related genes including cyclin E, cyclin A, CDKs and MCM proteins. Down-regulation of CK2 α results in accumulation of p27^{KIP1} and inhibition of CDK2-cyclin E complex causing delayed G1 to S cell cycle transition and reduced cell proliferation.

The expression/stability of p27^{KIP1} is regulated at multiple levels including post-translational modifications. It has been reported that activation of CK2 attenuates FoxO3A function thereby down-regulating p27^{KIP1} in human prostate cancer cells [63]. Our data show that CK2 α regulates FoxO3A levels in an AKT-dependent manner; however, we found that

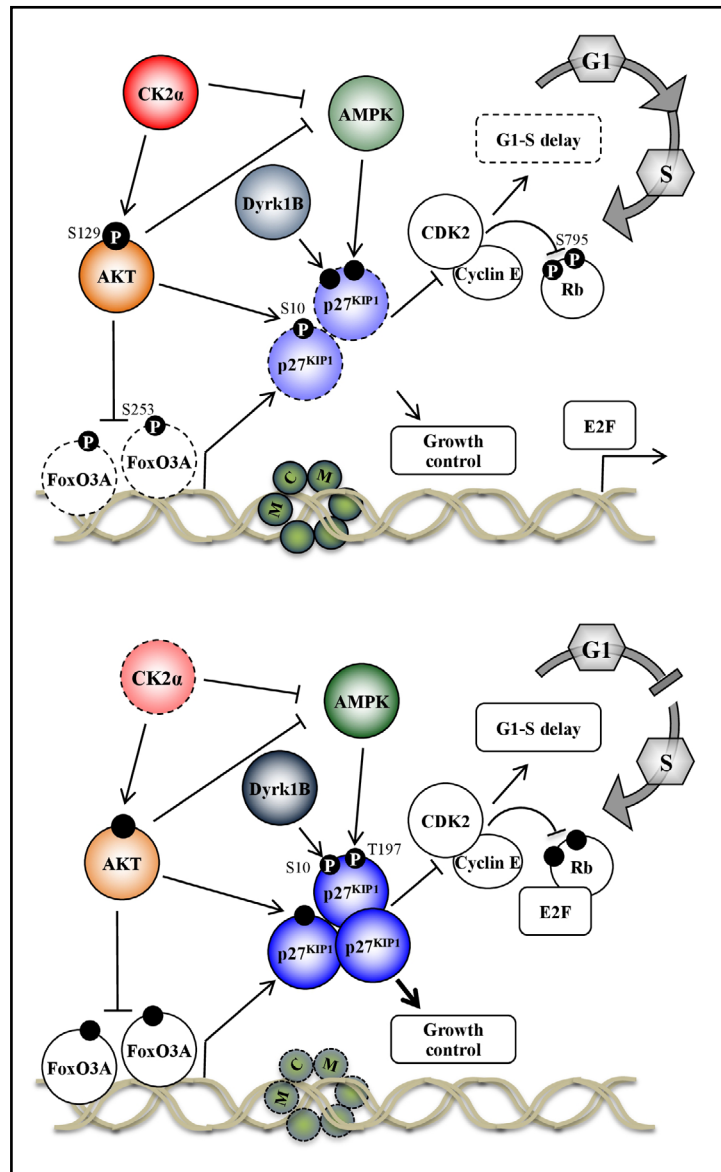
Fig. 8. Lack of proliferation potential following silencing of CK2 α results in up-regulation of p27^{KIP1}, which protects cells in circumstances sub-optimal for cell growth. (A) Cells were transfected with siRNA directed against p27^{KIP1} mRNA 24 hours after plating in the presence or absence of doxycycline for inducing CK2 α down-regulation. Cells were synchronized by serum withdrawal and harvested 48 hours afterward. It followed their analysis by flow cytometry. Sub-G1 denotes cells with fragmented DNA indicative of induction of cell death. Experiments were repeated four times. Average values are expressed in percentage \pm STDEV. *P = 0.0255, **P = 0.0007, ***P = 0.0054. (B) Twenty-four hours after plating in 6-well plates in the presence or absence of doxycycline, cells were transfected with scrambled or p27^{KIP1} siRNA. Cells were subsequently treated as shown in the Fig.. (C) 12 hours after serum withdrawal cell growth was analyzed by the IncuCyte S3 live-cell system (i.e. starting of the measurements). Measurements were performed every three hours. Cell pictures shown in the Fig. are representative and were taken at the end of the experiment. Data represent mean values \pm STDEV of n = 3 experiments, *P<0.0001.



the expression of p27^{KIP1} is mainly controlled by post-translational modifications and not by FoxO3A. This supports the notion that p27^{KIP1} is regulated in a complex manner and its expression might be controlled by different mechanisms depending on the cell type and cellular context.

Although partially understood, it has been reported that at the early stage of the cell cycle Dyrk1B, AKT, and AMPK are responsible for the stability of p27^{KIP1} through phosphorylation at S10 and T197 [41, 44]. As with S10, T197 is phosphorylated in early G1 [64]. Based on our results, it is unlikely that AKT acted as a major contributor to p27^{KIP1} stability as its inhibition by Wortmannin did not change the expression levels of p27^{KIP1}. Our data are in line with the findings reported by Larrea *et al.*, which showed that 6 hours of PI3K inhibition did not affect the levels of either AKT, phospho-p27^{KIP1}-S10 or total p27^{KIP1} in MCF-7 cells [65]. Conversely, inhibition of Dyrk1B and AMPK, respectively, led to partial destabilization of p27^{KIP1} (Fig. 7) supporting the notion that both protein kinases contribute to the regulation of p27^{KIP1} expression during G0/G1 phase. The involvement of other kinases cannot be excluded, in fact, in addition to Dyrk1B, ERK2 was reported to phosphorylate p27^{KIP1} at S10 and AKT, RSK1,

Fig. 9. Proposed model of G1/S cell cycle signaling controlled by CK2 α in myoblasts. We observed delayed cell cycle progression through G1 phase supported by low levels of cyclin E and cyclin A in response to CK2 α silencing. We additionally reported evidence of a marked increase in p27^{KIP1} protein levels and inhibition of the cyclin E-CDK2 complex. p27^{KIP1} inhibits the cyclin E-CDK2 complex which is one of the major upstream kinases of the retinoblastoma protein (Rb). This results in decreased phosphorylation of Rb at S795, which prevents subsequent release of E2F transcription factor bound to Rb. Increased phosphorylation at S10, and T197 of p27^{KIP1} contribute to the up-regulation of this cyclin kinase inhibitor. The study presented here, additionally underlines the protective role of p27^{KIP1} in myoblasts under growth conditions unfavorable for cell viability. Further details are discussed in the text.



RSK2 and PKC have been shown to phosphorylate p27^{KIP1} at T197 beside AMPK [44, 64]. One additional candidate is KIS a protein kinase which directly interacts with and phosphorylates p27^{KIP1} at S10 in leukemia cells [66]. However, contrary with previously published data [44] and our own results showing that compromised cell cycle progression correlates with up-regulation of p27^{KIP1} expression and phosphorylation levels at S10, Nakamura *et al.* [66] reported that KIS promotes cell cycle progression by binding and phosphorylating p27^{KIP1} at S10 promoting nuclear export and its inhibition.

Overall, although it remains to be determined whether these protein kinases are all relevant and under which circumstances (e.g. mitogenic signal, stress response, cell cycle phase, nutrients levels), our data demonstrate that phosphorylation of S10 and T197 is an important event ensuring increased stability of p27^{KIP1} early in G0/G1 phase. At present, it is not clear how CK2 α silencing results in increased Dyrk1B-mediated phosphorylation of p27^{KIP1} at S10. Future studies should address a possible crosstalk between these protein kinases in the regulation of cell cycle progression. Interestingly, data shown in Fig. 7C indicate that silencing of CK2 α results in increased AMPK-mediated phosphorylation of p27^{KIP1} at T197 suggesting a direct or indirect control of AMPK activity by CK2 α . This is in line with

work by Dixit *et al.*, demonstrating that inhibition of CK2 by small molecule inhibitors induces metabolic adaptation and survival in glioma cells by activating the PDK4-AMPK axis [67].

Although speculative at the moment, it is likely that the effects exerted by p27^{KIP1} might be largely cell-type dependent. The importance of p27^{KIP1} expression in the early stages of cell cycle transition is well documented. However, the outcome of different post-translational modifications remains to be further elucidated in view of the fact that up-regulation of p27^{KIP1} levels might result from the cooperation of distinctive signaling pathways linked to specific cellular circumstances. In this respect, Hauck *et al.*, showed that hypertrophic stimulation of cardiomyocytes results in induction of CK2 α' activity and progressive proteasomal-mediated degradation of p27^{KIP1} suggesting that CK2 α' supports hypertrophic signaling by blocking p27^{KIP1} growth-suppressive functions in non-proliferating cardiomyocytes [68]. Conversely, we found in our study that down-regulation of CK2 α was not accompanied by decreased expression of CK2 α' , yet, the levels of p27^{KIP1} were found up-regulated in myoblasts and CK2 α -knockout embryos. This supports the notion that the individual CK2 isoforms exert distinct effects according to certain cellular conditions (e.g. differentiation status, growth factors stimulation, cell type, etc.).

It has been shown that degradation of p27^{KIP1} associated with the transition of cells from G0 to G1/S phase, is mediated by the binding to the F-box protein Skp2 of the SCF^{Skp2} complex and subsequent ubiquitin-mediated degradation [4, 69, 70]. Hence, it is not to be excluded that up-regulation of p27^{KIP1} in cells depleted of CK2 α could also result from lack of Skp2/p27^{KIP1} regulation. This is plausible, as CK2 has been shown to play a critical role in polyubiquitination of the promyelocytic leukemia gene product PML [71] and Wee1 [22] in human cancer cells. On the other hand, Hara *et al.*, reported evidence that degradation of p27^{KIP1} at the G0/G1 transition is mediated by a Skp2-independent ubiquitination pathway [72]. We attempted to investigate the status of Skp2 in our model system based also on our recent findings by RNA-Seq showing lowered *Skp2* mRNA levels in cell with silenced CK2 α [20]. Unfortunately, Skp2 expression appeared beyond detection levels under the applied experimental conditions making it unlikely to have a significant role in p27^{KIP1} levels (data not shown). Moreover, we cannot exclude that up-regulation of p27^{KIP1} might arise from mechanisms independent from Skp2. Data from the literature showed that the Skp2-dependent pathway of p27^{KIP1} ubiquitination might play a more important role in S and G2 phases rather than in G1 phase [73]. Moreover, Skp2-independent degradation of p27^{KIP1} has been reported in Skp2^{-/-} cells involving other E3 ligases including KPC1, Pirh2 and DDB1 and possibly other proteins yet to be identified [74]. In many instances high Skp2 expression levels correlate with reduced p27^{KIP1}, however, loss of p27^{KIP1} seems to occur without increased Skp2 in most tumor types [41]. Finally, it is important to point out that decreased expression of CK2 β seen in cells with down-regulation of CK2 α , might contribute to regulate the expression of p27^{KIP1}. Tapia *et al.*, reported that p27^{KIP1} is an *in vitro* substrate of CK2 and interacts with CK2 β and that this phosphorylation event affects its secondary structure [75]. Hence, as p27^{KIP1} is a CK2-dependent binding partner of CK2 β (reviewed in [12]), a role of the latter with respect to modulation of p27^{KIP1} expression is not to be excluded. Apart from this, it is intriguing the correlation between CK2 α depletion and lowered expression of CK2 β . Initially reported by Allende and Allende [76], CK2 β contains sequences that resemble very closely the destruction box found on several cyclins and that play a role in mediating cell cycle dependent protein degradation (reviewed in [12, 19]). The parallels between CK2 β and cyclins is attractive, however, apart from the destruction box, it remains to be further elucidated whether other signals, such as phosphorylation, regulate the stability of CK2 β within the CK2 holoenzyme and as an independent CK2 isoform.

In this study, the expression of FoxO3A was found slightly up-regulated in cells with decreased levels of CK2 α and preceding the expression of FoxO1. This is in agreement with previous data showing that the level of FoxO1 is low in G0/G1 and then, increases towards G1/S phase while FoxO3A controls the expression of FoxO1 and -4 [77-79]. Moreover, the fact that FoxO1 appears to be strongly expressed in the perinuclear region in cells depleted

of CK2 α suggests that post-translational modifications may prevent full cytoplasmic translocation as previously reported [80]. In experiments where the phosphorylation status of FoxO3A at S253 was investigated following IGF-1-mediated stimulation of the PI3K/AKT signaling, we showed that dephosphorylation of FoxO3A was accompanied by a significant suppression of AKT phosphorylation at T308 and S129 in cells depleted of CK2 α . CK2-mediated phosphorylation of AKT at S129 results in increased association of heat shock protein 90 (HSP90) to AKT preventing dephosphorylation of the latter at the activating T308 in cancer cells. In support of a regulatory role of CK2 with respect to AKT kinase activity, it has been additionally shown that phosphorylation of AKT at T308 decreases when S129 is mutated into alanine [53, 81]. Hence, it is plausible that a similar regulatory mechanism might occur in myoblasts where a general cooperation between CK2 α , the PI3K/AKT signaling pathway and FoxO proteins contributes to regulate cell proliferation in the early phase of the cell cycle that will need to be further investigated in the future.

Regardless of the molecular mechanisms by which the levels of p27^{KIP1} is regulated in cells depleted of CK2 α , an important observation that emerged from previous work ([16, 20]) and the present study is that lack of CK2 α does not lead to induction of apoptosis in myoblasts. This is in contrast to what has been observed in cancer cells previously investigated (reviewed in [19, 82, 83]). This suggests that non-cancerous cells might not be strictly dependent on the expression of CK2 α , at least with respect to cell death mechanisms. However, it is not excluded that a compensatory mechanism mediated by CK2 α' might be activated in the absence of CK2 α , as previously observed [84].

We show that cells depleted of CK2 α preserve the capacity to proliferate, however, at a slower pace as compared to control cells. This effect might indicate that cells can traverse the cell cycle when conditions for proliferation are limited/critical (e.g. minimal CK2 activity, low expression of proteins normally present at origins of replication and implicated in DNA replication initiation). Up-regulation of p27^{KIP1}, which is seen both *in vitro* and *in vivo*, contributes to delay G1-to-S phase transition. However, lack of proliferation potential seen when p27^{KIP1} is down-regulated, reflects the protective role of p27^{KIP1} in myoblasts ensuring survival in conditions unfavorable for their growth. Finally, it is not to be excluded that elevated levels of p27^{KIP1} might be necessary for preventing replicative stress when cells lack CK2 α . This is conceivable, as down-regulation of CK2 α results in decreased levels of MCM proteins, which constitute the core of the replication origin recognition complex. In support of this, Quereda *et al.*, showed that complete abrogation of *KIP1/CIP1* cell cycle inhibitors in mice induce perinatal lethality in the absence of tumors due to high levels of replicative stress [20, 85].

Conclusion

There are still pending questions regarding the model proposed here which links CK2 α to the cell cycle machinery in non-cancerous cells. They concern the identity of the multiple post-translational modifications regulating stability, function and cellular localization of p27^{KIP1} and the possible contribution of additional proteins that regulate the cell transition through G1 phase yet to be identified. Nevertheless, the findings presented here provide novel insights into critical mechanisms, which control cell cycle progression in preparation of DNA replication and indicate that up-regulation of p27^{KIP1} in cells depleted of CK2 α ensure the viability of cells in a microenvironment that limit but is still permissive for growth.

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Author Contributions

BG designed the project, carried out a large part of the experiments, wrote the manuscript and provided financial support. M.D. contributed to results shown in Fig. 5 and Supplementary Fig. S3, M.A.S. and P.C. assisted with cell proliferation experiments using the IncuCyte S3 platform, I.D. supervised *in vivo* experimental work and assisted with collection of *in vivo* data, and B.S.A. participated in data analysis and critical discussion of the study.

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Statement of Ethics

Mice experiments were performed in accordance with relevant guidelines and regulations and approved by the Boston University Medical Center Institutional Animal Care and Use Committee (IACUC).

Disclosure Statement

The authors have no conflicts of interests to declare.

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