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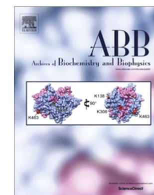

# GAP-43 slows down cell cycle progression via sequences in its 3′UTR

Karina De Moliner [a,1], Manuel Luis Wolfson [a,1], Nora Perrone-Bizzozero [b], Ana M. Adamo [a,*]

[a] Department of Biological Chemistry, IQUIFIB (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, C1113AAD Buenos Aires, Argentina
[b] Department of Neurosciences and Psychiatry, University of New Mexico School of Medicine, Albuquerque, NM, USA

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ABSTRACT

Growth-associated protein 43 (GAP-43) is a neuronal phosphoprotein associated with initial axonal outgrowth and synaptic remodeling and recent work also suggests its involvement in cell cycle control. The complex expression of GAP-43 features transcriptional and posttranscriptional components. However, in some conditions, GAP-43 gene expression is controlled primarily by the interaction of stabilizing or destabilizing RNA-binding proteins (RBPs) with adenine and uridine (AU)-rich instability elements (AREs) in its 3′UTR. Like GAP-43, many proteins involved in cell proliferation are encoded by ARE-containing mRNAs, some of which codify cell-cycle-regulating proteins including cyclin D1. Considering that GAP-43 and cyclin D1 mRNA stabilization may depend on similar RBPs, this study evaluated the participation of GAP-43 in cell cycle control and its underlying mechanisms, particularly the possible role of its 3′UTR, using GAP-43-transfected NIH-3T3 fibroblasts. Our results show an arrest in cell cycle progression in the $G_0/G_1$ phase. This arrest may be mediated by the competition of GAP-43 3′UTR with cyclin D1 3′UTR for the binding of Hu proteins such as HuR, which may lead to a decrease in cyclin D1 expression. These results might lead to therapeutic applications involving the use of sequences in the B region of GAP-43 3′UTR to slow down cell cycle progression.

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## Introduction

The growth-associated protein 43 (GAP-43)[2] is a neuronal phosphoprotein expressed in association with initial axonal outgrowth and remodeling of synaptic connections. GAP-43 has been shown to interact with other proteins including calmodulin [1], $G_0$ [41], rabaptin 5 [28] and actin [21]. In turn, its expression and phosphorylation are influenced by extracellular signals such as the neural cell adhesion molecule (NCAM) [22], growth factors [46], hormones [49] and lipids [2].

The expression of GAP-43 is complex and features both transcriptional and posttranscriptional components [9,40,34,45]. Transcriptional factors of the basic helix loop helix (bHLH) family are known to control the neural-specific expression of the gene [6,17]. However, in some conditions, the levels of transcription do not correlate well with the accumulation of mature GAP-43 mRNA [27], and GAP-43 gene expression is controlled primarily by changes in mRNA stability [35]. mRNA stabilization in turn depends on the interaction of specific instability-conferring sequences in the 3′untranslated region (3′UTR) of mRNAs with specific RNA-binding proteins (RBPs) [33]. In the case of GAP-43 mRNA, this process involves the PKC-dependent interaction of a highly conserved U-rich sequence with neuronal RBP HuD. HuD is a member of the Hu protein family, which is homologous to the Drosophila embryonic lethal abnormal vision (ELAV) RBPs and the best known mRNA stabilization factor [42].

In mammals, four proteins have been described as part of the ELAV/Hu family: HuB, HuC, HuD and HuR. HuR is expressed ubiquitously and HuB is expressed in neurons and gonads, whereas HuC and HuD are preferentially expressed in neurons [16,25]. Hu proteins bind to sequences rich in adenine and uridine residues known as the AU-rich element (ARE) sequences [37,5], which are present in the 3′UTR of specific mRNAs involved in cell growth and differentiation and which constitute key regulators of mRNA stability. The presence of this ARE sequence controls mRNA half-life and translation through its interaction with specific RBPs [36]. An important number of proteins involved in stress, immunological and inflammatory responses, as well as in cell proliferation, are encoded by ARE-containing mRNAs. For example, oncogenes (e.g. c-Myc and c-Fos), cell cycle-regulating proteins (cyclins D1, A1 and B1), cytokines (TNFα, IL2) and growth factors are codified

* Corresponding author. Fax: +54 11 4964 8288.
*E-mail address:* amadamo@qb.ffyb.uba.ar (A.M. Adamo).
[1] Equally contributed to this work.
[2] *Abbreviations used:* GAP-43, growth-associated protein 43; RBPs, RNA-binding proteins; AREs, Adenine and uridine (AU)-rich elements; Cdks; cyclin-dependent kinases; bHLH, basic helix loop helix; NGF, nerve growth factor.

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by these mRNAs. Thus, the steady-state levels of mRNA depend on the balance between mRNA transcription speed and the level of expression of RBPs responsible for its stabilization versus that of RBPs that promote its degradation.

Recent evidence suggests that GAP-43 may also have a role in cell proliferation. GAP-43 mRNA and protein expression are not detected in select human and mouse glioma cell lines, while their re-expression in deficient C6 glioma cells has been shown to inhibit growth *in vitro* [13]. Moreover, advanced neuroblastoma prognosis is associated to abnormal nerve growth factor (NGF)/high-affinity NGF receptor (TrkA) signal transduction, whose downstream genes include GAP-43 [26]. Finally, in a non-neural cell line, Zhao et al. demonstrated the lengthening of the cell cycle in transgenic fibroblasts expressing GAP-43 [48].

In this context, this study evaluated the participation of GAP-43 in cell cycle control and its underlying mechanisms, particularly regarding the possible participation of its 3′UTR sequence. Using GAP-43-transfected NIH-3T3 fibroblasts, we found an arrest in cell cycle progression and a decrease in cyclin D1 levels, which was mediated by a specific region within GAP-43 3′UTR containing a binding site for Hu proteins.

## Materials and methods

### *Materials*

All cell culture reagents were obtained from GIBCO (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Natocor (Cordoba, Argentina). The expression vector pcDNA3, Lipofectamine™ 2000 and Geneticin (G418) were from Invitrogen (Carlsbad, CA, USA). The expression vector pMEP4 is from Addgene (Cambridge, MA, USA).

Polyclonal anti-cyclin D1 and E, Cyclin-Dependent Kinase (Cdk) 2, 4 and 6, $p27^{Kip1}$, ERK1/2 and pERK1/2 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA, USA). The Cycletest™ Plus DNA Reagent Kit was from Becton Dickinson (San Diego, CA, USA). RNA*later* and RNAqueous-Micro were purchased from Ambion (Foster City, CA, USA). SYBR Green I dye, primers, the High-Capacity cDNA Reverse Transcription Kits, and the TaqMan RT Kit were obtained from Applied Biosystems (Foster City, CA, USA).

All other chemicals used were from Sigma–Aldrich (St. Louis, MO, USA) and of the highest purity commercially available.

### *Vector constructs*

The pcGAP construct was prepared by cloning rat GAP-43 cDNA [29] in the *HindIII/XbaI* sites of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). In addition, and in order to evaluate whether RBPs involved in RNA stability are implicated in the effects of GAP-43 expression on NIH-3T3 cell cycle progression, we conducted experiments using different chimeras including the GAP-43 3′UTR and the β-globin genes [45]. The Glob-Gap3′ chimera included the coding region of rabbit β-globin and the 3′UTR of GAP-43, while the Gap-Glob3′ chimera included the coding region of GAP-43 flanked by the β-globin 5′ and 3′UTRs from pSP64T [45]. In addition, to test the effect of specific sequences in the GAP-43 3′UTR, we used Glob-B, in which the B fragment of the GAP-43 3′UTR contains the HuD-binding site, and Gap-C [45], in which the C fragment contains a control region from GAP-43 3′UTR which does not include the HuD-binding site. All of these 3′UTR and chimeric constructs were cloned downstream of the human metallothionein-$II_A$ in the pMEP4 vector, which contains an inducible metallothionein promoter activated in the presence of $Cd^{2+}$.

### *Transfection experiments and cell culture treatments*

Wild type NIH-3T3 fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) low glucose, supplemented with 7.5% FBS and 50U penicillin/streptomycin/ml (complete medium) in a humidified atmosphere at 37 °C and 5% $CO_2$. Cell transfections were performed by lipofection using Lipofectamine™ 2000. After lipofection, cells were cultured in complete medium supplemented with G418 (0.5 mg/ml) at 37 °C. Clonal colonies were isolated, expanded and assayed for GAP-43 expression by Western blot or immunocytochemistry. Among several stably transfected cell lines, we selected T23G (expressing GAP-43) and T22p (transfected with pcDNA3 empty vector). Even if T23G cell clone exhibited the expression of GAP-43 protein, expression levels were low enough to be compatible with stable transfection. For cell cycle analysis, cells were arrested through a 48-h culture in a serum-deprived medium with 0.1% FBS. After 48 h, serum-deprived medium was replaced with complete medium for subsequent experiments.

In turn, for cyclin D1 protein, cyclin D1 mRNA and 3′UTR sequence quantification, NIH-3T3 cells were transiently transfected with the chimeras described above using Lipofectamine™ 2000 according to the procedure described in this section. Once transfection had been completed, an in order to achieve metallothionein promoter activation, cells were induced for 16 h in the presence of 5 μM $CdCl_2$. Exposure to $Cd^{2+}$ brings about an 8- to 10-fold activation of the metallothionein-IIA promoter in the pMEP4 vector and, as a consequence, increases construct expression [45]. Results were evaluated right after $CdCl_2$ removal (t0) and 4 h later (t4).

### *Cell adhesion and proliferation*

T22p and T23G clones were grown on 96-well plates ($1 \times 10^4$ cells/well) and incubated for different time periods in complete medium. To evaluate cell adhesion, cells were harvested every 15 min during 6 h, washed in PBS and fixed by addition of 100 μl paraformaldehyde (4% w/v) during 15 min. Plates were washed three times in deionized water, air-dried and stained by addition of 100 μl crystal violet solution (0.1% w/v) during 20 min at room temperature. Excess dye was removed by extensive washing with deionized water. Once the plates were air-dried, 100 μl acetic acid solution (10% v/v) was added to solubilize the dye. The absorbance of dye extracts was measured at 590 nm using a Biotrack II plate reader (Amersham Biosciences, NJ, USA). The same procedure was used to evaluate possible changes in cell proliferation induced by the expression of GAP-43, although cells were harvested at 6, 24, 48, 96 and 120 h.

### *Flow cytometry*

To synchronize cells, T22p and T23G clones were washed with PBS and then serum-starved for 48 h in DMEM containing 0.1% FBS. Next, re-entry into the $G_1$ phase of the cell cycle was initiated by replacing the starvation medium with complete medium. At different times (0, 12, 15, 18 or 24 h), cells were harvested and washed with PBS; the cell cycle was then analyzed through nuclear DNA quantification using the Cycletest™ Plus DNA Reagent Kit according to the manufacturer's instructions. Propidium-iodide-stained nuclei were analyzed by flow cytometry (Becton Dickinson) and the percentages of cells in the $G_0$–$G_1$, S and $G_2$–M phases of the cell cycle were determined by means of Mod Fit LT cell cycle analysis software (Verity Software, Topsham, ME, USA).

### *BrdU labeling*

To synchronize cells, T22p and T23G clones were washed with PBS and then serum-starved for 48 h in DMEM containing 0.1%

FBS. Next, re-entry into the $G_1$ phase of the cell cycle was initiated by replacing the starvation medium with complete medium. Sixteen hours after being released from $G_0$–$G_1$ blockade, cells were pulse-labeled by BrdU (10 μM) for 1.5 h, washed with PBS and fixed in a solution of paraformaldehyde 4% w/v in PBS. They were then treated with 6 M HCl in PBS containing 1% Triton X 100 during 15 min at room temperature. After blockade with a solution of 5% w/v albumin in PBS, cells were treated with a monoclonal anti-BrdU antibody (1:250) and then with a Texas Red goat anti-mouse secondary antibody (1:1000). Taking 300 cells for each experimental condition, BrdU-positive cells were analyzed by fluorescence microscopy using the Image Pro Plus Software.

*Western blot analysis*

The levels of cyclins D1 and E, and cyclin-dependent kinases (Cdks) 2, 4 and 6 and Cdk inhibitor $p27^{Kip1}$ were evaluated by Western blot. After a 24-h culture and in order to synchronize cells, T22p and T23G clones were washed with PBS and then serum-starved for 48 h in DMEM containing 0.1% FBS. Next, re-entry into the $G_1$ phase of the cell cycle was initiated by replacing the starvation medium with complete medium and cells were cultured for different times according to the experiments planned. Cells were harvested and washed twice with PBS; the cellular pellet was then resuspended in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) NP40 and protease inhibitors 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml antipain, 5 μg/ml aprotinin and 10 μg/ml chemostatin (Roche Cocktail). Samples containing 70 μg protein were separated by SDS–PAGE (10% acrylamide) [18] and blotted onto PVDF membranes. After 2-h blockade in 5% (w/v) non-fat milk in PBS, membranes were probed overnight at 4 °C with the different primary antibodies (polyclonal anti-cyclin D1, cyclin E, Cdks2, 4, 6 and Cdk inhibitor $p27^{Kip1}$, 1:500 for all antibodies). To evaluate ERK phosphorylation, T22p and T23G clones were synchronized as described above. After medium replacement, cells were harvested at 5, 10, 15 and 20 min, and aliquots containing 70 μg total protein were separated by SDS–PAGE. The levels of ERK1/2 and pERK1/2 were evaluated using specific antibodies (1:500). Membranes were subsequently incubated for 90 min at room temperature with the corresponding secondary horseradish peroxidase-conjugated antibody (1:15,000). Bands were visualized by enhanced chemiluminescence (ECL plus, Amersham Biosciences, Piscataway, NJ, USA) using a Phosphoimager 840 (GE Healthcare, Piscataway, NJ, USA).

*Cyclin-dependent kinase assay*

To synchronize cells, T22p and T23G clones were washed with PBS and then serum-starved for 48 h in DMEM containing 0.1% FBS. Next, re-entry into the $G_1$ phase of the cell cycle was initiated by replacing the starvation medium with complete medium. After 2 and 4-h culture, cells were harvested in Cdk4/Cdk6 buffer containing 50 mM HEPES pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT and 0.1% Tween 20, supplemented with the phosphatase and protease inhibitors 5 mM NaF, 0.1 mM sodium orthovanadate, 5 μg/ml of leupeptin, 50 μg/ml PMSF and 5 μg/ml of pestatin A; cells were then mechanically lysed by repeated passages through Pasteur pipettes. Cellular debris was removed from soluble extract by centrifugation at 16,000×*g* for 10 min at 4 °C.

Aliquots containing 100 μg total protein were used for immunoprecipitation of endogenous Cdk2, Cdk4 and Cdk6 using the above mentioned antibodies and 30 μl Protein A/G Plus-Agarose. Cdk4 and Cdk6 reactions were carried out in 50 μl of 50 mM HEPES buffer, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate and 1 mM NaF containing 10 μg Glutathione-S-transferase-Rb substrate (GST-Rb). Cdk2 reaction was assayed in 50 μl of a kinase buffer containing 50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT and 10 μg Histone I substrate.

In both cases, 2 mM EGTA and 10 μCi of [$\gamma$-$^{32}$P] ATP were added to the reaction media and assays were carried out for 30 min at 30 °C; non-radioactive ATP (final concentration 30 μM) was then added to each reaction mixture to reduce background signal. Reactions were stopped by the addition of Laemmli sample buffer and the reaction products were electrophoresed in SDS 12% polyacrylamide gel. The gel was then dehydrated using Slab Gel Dryer SGD 2000 Savant and the radioactive bands corresponding to different phosphorylated products were visualized by autoradiography using the Storm Phosphoimager 640 (GE Healthcare, Piscataway, NJ, USA).

*Real-Time PCR analysis*

NIH-3T3 cells transiently transfected with the chimeras described above were harvested and kept in RNA*later* solution at 4 °C and then samples stored at −80 °C. Total RNA was isolated with RNAqueous-Micro Kit. Samples were used for reverse transcription with random hexamer primers, using a High-Capacity cDNA Reverse Transcription Kit and a TaqMan RT Kit. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Real-Time PCR (RT-PCR) was performed using the SYBR Green PCR Master Mix on an Applied Biosystems 7500 Real Time PCR System. Primers were designed using Primer Express Software. The primer sequences were as follows:

| Gen | Forward primer | Reverse primer |
|---|---|---|
| GAPDH | 5′-TGTTCTAGAGACAGCC GCATCTT-3′ | 5′-CACCGACCTTCACCATCTT GT-3′ |
| Cyclin D1 | 5′-CCTCTCCTGCTACCGCA CAA-3′ | 5′-GCAGTCCGGGTCACACT TG-3′ |
| GAP-43 3′UTR | 5′-ACCCCGAGGCTGACCAA-3′ | 5′-CAACGTGGAAAGCCGTTT CT-3′ |

Raw data from at least three independent experiments were used to determine the relative expression levels of each transcript by employing the comparative $C_T$ method. In order to use the $\Delta\Delta C_T$ method to calculate the fold-differences in gene expression between samples, a validation experiment was done to determine that the efficiency of the target amplification and the efficiency of the reference (GAPDH) amplification are approximately equal. The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by: $2^{-\Delta\Delta C_T}$ (RQ).

*Statistical analysis*

Data are presented as the means ± SEM. The statistical analysis was performed either by Student's *t*-test for independent samples or ANOVA (one-way repeated) followed by Tukey's multiple comparison test.

## Results

*Involvement of GAP-43 expression in cell cycle progression*

In order to evaluate whether the expression of GAP-43 has effects on cell cycle progression and, as a consequence, on cell proliferation, we first studied possible changes in the adhesion properties of cultured NIH-3T3 cells stably transfected with GAP-43-expressing (T23G) or control vectors (T22p). For this assessment, an equal number of cells were seeded from the T22p and T23G

clones and cell adhesion was evaluated as described above. The amount of adhered cells was determined through crystal violet dying at 0.25, 0.50, 0.75, 2, 3, 4 and 6 h (Fig. 1A). Results showed no significant differences between clones in the amount of adhered cells, indicating that ectopic GAP-43 expression does not alter the adhesion properties of NIH-3T3 cells in culture. The same methodology was used to evaluate changes in cell proliferation at 6, 24, 48, 96 and 120 h. In this case, results showed no differences between clones up to 96 h; however, at 120 h, the amount of cells was significantly larger for the T22p clone (Fig. 1B), which suggests that the expression of GAP-43 may be involved in the control of cell cycle progression.

On the basis of these first results, we evaluated cell cycle progression by flow cytometry to determine the percentage of cells in the different phases of the cell cycle ($G_0/G_1$, S and $G_2/M$) at the times analyzed. Results showed no significant differences between cell populations after 48-h serum starvation (time 0), with approximately 80% cells in the $G_0/G_1$ phase in accordance with cell cycle arrest. After 6-h culture in complete medium, there were still no significant changes with regards to time 0. However, 12 h after complete medium implementation, a difference was observed in the percentage of cells in the S phase, with 30% for the T22p clone and 18% for T23G. At the same time, approximately 65% T22p cells were observed to be in the $G_0/G_1$ phase, as compared to 75% T23G ones. These differences became more evident after 18-h complete-medium culture, when a significant increase was observed in the proportion of T23G cells in the $G_0/G_1$ phase with regards to T22p cells (46.45% versus 22.99%), concomitantly with a decrease in the proportion of T23G cells in the S phase (50.93% versus 77.01%). These findings demonstrate that GAP-43 expression produces an arrest in NIH-3T3 cell cycle at the $G_0/G_1$ phase (Fig. 2A). Likewise, BrdU incorporation experiments after 16 h in complete-medium culture showed that the percentage of T23G cells in the S phase of the cycle was significantly lower than that of T22p cells ($p < 0.001$) (Fig. 2B).

### *Effects of GAP-43 expression on cyclins, Cdks and Cdk inhibitor $p27^{Kip1}$*

In order to evaluate the mechanisms involved in the arrest of the cell cycle induced by the expression of GAP-43, we studied the profile of proteins involved in cell cycle progression, particularly those participating in the $G_1/S$ transition. Results showed a significant decrease in cyclin D1 levels in T23G cells as compared to T22p cells at 6, 8 and 10 h, although no significant differences were observed at the other times points analyzed. In contrast, no significant differences in Cdk4 and Cdk6 levels were observed between GAP-43-expressing and control cells (Fig. 3A).

Regarding cyclin E levels, we found a significant decrease in cells expressing GAP-43 as compared to control cells between 6 and 12 h, which provides further support for cell cycle arrest in the $G_0/G_1$ phase. In agreement with results obtained for Cdk4 and Cdk6, no significant differences were observed in the levels of Cdk2 between GAP-43-expressing and control cells (Fig. 3B).

On the basis of these findings, and considering that cells expressing GAP-43 undergo cell cycle arrest during the $G_0/G_1$ phase, the activity of these three kinases was measured during the $G_1/S$ transition between 6 and 8 h in complete-medium culture. In agreement with the decrease observed in the levels of cyclins D1 and E as a consequence of GAP-43 expression, we found a significant decrease in the activity of Cdk2, 4 and 6 in cells expressing GAP-43 as compared to T22p cells (Fig. 3C).

As protein $p27^{Kip1}$, a Cdk inhibitor, plays an important role in the regulation of Cdk activity during $G_0/G_1$ progression and cell cycle arrest, we evaluated the levels of this protein along the cell cycle in both clones. While $p27^{Kip1}$ levels were higher in cells expressing GAP-43 at all times analyzed, differences were only significant after 6 and 8 h of complete-medium culture (Fig. 4).

### *Effects of GAP-43 expression on ERK1/2 activation*

As mitogenic growth factors initiate the cell cycle through pathways Ras/Raf/ERK, activating the transcription factors responsible for the expression of cyclin D, we next evaluated whether the decrease in cyclin D1 levels was due to changes in the activation of ERK1/2 as a consequence of GAP-43 expression. The levels of ERK1/2 and p-ERK1/2 were determined according to the protocol described above and the relationship between p-ERK1/2 and total ERK1/2 was evaluated as a parameter of MAPK pathway activation. Results showed no significant differences in early ERK1/2 activation (from 0 to 20 min) between cells expressing GAP-43 and control cells (Fig. 5).

### *Possible participation of RBPs in the effect of GAP-43 expression on cell cycle progression*

As mentioned before, numerous mRNAs have a 3′UTR-located ARE sequence, which is key in the regulation of their expression and which controls their half-life and translation by interacting with RBPs. Thus, the level of a certain mRNA in the steady state will depend on the balance between transcription velocity and the level of RBPs responsible for stabilization versus those promoting degradation. Considering this, together with the fact that GAP-43 expression may imply an increase in its mRNA and RBP binding, we evaluated whether the changes observed in cyclin D1 protein

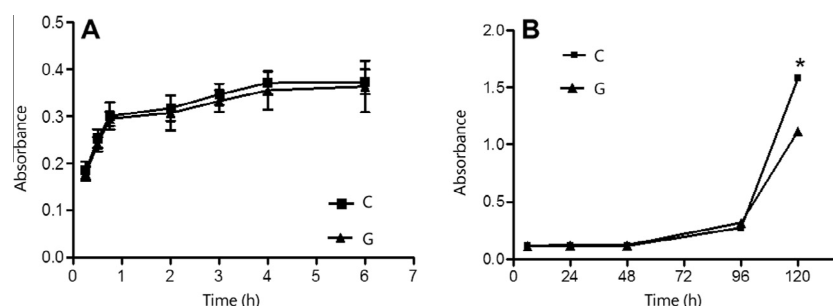


**Fig. 1.** Effects of GAP-43 expression on cell adhesion and proliferation. The same number of cells ($1 \times 10^4$) was plated for each clone, T22p (C) and T23G (G), in a 96-well plate. Results are expressed as 590 nm absorbance as a function of culture hours. Each point represents mean absorbance ± SEM of three independent experiments with each clone. (A) The number of adhered cells was determined through crystal violet staining between 15 min and 6 h, after which absorbance remained constant. No significant differences were observed in the number of adhered cells between clones at the time points analyzed. (B) The total number of cells was determined through crystal violet staining between 6 and 120 h. Significant differences: $^{***}p < 0.001$.

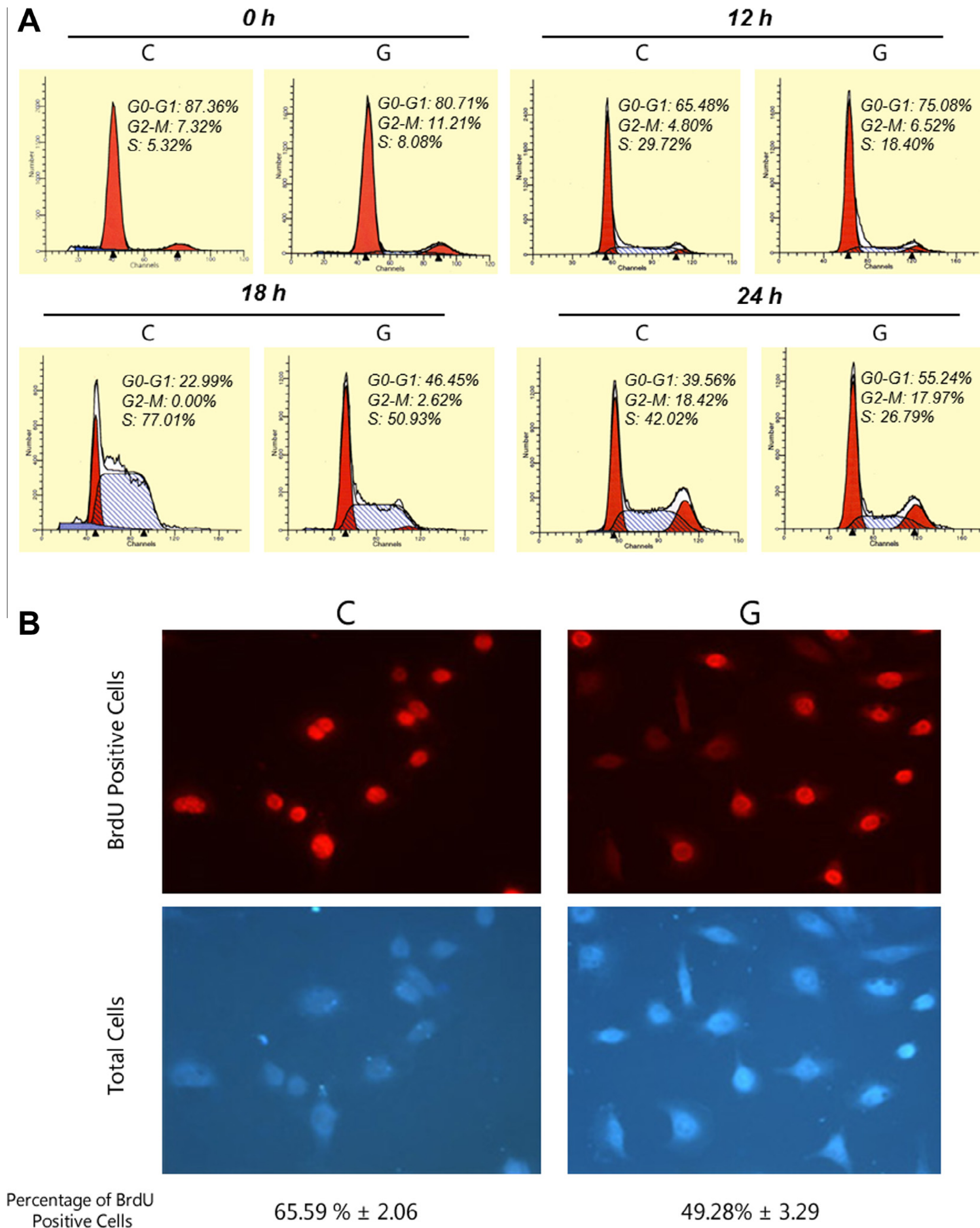


**Fig. 2.** Effects of GAP-43 expression on cell cycle progression (A) Synchronized T22p (C) and T23G (G) cells were analyzed at 0, 12, 18 and 24 h in complete medium. Cell cycle progression was evaluated by flow cytometry. Each histogram shows DNA staining by propidium iodide and the figure shows representative flow cytometry profiles of cell distribution in $G_0$–$G_1$, S, and $G_2$–M phases from four independent experiments. Percentages of cells in the $G_0$–$G_1$, S and $G_2$–M phases were determined through the Mod Fit LT cell cycle analysis software. (B) After 16 h in complete medium, synchronized C and G cells were pulse-labeled with BrdU during 90 min and BrdU-positive cells were analyzed by fluorescent microscopy using Image Pro Plus software. Three hundred cells were counted for each clone. Results are expressed as the percentage of BrdU-positive cells ± SEM from at least three independent experiments. Significant differences: $^{***}p < 0.001$.

levels may be associated to changes in its mRNA stability. In particular, we hypothesized that the decrease observed in cyclin D1 protein levels could be due to a decrease in cyclin D1 mRNA stability as the consequence of a decrease in the amount of RBPs available, in turn caused by RBP sequestration by GAP-43 mRNA.

We therefore studied the possible changes in cyclin D1 protein levels as a consequence of the overexpression of chimeras (Fig. 6A):

- Gap-Glob3′ (control, changes are not expected in the levels of cyclin D1 because this construct lacks the region B of GAP-43 3′UTR, which binds RBPs).
- Gap-C (changes are not expected in the levels of cyclin D1 because this construct lacks the region B of GAP-43 3′UTR, which binds RBPs).
- Glob-Gap3′ (a decrease is expected in the levels of cyclin D1 due to a decrease in its mRNA stability caused by RBP sequestration).
- Glob-B (a decrease is expected in the levels of cyclin D1 due to a decrease in its mRNA stability caused by RBP sequestration).

Results showed a significant decrease in cyclin D1 protein levels in cells transfected with the entire GAP-43 3′UTR (Glob-Gap3′) and

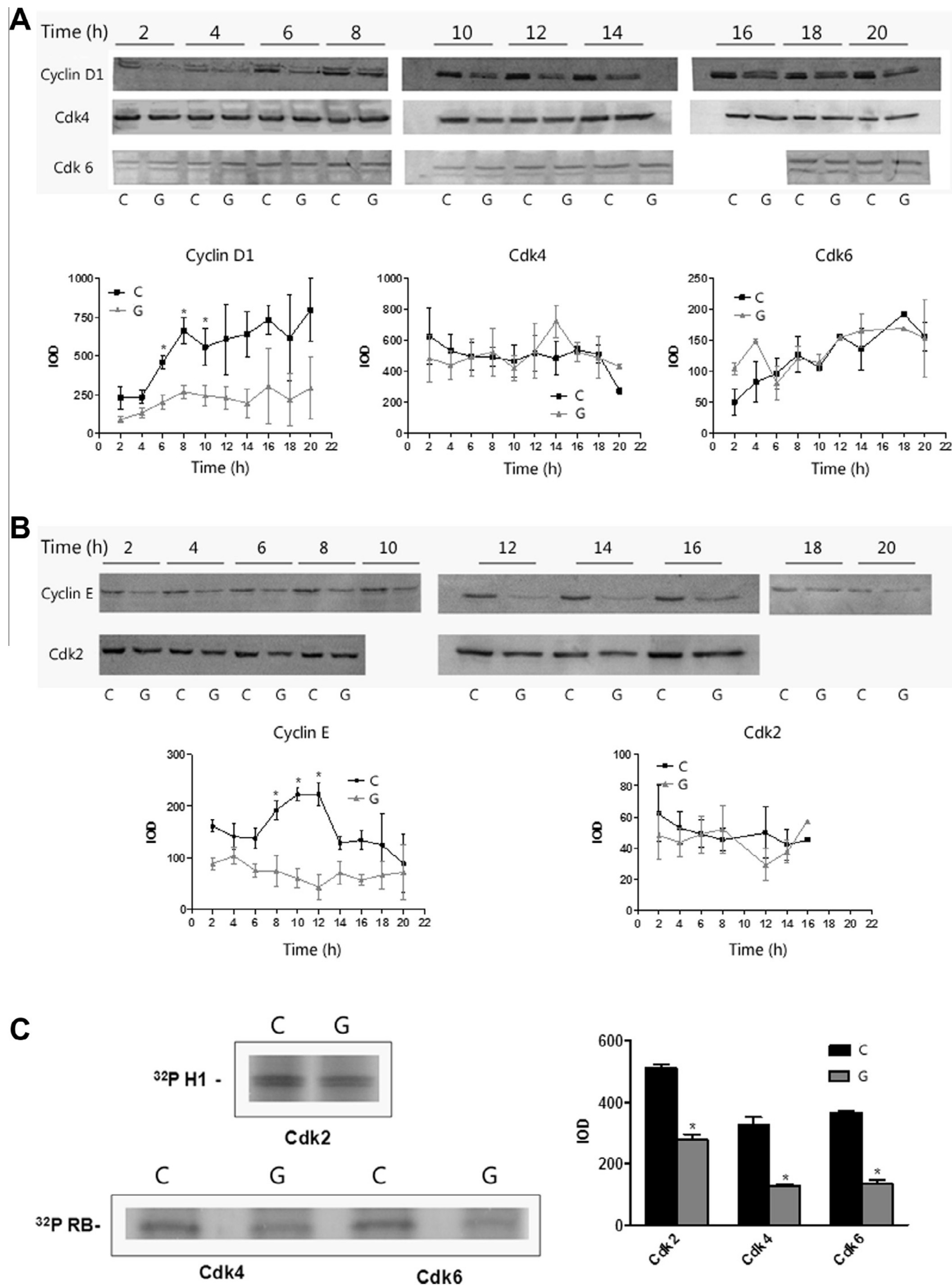


**Fig. 3.** Effects of GAP-43 expression on cyclins D1 and E and Cdk2, 4 and 6. (A and B) Synchronized T22p (C) and T23G (G) cells were harvested at 2 h intervals between 0 and 20 h in complete medium, and cyclin D1, Cdk4, Cdk6 (A), cyclin E and Cdk2 (B) were analyzed from cell extracts by Western blot analyses. Bands were visualized by chemiluminescence using Phosphoimager 840. After densitometric analyses, protein levels were normalized per total protein content in each lane. Results are expressed as the mean IOD ± SEM and each point is the average of at least three independent experiments. Significant differences: $^{***}p < 0.001$. (C) Synchronized C and G cells were harvested at 6 h in complete medium and cell extracts were immunoprecipitated using specific anti-Cdk2, anti-Cdk4 and anti-Cdk6 antibodies. After phosphorylation reaction in the presence of [$\gamma$-$^{32}$P] ATP and the specific substrates for each of the kinases analyzed, proteins were separated by SDS–PAGE (10% w/v acrylamide) and phosphorylated products were visualized by radioautography using Phosphoimager 840. Results are expressed as the mean IOD ± SEM and each point is the average of at least three independent experiments. Significant differences: $^{***}p < 0.001$.

just the B-region of the 3′UTR (Glob-B), both associated with the β-globin coding sequence, as compared to those transfected with Gap-Glob3′ (control). In contrast, no changes in cyclin D1 protein levels were found in cells transfected with the coding region of GAP-43 and the C region (Gap-C). It is worth pointing out that chimeras with GAP-43 3′UTR, i.e. Glob-Gap3′ and Glob-B, include the binding region of RBPs, in particular fragment B, which contains the binding site for RBP HuD (Fig. 6B).

With these results, we analyzed and correlated the levels of cyclin D1 mRNA and the GAP-43 3′UTR sequence in cells

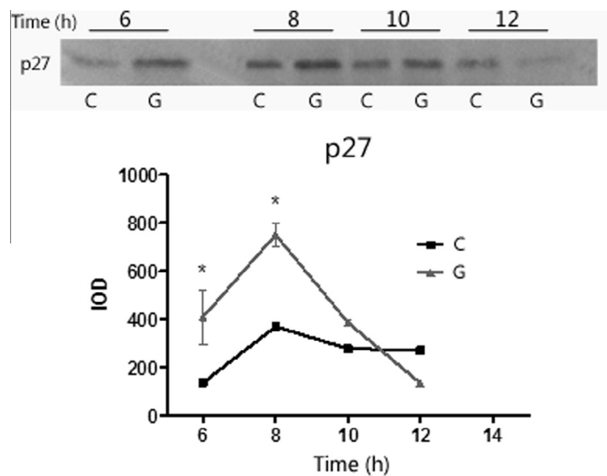


**Fig. 4.** Effects of GAP-43 expression on $p27^{Kip1}$ levels. Synchronized T22p (C) and T23G (G) cells were harvested at 6, 8, 10 and 12 h in complete medium and $p27^{Kip1}$ (p27) levels were analyzed from cell extracts by Western blot analyses. Bands were visualized by chemiluminescence using Phosphoimager 840. After densitometric analyses, protein levels were normalized per total protein content in each lane. Results are expressed as the mean IOD ± SEM and each point is the average of at least three independent experiments. Significant differences: $^{***}p < 0.001$.

transiently transfected with chimeras Glob-Gap3′ or Gap-Glob3′ (control) by RT-PCR. Cells were treated overnight with $CdCl_2$ to induce the metallothionein promoter in the constructs, and cells were harvested after 0 and 4 h of heavy metal depletion. At t0, results showed a 5-fold increase in GAP-43 3′UTR and a 50% decrease in cyclin D1 mRNA in cells transfected with Glob-Gap3′. At t4, the expression of GAP-43 3′UTR decreased in these cells and reached the levels of cells transfected with Gap-Glob3′; as a consequence, cyclin D1 mRNA levels increased and reached twice the levels of cells transfected with Gap-Glob3′ (Fig. 6C).

## Discussion

Nervous system formation relies on a fine balance between cell proliferation, cell cycle exit and the differentiation of several neural phenotypes [11,4,31]. An important number of factors controlling cell cycle progression have been implicated in the determination of cell fate and the differentiation of neural precursors. Conversely, molecules involved in the specification of certain phenotypes or their differentiation emerge as cell cycle regulators [7,12,8]. GAP-43 may then be regarded as a member of this category, i.e. a protein involved in neuronal regeneration which regulates cell cycle progression.

The expression of GAP-43, a protein specific to the nervous system, increases in differentiating neurons and glia. In undifferentiated neurons, the response of GAP-43 to extracellular signals plays an important role in guiding the axonal path, whereas its deficiency causes severe defects in cytoarchitecture. The presence of GAP-43 is also required in mitotic neuroblasts [3,10,15] and its absence causes an unsuitable onset of neural differentiation [20,38,39,24]. Shen et al. demonstrated that the expression of GAP-43 in multipotent precursors is necessary for both neurogenic and gliogenic commitment, while its absence alters neuronal and astrocyte differentiation [38]. In order to identify new genes associated with malignant astrocytoma formation, Huang et al. demonstrated that neither GAP-43 mRNA nor GAP-43 protein are present in cell lines derived from human and mouse gliomas. GAP-43 re-expression in these deficient cells has resulted in growth suppression and has been associated to a decrease in MAP kinase and to the activation of AKT, which suggests that GAP-43 may inhibit glioma growth through the modulation of mitogenic signaling pathways [13].

Considering this background information, we evaluated the possible participation of GAP-43 expression in cell cycle control in NIH-3T3 cells, a proliferating line which does not usually express this protein. The ectopic expression of GAP-43 produced no changes in cell viability or the adhesion properties of NIH-3T3 cells in culture. Taking this into account, the decrease in the number of cells corresponding to the GAP-43-expressing clone after 5 days in culture suggests a protein effect on cell cycle progression. In normally proliferating cells, growth arrest may be due to a decrease in the proportion of actively proliferating cells or an increase in cell cycle duration.

As these cells complete a cycle in 24 h, we analyzed the proportion of cells in each cell cycle phase, $G_0/G_1$, S and $G_2/M$ every 6 h in complete medium. In these experimental conditions and 18 h after medium replacement, we observed a significant decrease in the percentage of cells expressing GAP-43 in the S phase as compared to control cells. This decrease was at the expense of an increase in the proportion of cells in the $G_0/G_1$ phase, compatible with $G_0/G_1$ cycle arrest. Cell cycle withdrawal during the $G_1$ phase is a critical point when the cell must "decide" whether entering a new division cycle or differentiating. When a neural precursor commits to differentiation, it leaves the $G_1$ phase and enters the $G_0$ phase, a quiescent state. These findings are supported by results obtained from BrdU experiments, which showed lower incorporation in cells expressing GAP-43 as compared to control cells. In the same line, and using cumulative labeling of BrdU, Zhao et al. [48] determined that the time required for NIH-3T3 cells transfected with vector pLNCX2-GAP-43 to reach the proliferating stage was significantly longer than that of control cells, which is compatible with the increase observed in cell cycle duration.

In our experimental model, the expression of cyclin D1 was significantly lower in NIH-3T3 proliferating cells expressing GAP-43 than in control cells after 6, 8 and 10 h in complete medium. Although no significant changes in Cdk4 and Cdk6 protein levels

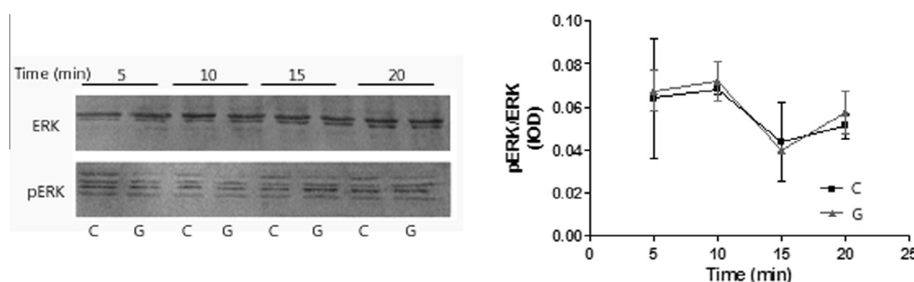


**Fig. 5.** Effects of GAP-43 expression on ERK1/2 activation. Synchronized T22p (C) and T23G (G) cells were harvested at 5, 10, 15, 20 min in complete medium and ERK1/2 and pERK1/2 levels were analyzed from cell extracts by Western blot analyses. Bands were visualized by chemiluminescence using Phosphoimager 840. Results are expressed as the mean IOD ± SEM and each point is the average of at least three independent experiments.

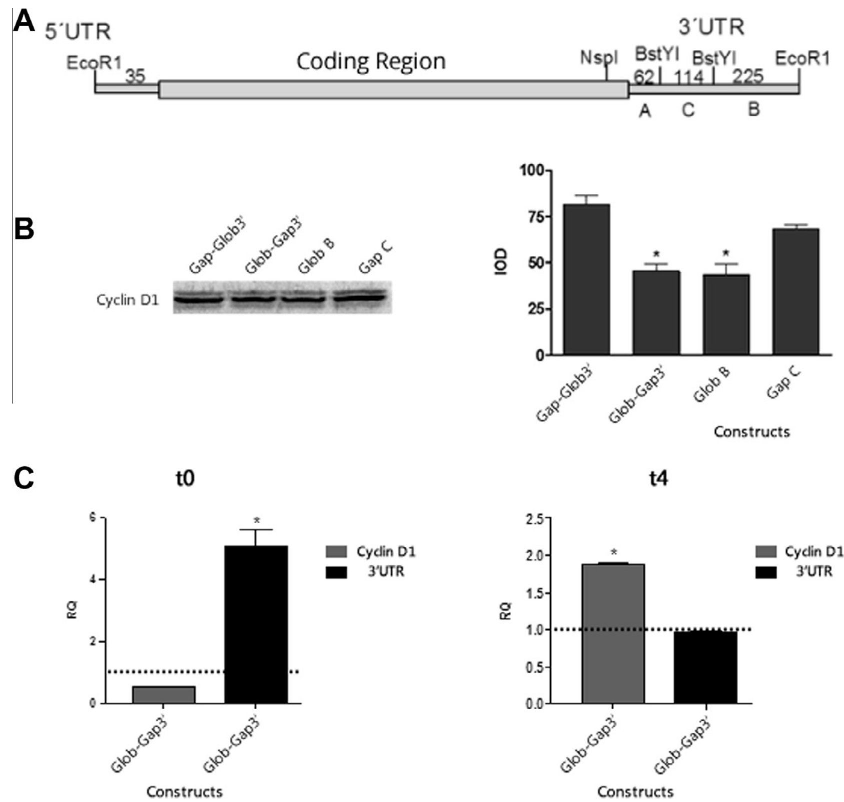


**Fig. 6.** Effects of Gap-Glob3′, Glob-Gap3′, Glob-B and Gap-C expression on cyclin D1 levels. (A) Schematic representation of wild-type GAP-43 cDNA showing regions A–C within 3′UTR, adapted from [45]. (B) NIH-3T3 cells were transiently transfected with constructs Gap-Glob3′, Glob-Gap3′, Glob-B and Gap-C. Once transfection had been completed, cells were induced for 16 h in the presence of 5 μM $CdCl_2$. Results were evaluated right after $CdCl_2$ removal (t0). Cyclin D1 protein levels were determined from cell extracts by Western blot analyses. Bands were visualized by chemiluminescence using Phosphoimager 840. Results are expressed as the mean IOD ± SEM and each point is the average of at least three independent experiments. Significant differences: $^{***}p < 0.001$. (C) NIH-3T3 cells were transiently transfected with constructs Gap-Glob3′ and Glob-Gap3′. Once transfection had been completed, cells were induced for 16 h in the presence of 5 μM $CdCl_2$. Results were evaluated right after $CdCl_2$ removal (t0) and 4 h later (t4). The levels of cyclin D1 mRNA and GAP-43 3′UTR sequence were then measured by RT-PCR. Reactions were done in a final volume of 25 μl containing SYBR Green 2× Master Green, primers F and R in a final concentration of 800 nM for cyclin D1, 500 nM for 3′UTR and 300 nM for GAPDH. Template mass used for each reaction was 1 ng. Universal thermocycling conditions were used. Bar graphs show the levels of cyclin D1 mRNA and GAP-43 3′UTR sequence in Glob-Gap3′-transfected cells at t0 and t4, respectively. Results are expressed relative to Gap-Glob3′-transfected cell values (dotted line).

were observed along the cell cycle or between control and GAP-43-expressing cells, we found a significant reduction in the activity of both kinases. These findings are in agreement with the decrease observed in cyclin D1 levels, and directly correlate with the decrease in cyclin E in cells expressing GAP-43 after 8–12 h in complete medium. Furthermore, while no changes were observed in the levels of Cdk2, the decline in cyclin E is in agreement with the decrease in the activity of this kinase as a consequence of the ectopic expression of GAP-43.

The generation of new neurons requires progenitor cells to abandon the cell cycle and activate specific differentiation and migration programs. A possible mechanism for the control of these events involves multifunctional proteins capable of controlling both cell cycle exit and differentiation [32]. Cdk inhibitor $p27^{Kip1}$, which regulates the cell cycle and promotes neuronal differentiation and migration, constitutes a good example. $p27^{Kip1}$ controls $G_1$–S transition and may, in that way, be involved in the lengthening of the $G_1$ phase [30,44]. In our studies, a significant increase in the expression of inhibitor $p27^{Kip1}$ was observed in cells expressing GAP-43 after 6–8 h in complete medium, which is in agreement with cell cycle arrest at the $G_1$ phase in NIH-3T3 cells expressing GAP-43, a protein directly involved in neuronal differentiation.

The ERK1 and ERK2 isoforms belong to a family of highly conserved serine/threonine kinases which are activated through phosphorylation. ERK activation is indispensable for cell proliferation and, in fibroblasts, it has been proven to take place over two phases: a quick initial phase in response to stimuli, and a late phase which takes several hours and is induced by the persistent presence of a mitogenic agent [23,14,43]. ERK activation is necessary for $G_1$/S transition [47], as it activates transcription factors responsible for cyclin D expression. ERK activation has been proposed as an event necessary for cell cycle progression induced by growth factors. The expression of GAP-43 in these cells produced no changes in early ERK1/2 phosphorylation levels, which suggests that the cell cycle arrest produced by the expression of GAP-43 in NIH-3T3 cells is not due to the inhibition of ERK1/2 signaling. Moreover, the absence of significant differences in ERK phosphorylation between GAP-43-expressing and control cells suggests that the decrease observed in cyclin D1 levels may be due to a posttranscriptional effect.

With the formation of the three germinal layers (ectoderm, mesoderm and endoderm), stem cells lose pluripotency and their function is restricted to the generation of specific cell types in different organs or systems derived from a single germinal layer. Along the cell cycle, $G_1$ is the most interesting phase in terms of the differentiation of stem cells and the one in which most changes take place during development. Interestingly, it has been suggested that the duration of the $G_1$ phase in embryonic, neural and hematopoietic stem cells determines their differentiation. The cell cycle hypothesis put forward by Lange and Calegari [19] states that a change in $G_1$ length may be a cause rather than a consequence of the differentiation of a progenitor cell. Therefore, changes in the

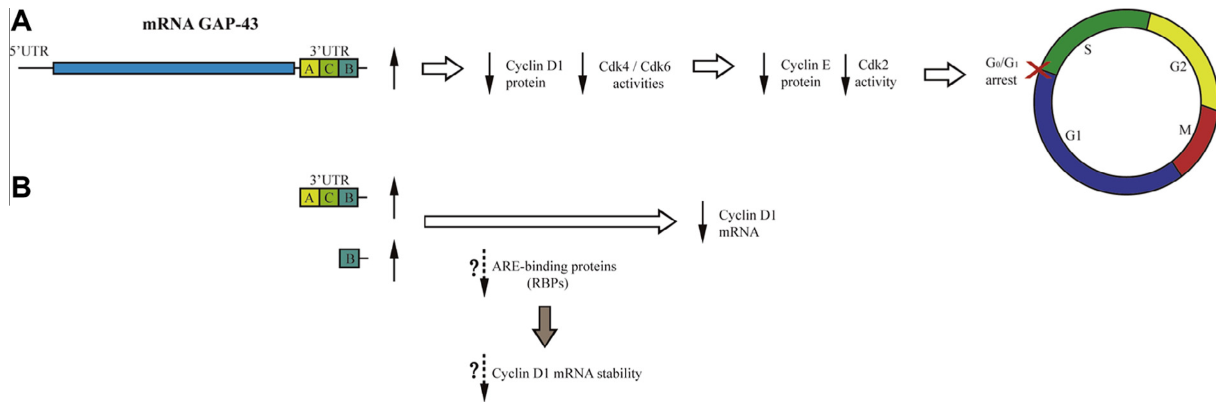


**Fig. 7.** Role of GAP-43 in the control of cell cycle progression: a proposed mechanism. (A) The transfection of proliferating NIH-3T3 cells with wild-type GAP-43 cDNA generated a decrease in cyclin D1 levels and, as a consequence, a decrease in Cdk4 and Cdk6 activity. These events in turn triggered a decrease in cyclin E levels and Cdk2 activity, which finally led to a cell cycle arrest in $G_0/G_1$. As no changes were observed in ERK1/2 phosphorylation, this arrest may respond to a posttranscriptional effect probably involving RBPs. (B) The expression of constructs Glob-Gap3′, which includes the coding region of rabbit β-globin and the 3′UTR of GAP-43, and Glob-B, in which fragment B in the 3′UTR of GAP-43 contains the HuD-binding site, induced a decrease in both cyclin D1 protein levels and mRNA expression. These findings hint at a mechanism involving a loss in cyclin D1 mRNA stability due to a decrease in RBP availability.

levels of cyclins/Cdks in the $G_1$ phase may control the proliferation versus the differentiation of a certain progenitor cell.

In this context, GAP-43 is implicated in neuronal differentiation during development and, according to our results and those of Zhao et al. [48], its expression in proliferating NIH-3T3 produces changes in cell cycle progression. In our experimental model, and in contrast to the results of Zhao et al., we proved an increase in the expression of $p27^{Kip1}$. In addition, we observed a decrease in the expression of cyclin D1 and a concomitant reduction in the activity of Cdk4 and Cdk6, which in turn triggered a decrease in cyclin E levels and in the activity of Cdk2, with no changes in the activation of ERK1/2.

When evaluating the possible mechanisms involved in cell cycle arrest by GAP-43 expression, we observed a significant decrease in the expression of cyclin D1 protein in cells transfected with constructs Glob-Gap3′, which contains the entire 3′UTR of GAP-43 mRNA, and Glob-B, which contains fragment B of the 3′UTR of GAP-43 mRNA where HuD binds, as compared to cells transfected with construct Gap-Glob3′, which exhibited the highest levels of cyclin D1 protein. This result shows that the consequence of the expression of the β-globin gene 3′UTR, which does not contain an ARE, is different from that of the expression of GAP-43 mRNA 3′UTR. In addition, no significant differences were observed in the levels of cyclin D1 in cells transfected with construct Gap-C, which only contains the coding region of GAP-43 and a region of the 3′UTR that does not contain the ARE or HuD binding site. The assessment of the levels of cyclin D1 mRNA and those of the 3′UTR of GAP-43 sequence in transfected NIH-3T3 cells revealed an inverse relationship, as evidenced by an increase in the expression of GAP-43 3′UTR concomitant with a decrease in the levels of cyclin D1 mRNA.

## Conclusions

Although further experiments need to be carried out to determine whether the 3′UTR of GAP-43 and cyclin D1 mRNA both bind to Hu proteins, these results suggest that the decrease observed in cyclin D1 protein levels as a consequence of GAP-43 expression may be due to an alteration in the stability of cyclin D1 mRNA. This alteration may in turn be due to a reduction in the availability of ARE-binding RBPs, which are required for its stabilization (Fig. 7). Should these results be confirmed, sequences in the B region of the GAP-43 3′UTR could be used to slow down cell cycle progression, which might imply promising therapeutic applications.

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## References

[1] K.A. Alexander, B.M. Cimler, K.E. Meier, D.R. Storm, J. Biol. Chem. 262 (13) (1987) 6108–6113.
[2] R.M. Bell, D.J. Burns, J. Biol. Chem. 266 (8) (1991) 4661–4664.
[3] P.A. Brittis, K. Meiri, E. Dent, J. Silver, Exp. Neurol. 134 (1) (1995) 1–12, http://dx.doi.org/10.1006/exnr.1995.1031.
[4] F. Cremisi, A. Philpott, S. Ohnuma, Curr. Opin. Neurobiol. 13 (1) (2003) 26–33. doi:S0959438803000059.
[5] C.Y. Chen, A.B. Shyu, Trends Biochem. Sci. 20 (11) (1995) 465–470. doi: S0968000400891021.
[6] A. Chiaramello, T. Neuman, D.R. Peavy, M.X. Zuber, J. Biol. Chem. 271 (36) (1996) 22035–22043.
[7] C.L. Dou, S. Li, E. Lai, Cereb. Cortex 9 (6) (1999) 543–550.
[8] M.A. Dyer, C.L. Cepko, Development 127 (16) (2000) 3593–3605.
[9] B.J. Eggen, H.B. Nielander, M.G. Rensen-de Leeuw, P. Schotman, W.H. Gispen, L.H. Schrama, Brain Res. Mol. Brain Res. 23 (3) (1994) 221–234.
[10] C. Esdar, S.A. Oehrlein, S. Reinhardt, A. Maelicke, T. Herget, Eur. J. Neurosci. 11 (2) (1999) 503–516.
[11] A. Gilardini, P. Marmiroli, G. Cavaletti, Curr. Med. Chem. 15 (29) (2008) 3025–3035.
[12] N. Heins, F. Cremisi, P. Malatesta, R.M. Gangemi, G. Corte, J. Price, G. Goudreau, P. Gruss, M. Gotz, Mol. Cell. Neurosci. 18 (5) (2001) 485–502, http://dx.doi.org/10.1006/mcne.2001.1046.
[13] Z.Y. Huang, Y. Wu, S.P. Burke, D.H. Gutmann, Cancer Res. 63 (11) (2003) 2933–2939.
[14] C. Kahan, K. Seuwen, S. Meloche, J. Pouyssegur, J. Biol. Chem. 267 (19) (1992) 13369–13375.
[15] S. Kanazir, S. Ruzdijic, S. Vukosavic, S. Ivkovic, A. Milosevic, N. Zecevic, L. Rakic, Brain Res. Mol. Brain Res. 38 (1) (1996) 145–155. doi: 0169328X96000083.
[16] J.D. Keene, Proc. Natl. Acad. Sci. U.S.A. 96 (1) (1999) 5–7.
[17] W.R. Kinney, R.K. McNamara, E. Valcourt, A. Routtenberg, Brain Res. Mol. Brain Res. 38 (1) (1996) 25–36. doi: 0169328X95002873.
[18] U.K. Laemmli, Nature 227 (259) (1970) 680–685.
[19] C. Lange, F. Calegari, Cell Cycle 9 (10) (2010) 1893–1900. doi: 11598.
[20] S. Mani, Y. Shen, J. Schaefer, K.F. Meiri, Mol. Cell. Neurosci. 17 (1) (2001) 54–66, http://dx.doi.org/10.1006/mcne.2000.0931 S1044-7431(00)90931-X.
[21] K.F. Meiri, P.R. Gordon-Weeks, J. Neurosci. 10 (1) (1990) 256–266.
[22] K.F. Meiri, J.L. Saffell, F.S. Walsh, P. Doherty, J. Neurosci. 18 (24) (1998) 10429–10437.
[23] S. Meloche, K. Seuwen, G. Pages, J. Pouyssegur, Mol. Endocrinol. 6 (5) (1992) 845–854.
[24] R. Mishra, S.K. Gupta, K.F. Meiri, M. Fong, P. Thostrup, D. Juncker, S. Mani, Cell Cycle 7 (3) (2008) 348–357. doi: 5235 [pii].

[25] C.D. Mobarak, K.D. Anderson, M. Morin, A. Beckel-Mitchener, S.L. Rogers, H. Furneaux, P. King, N.I. Perrone-Bizzozero, Mol. Biol. Cell 11 (9) (2000) 3191–3203.
[26] T. Nakayama, S. Asami, S. Ono, M. Miura, M. Hayasaka, Y. Yoshida, M. Toriyama, S. Motohashi, T. Suzuki, Jpn. J. Clin. Oncol. 39 (4) (2009) 251–259, http://dx.doi.org/10.1093/jjco/hyp011.
[27] U. Namgung, A. Routtenberg, Eur. J. Neurosci. 12 (9) (2000) 3124–3136. doi: ejn196.
[28] R.L. Neve, R. Coopersmith, D.L. McPhie, C. Santeufemio, K.G. Pratt, C.J. Murphy, S.D. Lynn, J. Neurosci. 18 (19) (1998) 7757–7767.
[29] R.L. Neve, N.I. Perrone-Bizzozero, S. Finklestein, H. Zwiers, E. Bird, D.M. Kurnit, L.I. Benowitz, Brain Res. 388 (2) (1987) 177–183. doi: S0006-8993(87)80012-4.
[30] L. Nguyen, A. Besson, J.M. Roberts, F. Guillemot, Cell Cycle 5 (20) (2006) 2314–2318. doi: 3381 [pii].
[31] S. Ohnuma, W.A. Harris, Neuron 40 (2) (2003) 199–208. doi: S0896627303006329.
[32] S. Ohnuma, A. Philpott, W.A. Harris, Curr. Opin. Neurobiol. 11 (1) (2001) 66–73. doi: S0959438800001756.
[33] N. Perrone-Bizzozero, F. Bolognani, J. Neurosci. Res. 68 (2) (2002) 121–126, http://dx.doi.org/10.1002/jnr.10175.
[34] N. Perrone-Bizzozero, R.L. Neve, N. Irwin, S. Lewis, I. Fischer, L.I. Benowitz, Mol. Cell. Neurosci. 2 (1991) 402–409.
[35] N.I. Perrone-Bizzozero, V.V. Cansino, D.T. Kohn, J. Cell Biol. 120 (5) (1993) 1263–1270.
[36] J. Ross, Microbiol. Rev. 59 (3) (1995) 423–450.
[37] G. Shaw, R. Kamen, Cell 46 (5) (1986) 659–667. doi: 0092-8674(86)90341-7.
[38] Y. Shen, S. Mani, K.F. Meiri, Mol. Cell. Neurosci. 26 (3) (2004) 390–405, http://dx.doi.org/10.1016/j.mcn.2004.03.004 S1044743104000697.
[39] Y. Shen, R. Mishra, S. Mani, K.F. Meiri, Cerebellum 7 (3) (2008) 451–466, http://dx.doi.org/10.1007/s12311-008-0049-5.
[40] R.G. Starr, B. Lu, H.J. Federoff, Brain Res. 638 (1–2) (1994) 211–220. doi: 0006-8993(94)90652-1.
[41] S.M. Strittmatter, D. Valenzuela, M.C. Fishman, J. Cell Sci. 107 (Pt 1) (1994) 195–204.
[42] A. Szabo, J. Dalmau, G. Manley, M. Rosenfeld, E. Wong, J. Henson, J.B. Posner, H.M. Furneaux, Cell 67 (2) (1991) 325–333. doi: 0092-8674(91)90184-Z.
[43] H. Tamemoto, T. Kadowaki, K. Tobe, K. Ueki, T. Izumi, Y. Chatani, M. Kohno, M. Kasuga, Y. Yazaki, Y. Akanuma, J. Biol. Chem. 267 (28) (1992) 20293–20297.
[44] T. Tarui, T. Takahashi, R.S. Nowakowski, N.L. Hayes, P.G. Bhide, V.S. Caviness, Cereb. Cortex 15 (9) (2005) 1343–1355, http://dx.doi.org/10.1093/cercor/bhi017.
[45] K.C. Tsai, V.V. Cansino, D.T. Kohn, R.L. Neve, N.I. Perrone-Bizzozero, J. Neurosci. 17 (6) (1997) 1950–1958.
[46] C.O. Van Hooff, J.C. Holthuis, A.B. Oestreicher, J. Boonstra, P.N. De Graan, W.H. Gispen, J. Cell Biol. 108 (3) (1989) 1115–1125.
[47] T. Yamamoto, M. Ebisuya, F. Ashida, K. Okamoto, S. Yonehara, E. Nishida, Curr. Biol. 16 (12) (2006) 1171–1182, http://dx.doi.org/10.1016/j.cub.2006.04.044.
[48] J. Zhao, Y. Yao, C. Xu, X. Jiang, Q. Xu, Int. J. Dev. Neurosci. 27 (6) (2009) 531–537, http://dx.doi.org/10.1016/j.ijdevneu.2009.06.013.
[49] H. Zwiers, P.J. Coggins, Peptides 11 (5) (1990) 951–954.