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# Stabilization of cellular mRNAs and upregulation of proteins by oligoribonucleotides homologous to the Bcl2 ARE motif

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**Running title**

Inducible RNAs stabilized by ORNs

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**Abbreviations**: ARE, Adenine-uridine Rich Elements; AUBPs, Adenine-Uridine Binding Proteins; ORN, oligoribonucleotides; UTR, untranslated region; b-ARE, bcl2 ARE; b-RNA, 3'untranslated region of Bcl2 RNA; HEK 293, human embryonic kidney 293; DMEM, Dulbecco's modified Eagle's Medium; FCS, Fetal Calf Serum; degORNs, 2'-*O*-methyl degenerated oligoribonucleotides; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; DRB, 5,6-dichloro-1-β-D-ribofuranosyl - benzimidazole; GST, Glutathione S Transferase; GSH-sepharose, Glutathione affinity sepharose; RT-PCR, Reverse Transcription Polymerase Chain Reaction; RIP, RNA immunoprecipitation; MoAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGFR, insulin-like growth factor-I receptor; SD, Standard Deviation.

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## ABSTRACT

A-U Rich Elements (ARE) play an important role in modulating mRNA stability, being the target site of many ARE-binding proteins (AUBPs) that are involved in the decay process. Three 26mer 2'O-methyl oligoribonucleotides (ORNs) homologous to the core region of ARE of bcl2 mRNA have been studied for decoy-aptamer activity in UV cross-linking assays. Sense-oriented ORNs competed with the ARE motif for the interaction with both destabilizing and stabilizing AUBPs in cell free systems and in cell lines. Moreover, ORNs induced mRNA stabilization and upregulated both Bcl2 mRNA and protein levels in the cells. Bcl2 ORNs stabilized other ARE-containing transcripts and upregulated their expression. These results indicate that Bcl2 ORNs compete for AUBP-ARE interactions independently of ARE class and suggest that in the cell the default labile status of ARE-containing mRNAs depends on the combined interaction of such transcripts with destabilizing AUBPs.

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## INTRODUCTION

In mammalian cells each step in the pathway of gene expression, from DNA to protein, is efficiently regulated. Although gene expression depends primarily on transcription, mRNA is regulated by various post-transcriptional events (Riddihough, 2005; Moore, 2005; Kindler et al., 2005). After the relatively slow process of transcription, the basal level of mRNA is mainly determined by the rate of degradation (Wagner and Lykke-Andersen, 2002; Cougot et al., 2004). Many messengers are further fine-tuned in response to environmental and physiological conditions (Shim and Karin, 2002).

Most of inducible genes, including those coding for growth factors, receptors or regulatory proteins, have unstable mRNAs because cells may have to modulate the corresponding protein level rapidly (Chen and Shyu, 1995). For these genes, the rate of mRNA turnover is the crucial step in regulating basic functions, including cell cycle, cell viability and reaction to stress (Winzen et al., 2004; Cheadle et al., 2005).

The differences in phenotypes among cells are mainly due to untranslated sequences involved in regulation rather than in coding sequences. Regulatory factors include RNA-binding proteins that heavily influence individual's proteome by regulating RNA stability and translation. These proteins associate with multiple messenger RNAs according to a model of co-ordinated gene expression of functionally related transcripts (Keene and Lager, 2005).

At the level of individual transcript, we have shown that the turnover of the bcl2 messenger can be regulated by the protein Bcl2 itself. A combinatorial mechanism is emerging of RNA regulation by multi-target proteins responsible for different levels of specificity and functionality.

Two major degradation mechanisms have been shown to affect mammalian mRNAs half-life: deadenylation-decapping mediated decay (Bail and Kiledjian, 2006; Gao et al., 2001) and

decay mediated by the ARE motives (Raijmakers et al., 2004; Guhaniyogi and Brewer, 2001). In both pathways gene-specific stretches, usually located in the 3' UTR and 5' UTR, are needed for degradation (Stoecklin et al., 2006).

The AREs are discrete nucleotide structures located in the 3' UTR, at present identified in more than 4000 inducible genes including cyclins, kinases, cytokines, receptors, stress genes and proto-oncogenes (Bakheet et al., 2006). These elements have an important role in mRNA stability, being the target site of many AUBPs, involved in the decay processes and being able to recruit the exosome (Barreau et al., 2006; Bevilacqua et al., 2003b).

Recently, mRNA turnover have been found to be located in discrete cytoplasmic foci, the processing bodies. Under stress conditions that lead to stalled translation, preinitiation complexes can aggregate in cytoplasmic granules from which selected transcripts are degraded (Kedersha et al., 2005; Brengues et al., 2005).

Understanding the molecular mechanisms that regulate the rate of degradation and learn how each mRNA is maintained at the desired level in the cellular cytoplasm is a crucial issue. Future studies might unravel new pathogenic pathways leading to many diseases, including cancer (Denkert et al., 2004; Audic and Hartley, 2004) or neurodegenerative disorders (Si et al., 2004) and disclose new therapeutic strategies to regulate gene expression by exogenous means. Moreover, inhibition of the degradation process increases the cellular amount of RNA and of its relative protein.

The regulation of inducible proteins causes obvious phenotypes. For instance, increasing the cellular level of the antiapoptotic protein Bcl2 can protect cells from apoptotic stimuli, although it might have transforming effects (Meijerink et al., 2005). The degradation of Bcl2 mRNA is an ARE-dependent process (Schiavone et al., 2000) mediated by trans-acting elements (Donnini et al., 2004). The rate of Bcl2 mRNA degradation is also dependent on the amount of the Bcl2 protein in the cells (Bevilacqua et al., 2003a).

In the present work, three ORNs designed on the ARE motif (b-ARE) of Bcl2 mRNA have been studied through UV cross-linking assays to prove their AUBP inactivation according to a decoy-aptamer mechanism.

Several studies indicate that AUBPs, including KSRP, TTP, AUF1, elicit a destabilizing activity, promoting the rapid decay of unstable mRNAs, though HuR may exhibit an opposite effect. Sense-oriented ORNs were used to compete for the binding of these AUBPs to synthetic b-ARE and studied for their effects on the rate of ARE-dependent RNA decay. The rate of RNA degradation, the level of RNA expression and the amount of protein synthesized were evaluated in cell lines exposed to ORNs. The present study aims at uncovering a new mechanism for the upregulation of gene expression based on competition for AUBPs activity.

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## RESULTS

*Displacement of ARE binding proteins.*

The turnover of many inducible RNAs is regulated by AUBPs. Three 26mer ORNs, each homologous to one-third of b-ARE core, shown in Fig. 1A, were used in UV cross-linking assays in an effort to obtain deeper insight into the mechanisms modulating bcl2 mRNA stability. Four relevant human recombinant AUBPs, KSRP, p37-AUF1, TTP and HuR, were incubated with a radiolabeled fragment of bcl2 3' UTR (b-RNA) encompassing the related b-ARE motif.

Oligoribonucleotides were added at 1μM to the RNA-AUBP mixture in the reaction tube of the UV cross-linking assay. Fig. 1B shows that each ORN inhibited the binding of AUBPs to the b-RNA in a sequence-specific fashion.

ORN2 was the most effective in displacing KSRP, HuR and AUF1. In the TTP assay, the three ORNs inhibited the binding event with similar efficacy. Although UV cross linking is only a semi-quantitative assay, ORNs were able to displace AUF1, an effective regulator of the b-RNA half-life (Lapucci et al., 2002), in a dose-response fashion (Fig. 1C). To address the displacement activity in cells, HEK 293 cells stably transfected with pGL4.71P bARE plasmid expressing chimeric mRNA Rluc-bARE were treated with ORNs 1.5 μM. RNA immunoprecipitation assays were performed 30 h after transfection with ORNs to evaluate the effect on AUBPs binding to b-ARE. The amount of the relevant RNA was significantly reduced in cells treated with sense ORNs, as shown in Fig. 1D.

*In vitro inhibition of b-RNA degradation.*

Whether displacement of AUBPs might change the rate of b-RNA turnover was analyzed by a cell free degradation assay. The b-RNA fragment including b-ARE, labeled by incorporation of the precursor DlG-UTP was transcribed as reported (Bevilacqua et al., 2003a). An IGFR

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RNA fragment, labeled as above, was added to the degradation mixture as a control. For the decay assays, cell lysates from DOHH2 lymphoma cells were mixed with the relevant transcripts and incubated at 37°C for the indicated times.

The three ORNs, used in combination to span the entire b-ARE motif at a final concentration of 10 μM, proved highly effective (Fig. 2A). Fig. 2B shows that the half-life of the b-RNA was more than twice that of samples containing degORNs. In contrast, the degradation rate of the IGFR transcript, not expressing ARE motifs, was unaffected. The slow down of b-RNA turnover by the ORNs was dose-dependent: the activity increased from 1 μM to 10 μM and dropped off at higher doses (Fig. 2C and 2D). Oligos designed in between ORN1 and ORN2 or ORN2 and ORN3 or flanking the core sequence did not display a significant activity (data not shown).

The displacing activity of each ORN at the optimal concentration of 10 μM was correlated with the rate of b-RNA degradation. As shown in Fig. 3A, at each time point, the rate of b-RNA degradation in the presence of each ORN at the optimal concentration of 10 μM. At each time point, the rate of b-RNA decay in the presence of ORNs was lower than with inactive degORNs. The most effective, ORN2, extended the half-life of b-RNA from 35 min to more than 60 min (Fig. 3B). ORN activity closely correlated with the displacing activity shown in the UV cross-linking assays.

*Increased level and stabilization of RNAs in cell lines*.

Raji lymphoma cells and HL60 promyelocytic cells were exposed to three ORNs mixture to a total concentration of 1.5 μM in DOTAP. The amount of Bcl2 mRNA in the cells was measured by real-time RT-PCR (TaqMan Probe System) at the indicated times.

As shown in Fig. 4A and 4C, ORNs were able to increase the amount of Bcl2 mRNA in both cell lines, the highest activity being obtained 48 h after treatment. The possibility that ORNs might stabilize Bcl2 mRNA was studied by analyzing the amount of Bcl2 mRNA in extracts

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from cells treated with the transcription inhibitor DRB. Using the real-time RT-PCR, the Bcl2 mRNA half-life was calculated 4.5 h in untreated, more than 7 h in ORN-treated Raji cells (Fig. 4B); it was 3.5 h in untreated versus 6 h in ORN-treated HL60 cells (Fig. 4D).

Individual AUBPs can interact and regulate the mRNA decay of many genes (Chen and Shyu, 1995), it is thus possible that the AUBPs displacement can stabilize ARE-expressing genes other than bcl2 (Supplemental Data on-line). Therefore, we measured the amount of c-myc RNA in HL60 cells (Fig. 4E) as well as the amount of Mitogen-activated Protein Kinase Kinase 6 (MKK6) and p27 RNA in Raji cells (Fig. 4F and 4G).

The amount of all three RNAs was significantly increased in cells treated with sense-oriented ORNs.

*Level of relevant proteins in cells treated with ORNs.*

The increased level of RNAs in ORN-treated cells prompted us to study the expression of the relevant proteins in two lymphoid cell lines. Fig. 5A shows the increased expression of Bcl2 protein measured on day 3 or on day 5 from the initial exposure to the ORNs. Proteins were quantified by densitometry and plotted in histograms (Fig. 5B). The dose-related activity peaked at 1 μM, was lower at 3 μM and higher on day 5 (after two ORN treatments).

In a similar way, the level of p27 protein, whose RNA expresses an ARE motif, was significantly increased by the ORN treatments (Fig. 6A and 6B).

**DISCUSSION**

Synthetic single-strand oligoribonucleotides, chemically modified to resist nuclease degradation, were prepared to compete with b-ARE for some relevant AUBPs, in accordance with the decoy-aptamer strategy (Nimjee et al., 2005; Tomita et al., 2002). We also examined whether sense-oriented ORNs would change the rate of b-RNA degradation in *in vitro* assays and in viable cells.

In direct UV cross-linking assays, ORNs successfully competed with the b-ARE motif, likely inhibiting the interaction of recombinant HuR, KSRP, AUF1 or TTP with b-RNA. Though both stabilizing and destabilizing AUBPs were displaced by oligos, RNA stabilization turned out as the final effect of sORNs treatment.

ORN2, designed on the central region of the ARE motif, was the most effective to inhibit the binding of all four AUBPs. In this sub-region are located relevant determinants for the degradation machinery, determinants that are required for the binding of important AUBPs. The binding activity of HuR and KSRP to the b-ARE was strongly decreased also in HEK 293 upon ORNs treatment, as shown by RNA immunoprecipitation.

Each AUBP, included those studied here, can bind AREs present in several mRNAs (Lu and Schneider, 2004; Good, 1995). How one AUBP can bind different nucleotide structures is not yet known. A possibility might be the recognition of characterizing elements such as the AUUUA pentamer or U rich stretches expressed in the 3' UTR of many messengers (Fialcowitz et al., 2005; Winzen et al., 2004). According to the post-transcriptional operon model (Keene and Lager, 2005) RNA-binding proteins coordinately regulate the expression of multiple mRNAs encoding functionally related proteins. These mechanisms provide combinatorial regulation of genetic information including the evolution of multifunctionality of eukaryotic proteins. Recently, systems and highthroughput approaches have provided

insight into coordinated post-transcription regulation of gene expression. Multiple interactions have been shown by microRNAs, which can also complement short 3' UTR stretches (5-8 nt) within a variety of RNA targets contributing to regulate protein synthesis along a coordinated fashion (Kim and Nam, 2006; Yoon and De Micheli, 2005).

This study, while not involved directly into this matter, suggests that characterizing structures expressed in most AREs may behave as shared targets for AUBPs.

The binding of a protein to different AREs of unrelated transcripts was inhibited by sequence-specific ORNs mimicking endogenous structures. Meanwhile, one single ORN can compete with different AUBPs.

The decoy activity in vitro and in cell lines actually turned out to regulate the rate of b-RNA degradation. In the cell-free system, ORNs protected b-RNA from the degradative action of an effective cell extract. Individually, the most effective was ORN2, while ORN3 showed no detectable activity. Thus, the stabilizing activity mirrored the decoy activity. Moreover, the effect of the triple ORN mixture on the rate of b-RNA degradation was dose-dependent. This suggest that the decreased interaction of destabilizing AUBPs with Bcl2 mRNA transcript, due to the ORNs presence, leads to an mRNA stabilization.

In different human cell lines ORNs were able to significantly increase the level of Bcl2 mRNA. The biochemical mechanism might be dependent on the ORN ability to slow down the rate of degradation, as shown by the time course determinations of the Bcl2 mRNA in cells treated with a transcription inhibitor.

Not entirely unexpected, ORNs also inhibited the rate of degradation of unrelated mRNAs whose decay is dependent on AREs and AUBPs. These observations might figure out the coordinated and temporally linked regulation of functionally related transcripts.

Consistent with the increased amount of mRNA, the level of Bcl2 protein was significantly and dose-dependently higher in cells exposed to ORNs. Moreover, as predicted above, the

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level of proteins encoded by stabilized RNAs were increased as well. It is shown that the p27 protein, which fluctuates during the cell cycle often in coordination with the level of Bcl2 (Greider et al., 2002; Calastretti et al., 2001), was also upregulated.

Although the mechanism of RNA augmentation by ORNs homologous of the b-ARE motif is only in part understood, the findings described here are in keeping with a model in which ARE is regulated at different levels of specificity and coordination (Keene and Lager, 2005). The ARE of the bcl2 gene, characterized by a unique nucleotide sequence, might be targeted specifically by the relevant protein that actually regulates the rate of degradation of its own RNA, according to a negative feed-back mechanism (Bevilacqua et al., 2003a). The bcl2 transcript can also be regulated specifically by antisense oriented oligoribonucleotides targeting the b-ARE motif (Ghisolfi et al., 2005) supporting the concept of the individual regulation.

Conversely, the coordinated regulation of multiple related transcripts, as shown here by the use of sense-oriented ORNs, is not in contrast with the single transcript regulation. On the basis of studies on AUBP displacement, sequence-specific ORNs, by competing for the docking sites of proteins that recognize common elements within the ARE structures, might exert a multi-transcript activity. These findings support a combinatorial modality for the co-ordinating transcript regulation based on the interaction of *trans* acting factors and *cis* elements.

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## MATERIALS AND METHODS

*Cell Cultures and Chemicals* - Viable human lymphoma cell lines, DOHH2, HL60, Raji in RPMI 1640 or viable monolayer HEK 293 cells were grown in complete DMEM, additioned with 10% FCS. Three 2'-O-methyl ORNs, designed on the b-ARE motif as shown in Fig. 1A (nt 942 to 1050, GenBank M14745), or 26-mer degenerated ORNs (degORNs) purchased from Dharmacon were delivered to cells by DOTAP (Roche Diagnostics). The transcription blocker 5,6-dichloro-1-β-D-ribofuranosyl - benzimidazole (DRB) was purchased by Sigma-Aldrich.

*Plasmids, synthetic transcripts and recombinant proteins* - The Kpn I – Hind III 240-bp fragment containing the SV40 Promoter was excised from the pGL3-Promoter Vector and cloned into the corresponding restriction sites of the pGL4.71 Vector (Promega) to obtain the pGL4.71P plasmid. The 5'-primer CGTCTAGAGTCAACATGCCTGC and the 3'-primer CGTCTAGAGGTGATCCGGCCAA (flanked at the 5' end by a CG overhang followed by the Xba I restriction site) were used to amplify the 400 bp segment containing the 3' UTR ARE sequence from the human bcl2 cDNA fragment. This fragment was cloned into the Xba I cloning site at the 3' end of the hRlucP gene coding for *Renilla luciferase* of the pGL4.71P plasmid, to produce the pGL4.71P bARE plasmid. Synthetic Bcl2 RNA (b-RNA) (540 nt) used for in vitro degradation assays was prepared as previously described (Bevilacqua et al., 2003a). For UV cross linking assays, radiolabeled bcl2 transcript was synthesized as described (Chen et al., 2001). Proteins GST–KSRP, GST–HuR, GST–AUF1(p37) were produced in BL21 and purified using GSH–Sepharose 4B (Amersham) resin, whereas histidine-tagged TTP were produced in *Escherichia coli* and purified using GSH–Sepharose 4B or Ni-NTA (Qiagen).

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*UV cross-linking* - UV cross-linking assays were performed as described (Chen et al., 2001) with minor modifications. Each AUBP, KSRP 400ng, TTP 300ng, HuR or p37-AUF1 200ng, and $^{32}$P-labeled b-RNA (0.5 ng = $2 \times 10^5$ cpm) were incubated at room temperature for 20 min in a RNA-binding buffer (20 µL) containing 10 mM HEPES (pH 7.6), 3 mM $MgCl_2$, 100 mM KCl, 2 mM DTT, 5% glycerol, 0.5% NP-40, yeast RNA (1 µg), and heparin (1 µg). For competition experiments, unlabeled ORNs 1µM, 10 µM and 100 µM, were incubated with lysates for 10 min before addition of the RNA probe. Unbound RNA was digested with RNAase T1 (200 units per reaction) for 15 min at 37°C. Reaction mixtures were transferred to a 96-well plate and irradiated at 4°C for 10 min with a UV Stratalinker (Stratagene). After digestion with RNase A (200 ng per reaction) for 10 min at 37°C, samples were separated by electrophoresis on reducing 10% SDS–polyacrylamide gels. Gels were dried, and $^{32}$P-labeled proteins were made visible by autoradiography.

*RNA degradation* – The assays were performed as described with minor modifications (Ghisolfi et al., 2005). The cell-free reaction mixture was maintained 45 min at 4°C, before the activation at 37°C. Cellular degradation was studied in ORN treated cells at day 0 and treated with DRB on day 1. After 0, 3, 5 and 7 hours total RNA was extracted with NucleoSpin RNA II columns (Macherey-Nagel) and amplified by TaqMan real-time RT-PCR (Applied Biosystem). The cellular amounts of Bcl2, c-myc, MKK6, p27 mRNAs, normalized to the GAPDH housekeeping gene, were determined as above.

*Cell transfection* – HEK 293 cells were co-transfected with pcDNA3 and pGL4.71P bARE plasmid expressing the chimeric mRNA Rluc-bARE by using Lipofectamine 2000 (Invitrogen). After 48 h, cells were replated in selective media containing 0.8 mg/ml antibiotic G418 (Sigma-Aldrich). Single clones were selected for their resistance to G418 and luciferase activity.

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*RNA Immunoprecipitation assay* - RIP was performed according to Gherzi et al. (2004) with slight modifications. Briefly, HEK 293, stably expressing pGL4.71P bARE plasmid, were transfected with ORNs at 1.5 μM. After 48 hours, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 100 mM NaF, 1 M Na3VO4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 2 mM PMSF, 10 mM vanadyl ribonucleoside complex, and 1 mg/ml aprotinin for 15 min at 4°C. Lysates, spun at 14,000g for 15 min at 4°C and supernatants, were incubated overnight with rabbit αKSRP (Gherzi et al., 2004) or TTP (purchased from Dr.W.Rigby, Dartmouth Medical School) or αHuR MoAb (Santa Cruz Biotechnology) at 4°C under rotation and purified with protein A (rabbit antibodies) or G Sepharose (MoAb). RNA, extracted with RNAzol, was determined by RealTime RT-PCR using a Rluc-bARE chimeric probe.

*Western Blot analysis* – The assays were performed under standard conditions, blots were probed with αBcl2 (Dako), αp27 and αHuR (Santa Cruz) MoAbs or rabbit αKSRP, αTTP and αβ-actin (Sigma-Aldrich), and detected by ECL-Plus kit (Amersham Biosciences).

*Statistics* – The data were expressed as means ± SD, statistical significance was calculated by using a Student's t test. * $P \leq 0.05$; ** $P \leq 0.01$.

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**FOOTNOTES**

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**FIGURE LEGENDS**

Fig. 1. ORN displacement of AUBPs from b-ARE. *A,* Schematic structure of b-ARE and nucleotide positions from which ORNs were designed. Human b-ARE sequence (bases 942-1020, GenBank™ accession number M14745), and three homologous 2'-O-methyl modified ORNs are depicted. *B,* Competition by ORNs for AUBPs in the UV cross-linking assays. Radiolabeled b-RNA was incubated with the AUBPs KSRP, HuR, TTP, or AUF1. Before the UV exposure, degORNs or individual ORNs were added at 1 μM to the reaction mixture. *C,* Dose-response relationship of ORNs. The displacement of AUF1 from the target b-ARE using increasing amounts of ORNs in the UV cross-linking assays is shown. *D,* RIP assays. Extracts from HEK 293 cells stably transfected with the chimeric Rluc-bARE construct and treated with 1.5 μM ORNs were immunoprecipitated with αHuR moAb or αKSRP and αTTP polyclonal Abs. The amount of Rluc-bARE in the HuR, KSRP or TTP immunocomplex was evaluated by real-time PCR and normalized to input RNA of corresponding samples. *Hatched columns:* untreated; *white columns:* degORNs; *black columns*: senseORNs. Equal amounts of HuR, KSRP or TTP were detected in ORNs treated and untreated samples, as shown in Western Blot lower panels. The data are representative of one out of three independent experiments.

Fig. 2. Inhibition of b-RNA degradation by ORNs *in vitro*. *A,* The mixture of the three ORNs or the degORNs, total dose10 μM, was added to the degradation mixture containing the total extract from the DOHH2 lymphoma cells, b-RNA (530 nt) and IGFR-RNA (350 nt). Incubation times with and without cell extract are indicated. *B,* time course of decay of b-RNA estimated by laser densitometry of the gel bands. Mean ± SD from three assays. *C,* dose response assay of ORNs, experimental conditions as in *A. D,* Histograms of C (incubation for

60 min at the indicated doses) obtained by densitometry (mean ± SD, n = 3). Statistical significance has been calculated versus degORN 10 μM treatment.

Fig. 3. Inhibition of b-RNA degradation by individual ORNs. *A*, As for Fig. 2, with individual ORNs or degORNs at 10 μM. *B*, time course of decay of b-RNA estimated by laser densitometry of the gel bands (mean ± SD, n = 3).

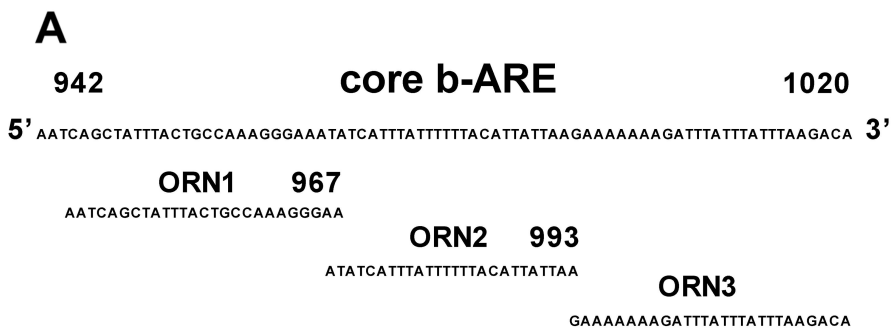
Fig. 4. ORN inhibition of Bcl2 RNA degradation in two cell lines. *A*, Bcl2 mRNA relative amount at different times in Raji cells exposed to ORNs. Cellular Bcl2 mRNA was determined by real-time RT-PCR in extracts from cells transfected with 1.5 μM ORNs at time 0, normalized with GAPDH cDNA (mean ± SD, n = 3). *B*, Rate of bcl2 mRNA degradation in Raji cells after treatment with the transcription inhibitor DRB, 20 μg/mL, *C*, Relative amount of bcl2 mRNA at different times in HL60 cells since the ORN treatment. *D*, Rate of Bcl2 mRNA degradation in HL-60 cells after treatment with the transcription inhibitor DRB, 20 μg/mL. *E*, Amount of c-myc mRNA in HL-60 cells at different times since the ORN treatment. *F*, Time course of MKK6 mRNA in Raji cells. *G*, Time course of p27 mRNA in Raji cells. A, C, E, F and G *hatched columns* untreated, *blank columns* degORN, *solid columns* ORNs. For all panels data are expressed as mean ± SD, n = 3.

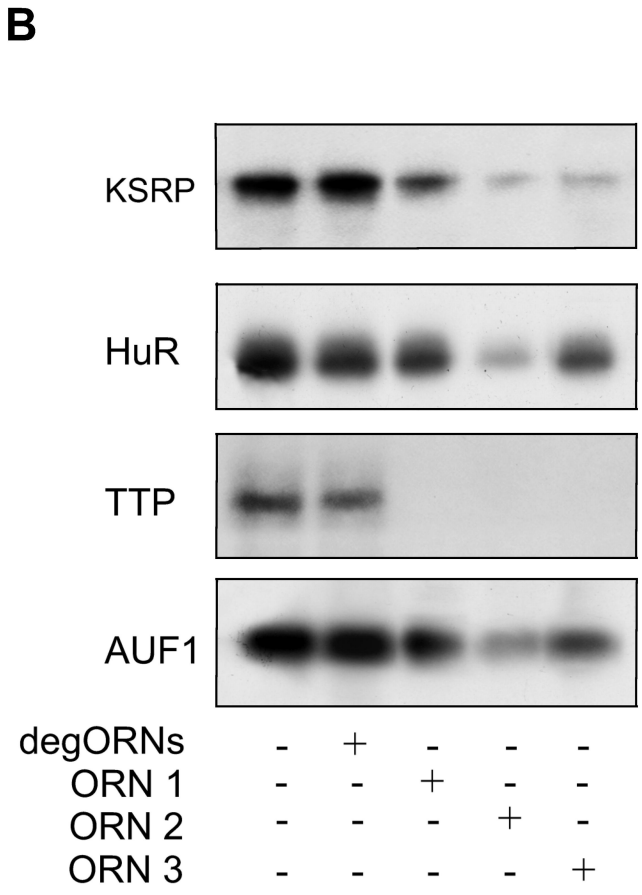
Fig. 5. Bcl2 protein content in HL60 cell treated with ORNs. *A*, ORNs or degORNs were transfected into HL60 cells, at the indicated dose, at day 0 and at half-dose at day 3 in the 5-day assays, under the same conditions as in Fig. 4. Cell samples were analyzed for Bcl2 protein levels by Western Blot assays on day 3 and on day 5. *B*, The histograms show the relative intensity versus untreated (open bars degORNs, solid bars ORNs) of the gel bands in *A*, estimated by laser densitometry (mean ± SD, n = 4).

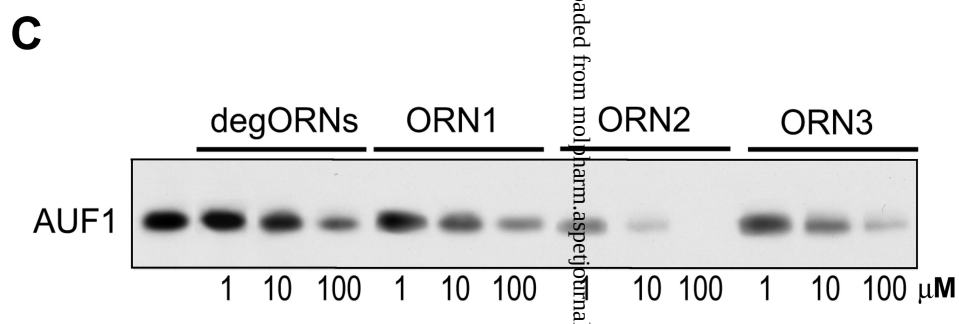
Fig. 6. Amount of p27 protein in Raji cell treated with ORNs. *A*, Western blot analisys of p27 protein amount in Raji cells; experimental conditions as for Fig. 5, assays on day 3. *B*, The

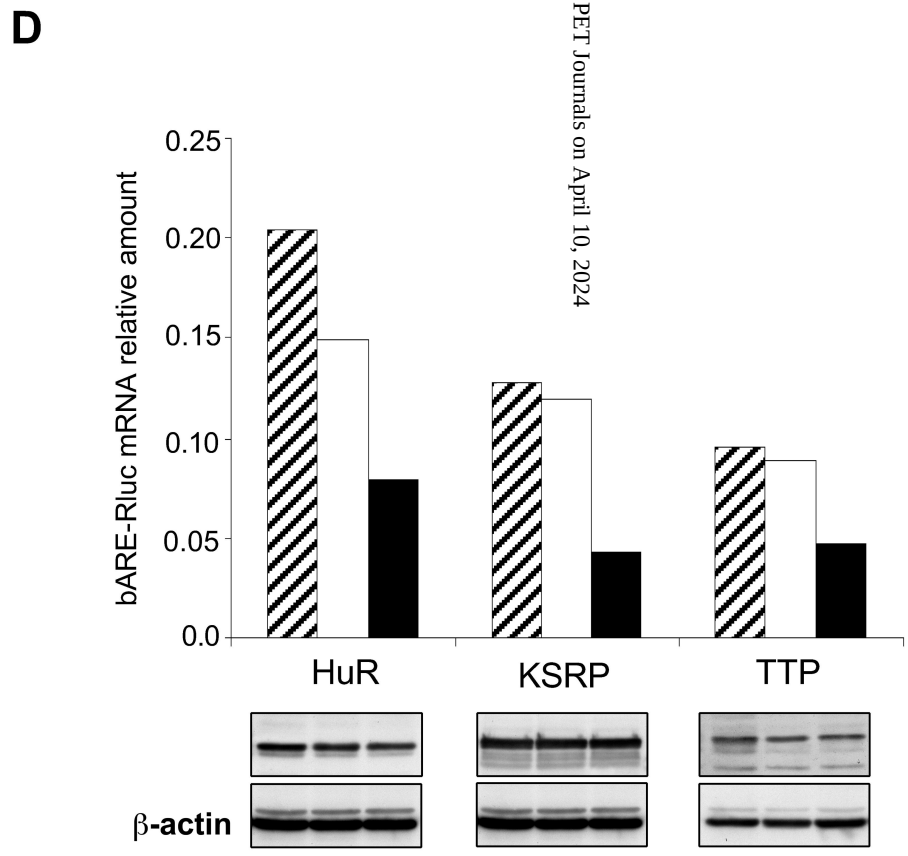
histograms show the relative intensity versus untreated (open bars degORNs, solid bars ORNs) of the gel bands in *A*, estimated by laser densitometry (mean ± SD, n = 3).

FIGURE 1



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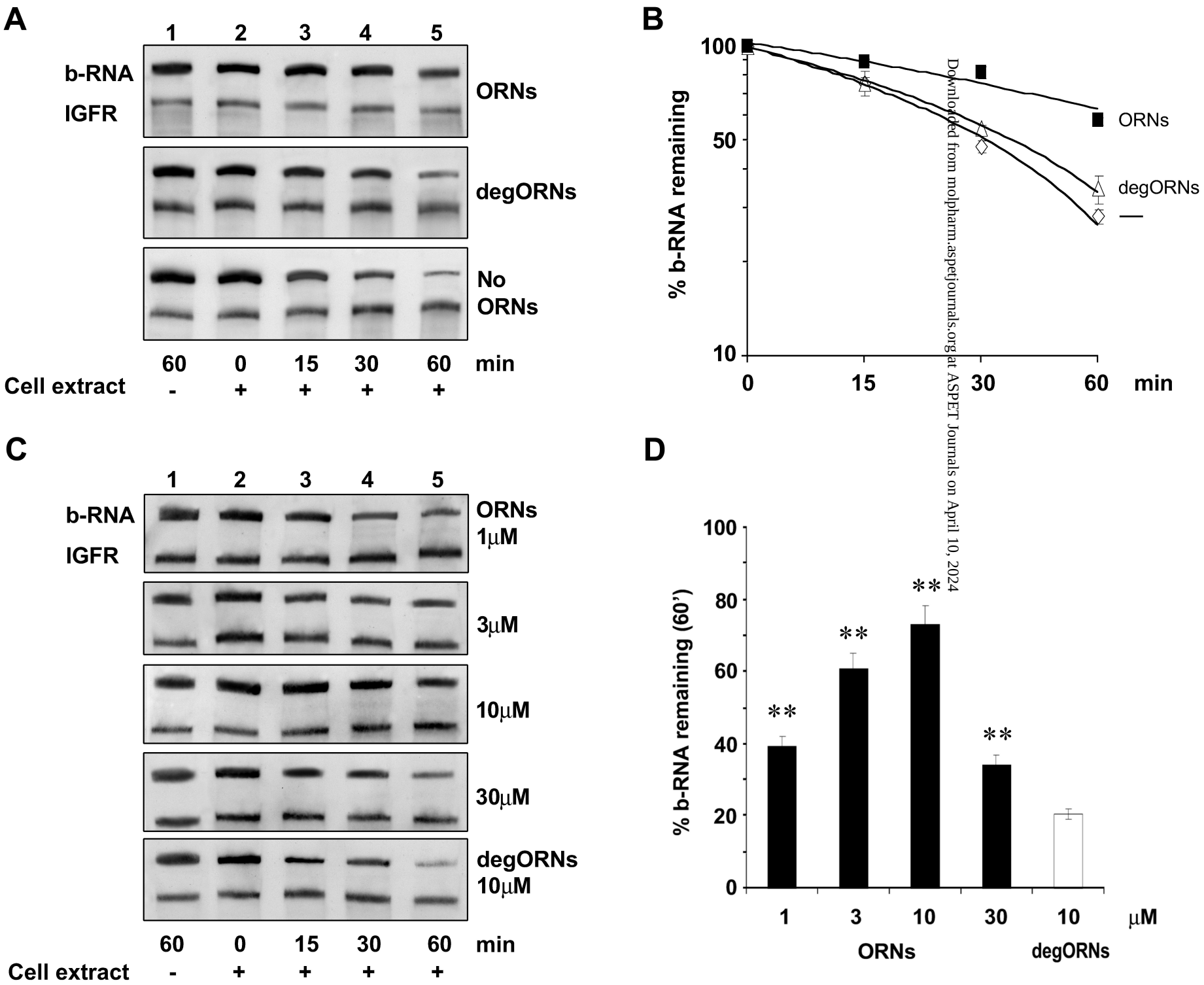

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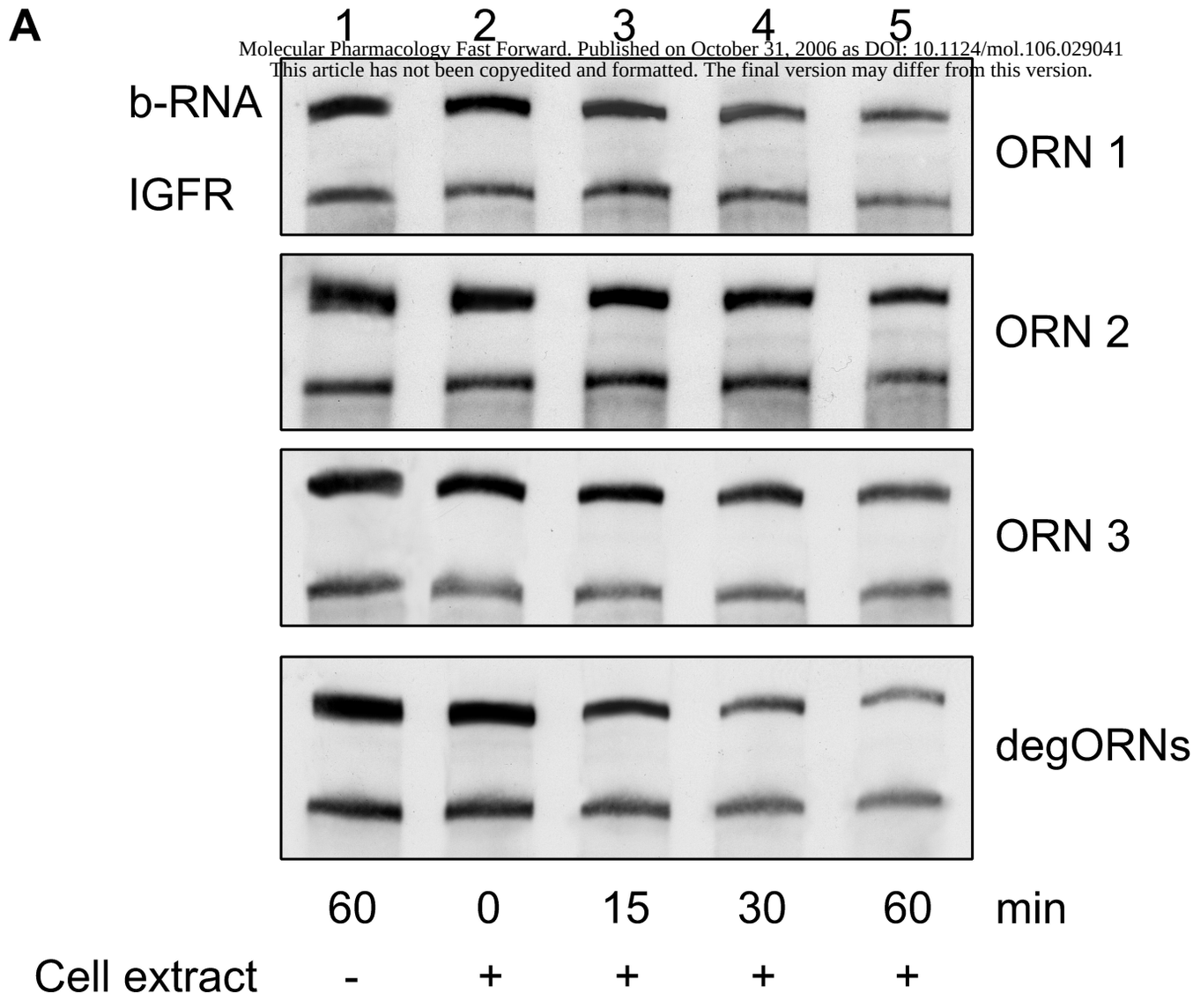


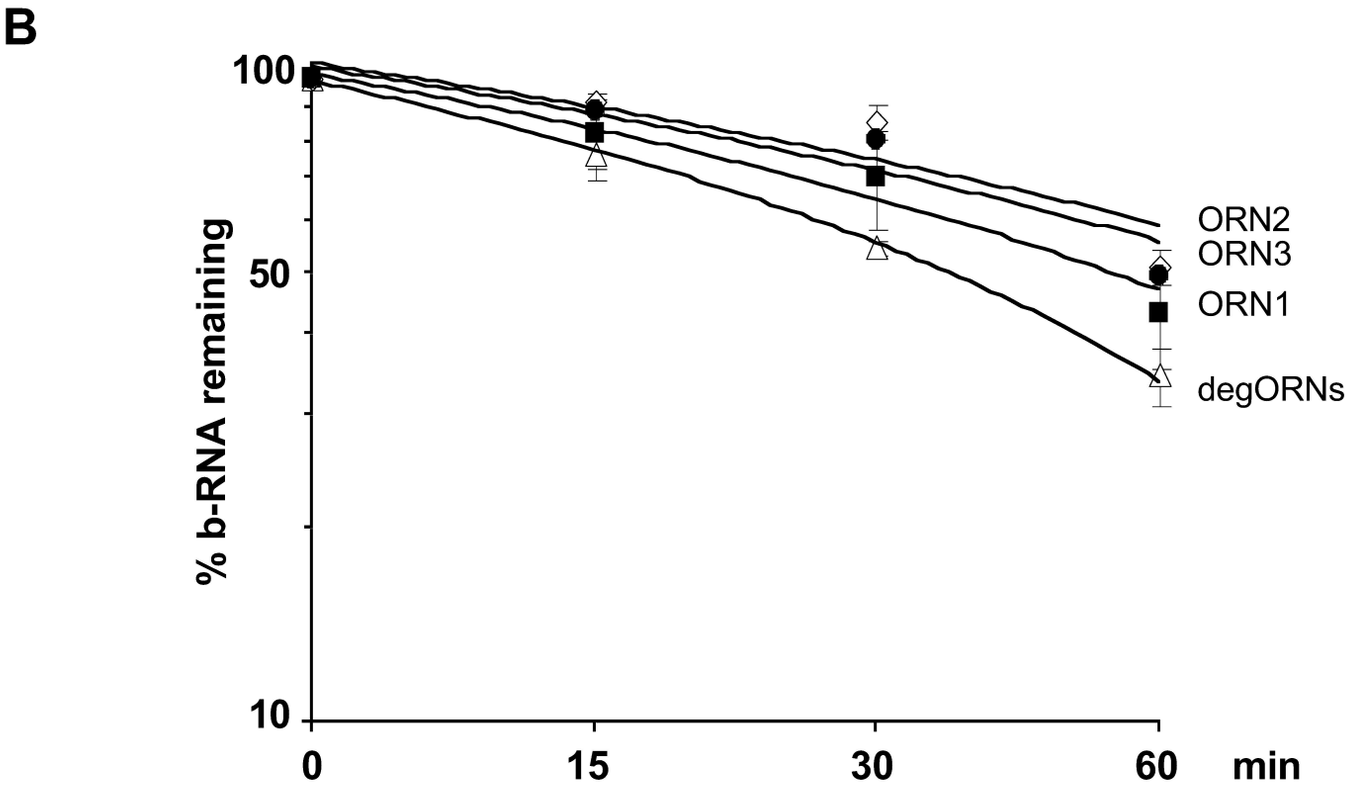

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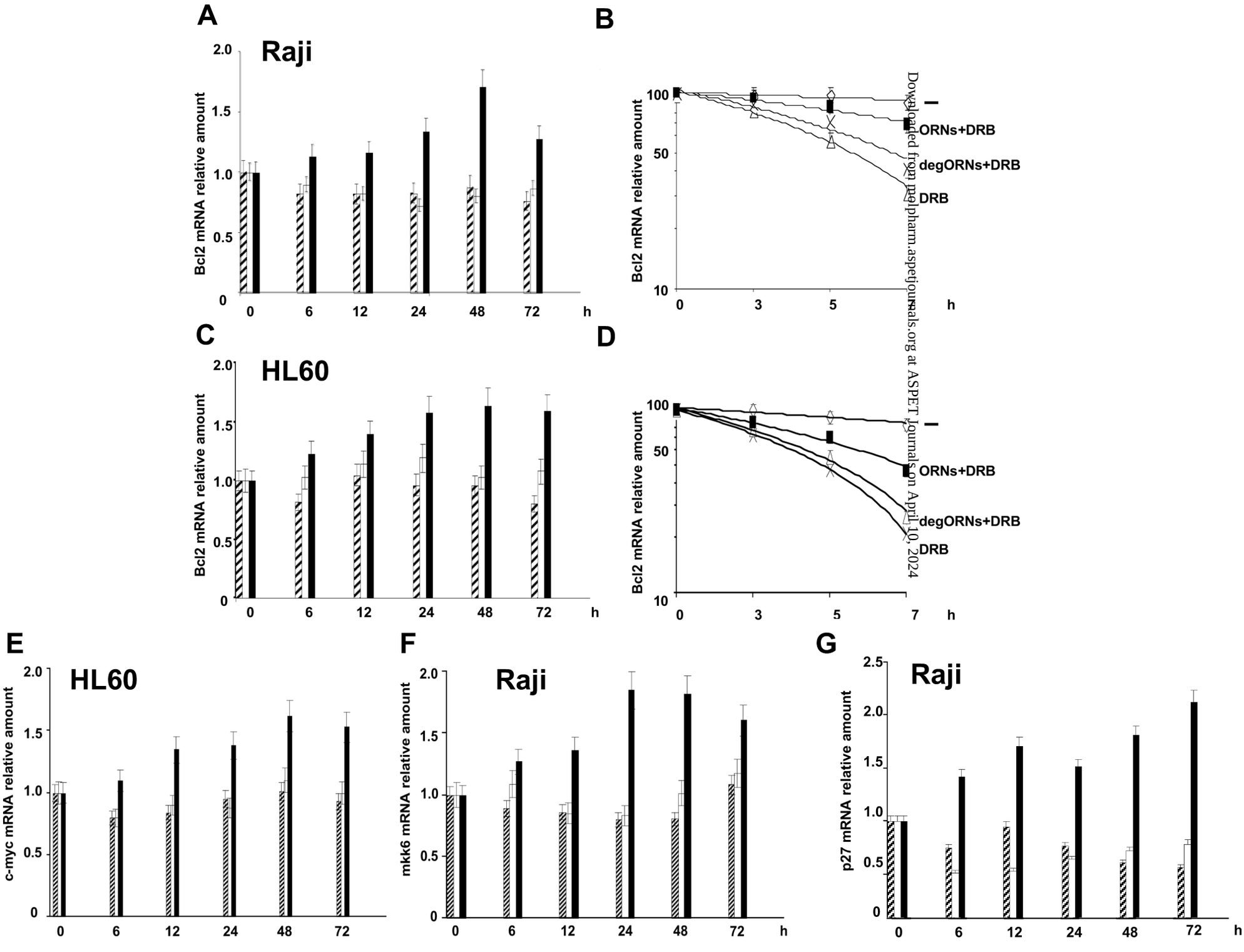

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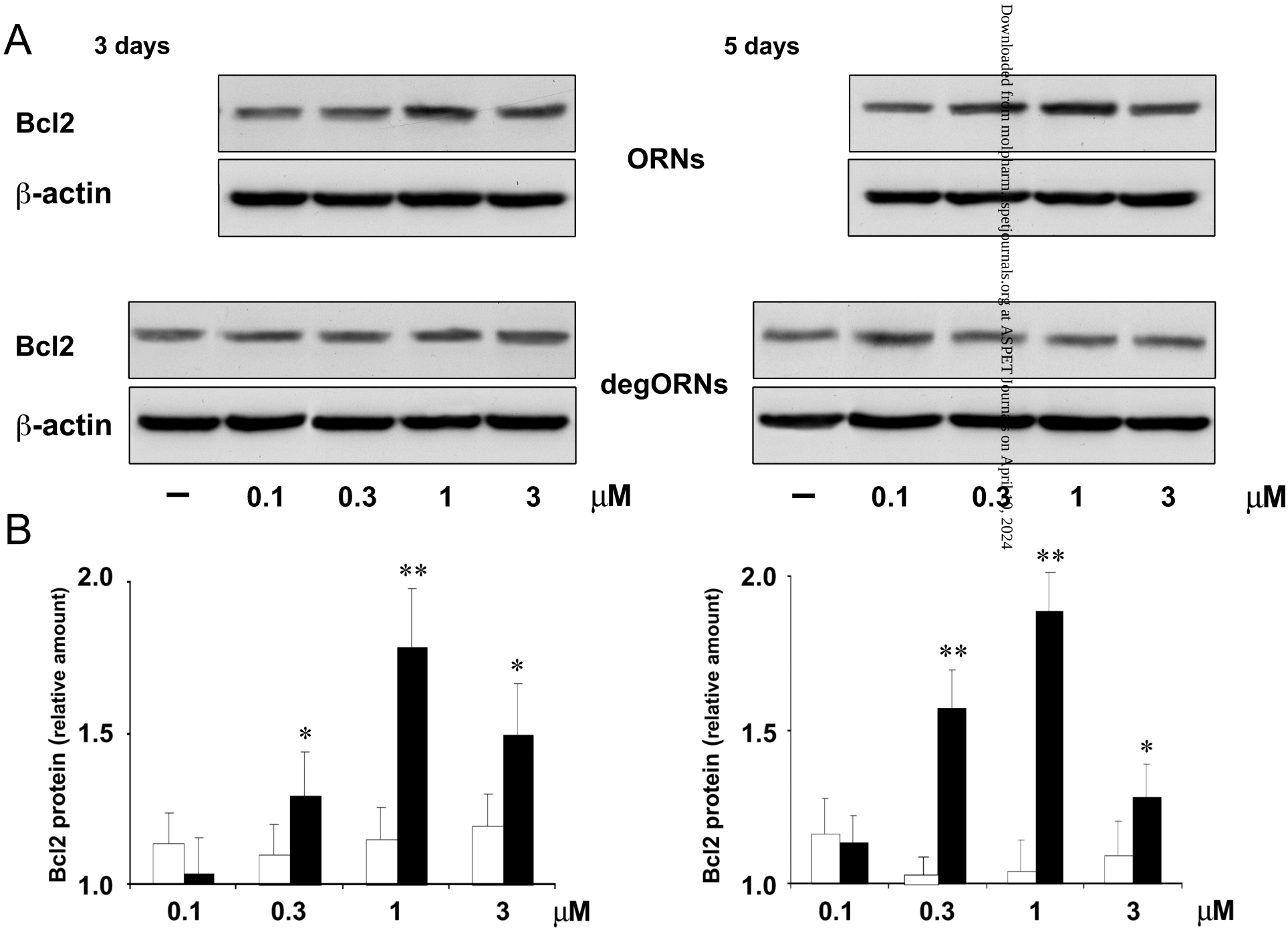

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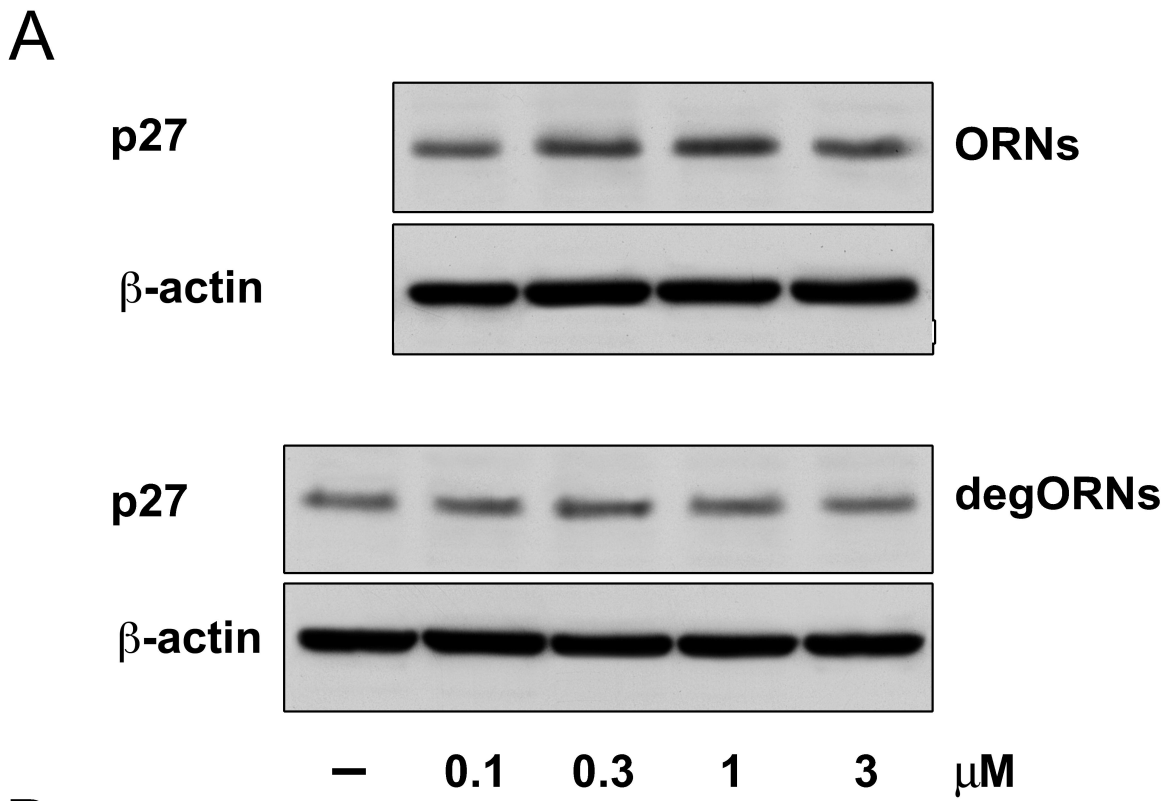
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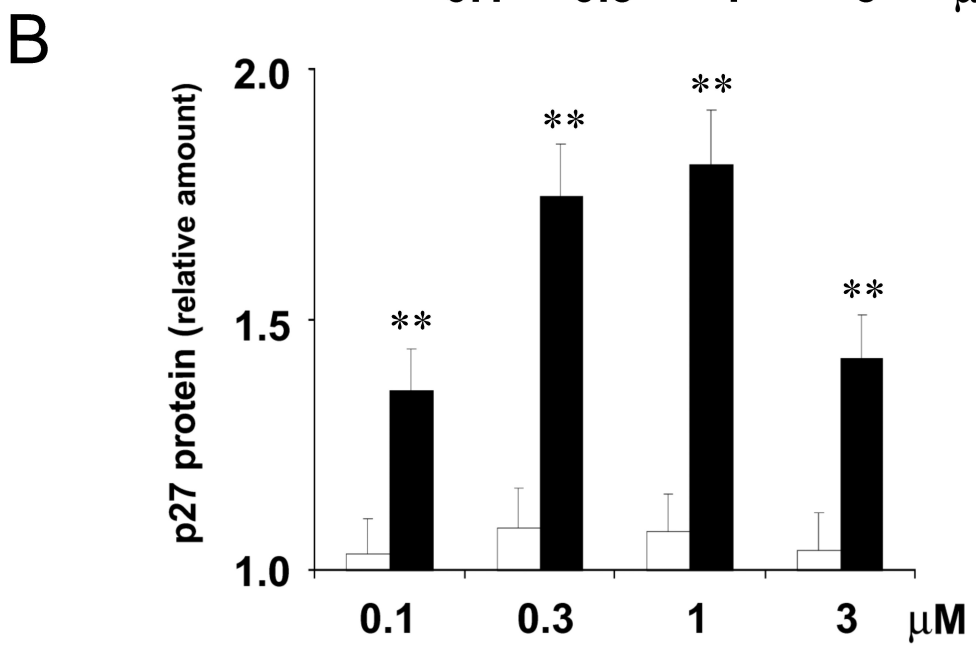
β-actin
p27
degORNs
β-actin
–
0.1
0.3
1
3
μM


B
p27 protein (relative amount)
2.0
1.5
1.0
**
**
**
**
0.1
0.3
1
3
μM