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# Functional characterization of ribosomal P1/P2 proteins in human cells

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Abbreviations used: Dox, doxycycline; RNAi, RNA interference; shRNA, short hairpin RNA

## SYNOPSIS

The stalk is a large ribosomal subunit domain that regulates translation. In this study, the role of the ribosomal stalk P proteins in modulating ribosomal activity has been investigated in human cells using RNAi. A strong down-regulation of P2 mRNA and a drastic reduction of P2 protein in a stable human cell line was achieved using a Dox-inducible system. Interestingly, the amount of P1 protein was similarly reduced in these cells, in contrast to the expression of P1 mRNA. The loss of P1/P2 proteins produced a reduction in the growth rate of these cells, as well as an altered polysome pattern with reduced translation efficiency but without affecting the free 40S/60S subunit ratio. A decrease in the ribosomal subunit joining capacity was also observed. These data indicate that P1/P2 proteins modulates cytoplasmic translation by influencing the interaction between subunits, thereby regulating the rate of cell proliferation.

## INTRODUCTION

Eukaryotic ribosomes contain four RNA molecules and approximately eighty proteins make up the small 40S and large 60S subunit. As key components of the translation machinery, ribosomes are central to many cellular processes [1]. Both in prokaryotes and eukaryotes, a lateral protuberance projects from the large subunit known as the ribosomal stalk [2]. This structure is an active ribosomal domain that directly interacts with the soluble translation factors, regulating their activity during the course of protein synthesis [3]. The eukaryotic ribosomal stalk is composed of ribosomal P proteins that usually exist as phosphoproteins in the cell. A 32 kDa protein P0 is located at the stalk base and it forms a complex with several highly conserved 12 kDa proteins with very acidic isoelectric points (pI 3-4). On the basis of primary sequence similarities, these proteins are classified as P1 and P2 proteins [4], and an additional P3 group is also found in plants [5]. The stalk structure is formed by two heterodimers (P1/P2)<sub>2</sub> [6, 7], with P1 anchoring the complex to the P0 core ribosomal protein [8-10], which is in turn attached to 28S RNA [11]. These acidic ribosomal P proteins are the only molecules in the ribosome that exist in multiple units, although they are also found in a free state in the cytoplasm. In yeast [12, 13], plants [14], and mammals [15], the free proteins have been shown to participate in an exchange process between the ribosome-bound P1/P2 and a cytoplasmic pool of these proteins. Indeed, this exchange seems to be connected with ribosome-modulating mechanisms in which the ribosomal stalk participates in yeast [16]. Therefore, the stalk can exist in multiple configurations [17-19] and altering the P1/P2 composition of the stalk, mainly through its acidic P protein content [20] and/or the phosphorylation of these components [21, 22], can influence the activity of the ribosome and provoke global changes in protein synthesis [19, 23].

Most information on the possible regulatory functions of the ribosomal stalk has been obtained in *Saccharomyces cerevisiae*. Intrinsic difficulties to perform genetic manipulation in higher eukaryotes has made it difficult to investigate whether the stalk fulfils similar roles in mammalian cells. However, gene silencing with RNA interference (RNAi) enables the function of specific proteins to be investigated in established cell lines. Thus, permanent transfected cell lines expressing plasmid-encoded short hairpin RNAs (hRNAs) can lead to partial silencing of genes.

Using direct transient transfection of carcinoma cell lines with antisense oligonucleotides, approximately 60% silencing of the P2 protein has been achieved [24]. However, this level of inhibition is probably not sufficient to perform a convincing analysis of the function of the silenced proteins. Here, the importance of the stalk acidic proteins in translation has been investigated by generating stable human cell lines in which the P2 protein could be conditionally suppressed by up to 95% with RNAi. Using this approach, we show that RNAi expression directly reduces P2 mRNA and protein levels, which also results in a parallel decrease in the ribosomal P1 protein. Moreover, conditional depletion of P2 mRNA and protein also reduces the growth rate of the cells. We present evidence that the phenotype produced by P1/P2 depletion is due to the effects on the capacity of the 60S ribosomal subunit to associate with the 40S subunit.

## METHODS

### Cell lines and culture conditions

The 293T (a human embryonic kidney cell line), TTR4 and SP23 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco Cell Culture Systems, Invitrogen) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), 100 IU/ml penicillin G, and 100 µg/ml streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% (v/v) CO<sub>2</sub>, and they were passaged regularly at sub-confluence.

### Plasmid construction

The target-specific siRNA duplexes were designed with the online siRNA Target Finder software (Ambion; [http://www.ambion.com/techlib/misc/siRNA\\_finder.html](http://www.ambion.com/techlib/misc/siRNA_finder.html)), and the sequence selected to generate siRNA corresponds to the 369-387 region of the P2 mRNA sequence [25]. The pSUPERIOR.puro (OligoEngine) vector for inducible expression of siRNA was digested with BglII and HindIII, and the oligonucleotides (GATCCCCGGAGGAGTCTGAAGAGTCATTCAAGAGATGACTCTTCAGACTCCTCC TTTTA and AGCTTAAAAGGAGGAGTCTGAAGAGTCATCTTGAATGACTCTTCAGACTCCT CCGG) were ligated into the vector to generate pSUPERIOR.puro-P2. The 19-nucleotide target sequence for P2 is indicated in boldface within the oligonucleotide sequences.

### Generation of a stable TTR4 cell line

Human 293T cells were transfected with the tetracycline (*TetR*) repressor-expressing vector pcDNA6/TR (Invitrogen), which contains a blasticidin selection marker, using the jetPEI™ (Qbiogene) transfection reagent according to the manufacturer's instructions. After 48 hours had passed, the cells were transferred to medium containing 10 µg/ml blasticidin hydrochloride (Sigma) for 2 weeks. Single clones were then isolated and expanded for an additional 2 months in media containing 10 µg/ml blasticidin, and analyzed by PCR.

### Generation of doxycycline-inducible P2 protein silencing cell line (SP23 clones)

TTR4 cells were transfected with pSUPERIOR.puro-P2 plasmid and selected with 1 µg/ml puromycin (Sigma) for 2 weeks. Single clones were isolated and expanded for an additional two months in the presence the antibiotic and analyzed by PCR. The cells (stable clones) were treated with different concentrations of doxycycline (Dox, Sigma) for the times indicated, and the P1 and P2 mRNA and protein they expressed was then analyzed.

### Western blot analysis

After treatment with and without Dox for the different times indicated, subconfluent cell monolayers were washed twice with ice-cold PBS (Phosphate Buffered Saline) before they were removed from the dish by trypsinization. The cells were resuspended in a buffer containing 15 mM Tris-HCl pH 7.4, 80 mM KCl, 5 mM MgCl<sub>2</sub>, 1% (v/v) Triton X-100 and protease inhibitors (2.5 µg/ml leupeptin, pepstatin, bestatin, aprotinin, chymostatin and antipain (Sigma), and 1 mM phenylmethylsulfonyl fluoride (PMSF)), and they were disrupted by 10 passages through a 20-gauge syringe needle. SDS-PAGE gels (12.5% (w/v)) were loaded with 10 µg of protein or 20 µl of the sucrose fractions and the separated proteins were then transferred to PVDF membranes (Immobilon-P, Millipore), which were probed with a monoclonal antibody specific to the highly

conserved carboxyl terminus of eukaryotic acidic P-proteins 3BH5 [26]. A horseradish peroxidase-conjugated rabbit anti-mouse antiserum was used as a secondary antibody. The amounts of P0, P1 and P2 protein were quantified from the Western-blot by scanning densitometry using NIH Image version 1.29 software. All results were calculated as the percentage of protein expression (P1 or P2 protein normalized to the P0 protein) in Dox-induced cells *versus* control cells.

### Real time quantitative RT-PCR analysis

Total RNA was isolated using a RNeasy mini kit (Qiagen) according to the manufacturer's instructions, and the RNA recovered was quantified by measuring the absorbance at 260/280 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). An equal amount of purified RNA was examined in triplicate from each sample in each experiment. Real-time quantitative reverse transcription polymerase chain reaction was performed using LightCycler RNA Master SYBR Green I (Roche) on the LightCycler 2.0 System (Roche). The primers used in this study were those retrieved from PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>; PrimerBank IDs: P0 (16933546a1), P1 (4506669a1) and P2 (4506671a1)) and they were purchased from Isogen. The reaction was carried out in a 20  $\mu$ l volume and it included an initial reverse transcription at 61°C for 20 min, followed by an incubation at 95°C for 30 s and 45 amplification cycles of: 1 s at 95°C followed by 5 s at 60°C and finally 7 s at 72°C. The products amplified from each reaction were analyzed using LightCycler Relative Quantification Software v.1.0 (Roche). PCR efficiencies (E) were calculated based on the slope of the relationship between the log input RNA versus the threshold cycle ( $C_t$  is defined as the point where the fluorescence exceeds the background threshold level and it is determined as the second derivative maximum):

$$E = 10^{-1/\text{slope}}$$

The relative expression between Dox-treated and control cells was quantified by the Pfaffl method [27] using P0 as a reference gene:

$$\text{Relative expression} = E_{\text{target(P1 or P2)}}^{\Delta C_t(\text{Control-Dox})} * E_{\text{reference(P0)}}^{\Delta C_t(\text{Dox-Control})}$$

The following equation was used to determine the relative quantification of a target gene in comparison to a reference gene (P0) in control cells:

$$\text{Relative expression} = E_{\text{target(P1 or P2)}}^{-C_t(\text{Control})} * E_{\text{reference(P0)}}^{C_t(\text{Control})}$$

### Cell proliferation assay

To analyze cell proliferation,  $5 \times 10^5$  cells were cultured for 3 days before exposing them to different concentrations of Dox. The number of cells was counted daily using a Neubauer cell counter and the doubling time (DT) was calculated from the regression analysis of the growth curves ( $N = N_0 e^{kt}$ , where t indicates the length of time and N denotes the cell number), where  $DT = \ln 2/k$ , and k is a proliferation rate coefficient that was estimated individually for each exponentially growth curve.

### Cell viability

The effect of silencing P2 by Dox-induction on cell viability was assessed using a trypan blue exclusion assay. Prior to trypsinization, medium was collected from all the cultures in order to include any cells that had detached during treatment. Cells were then combined with 0.4% trypan blue at a 1:1 ratio and counted to assess their viability.



### Flow cytometry

Cells were harvested after the treatments and time periods indicated, and they were analyzed by flow cytometry. The cells were fixed with methanol, treated with 50 µg/ml Ribonuclease A and stained with 50 µg/ml of propidium iodide (PD Pharmigen), and their DNA content was analyzed on a FACS Calibur flow cytometer (BD Biosciences). For each sample 20,000 cells were used and the data were analyzed with the CELLQuest software (Beckton-Dickinson). The percentage of cells in each phase of the cell cycle was determined as a ratio of the fluorescent area of the appropriate peaks to the total fluorescent area.

### Analysis of polysomes

Ribosome profiles were prepared from a total of  $2 \times 10^7$  cells at 80% confluence, which were washed with ice-cold PBS containing 100 µg/ml cycloheximide (Sigma) to block ribosomes in the elongation step. Cells were lysed with buffer A (15 mM Tris-HCl pH 7.4, 80 mM KCl, 5 mM MgCl<sub>2</sub> and 100 µg/ml cycloheximide), containing 1% (v/v) Triton X-100, 40 U/ml RNasin and protease inhibitors (see above). Cytoplasmic extracts were obtained after centrifugation at 15,000 x g for 30 min at 4°C, they were loaded onto a linear 10 to 50% (w/v) sucrose gradient in buffer A and then centrifuged at 270,000 x g for 2 h 15 min at 4°C. Gradients were fractionated by upward displacement with 87% (v/v) glycerol in an ISCO density gradient fractionator, and the absorbance at 260 nm was monitored continuously using an ISCO UA-5 UV monitor. The area under the profile in each peak (subunits, monosomes and polysomes) was calculated and the background signal was subtracted. The overall translation efficiency was calculated as the area under the curve representing two or more ribosomes (polysomal RNA) divided by the total area under the curve [28]. The average number of ribosomes per mRNA in the polysomes (with two or more ribosomes attached) was estimated from the polysome profiles, summing the product of the area of each peak multiplied by the number of ribosomes at that peak, and dividing by the total area of polysomes. The relative ribosome content was calculated as the area under the curve representing ribosomes (one or more ribosomes) divided by the area under the subunit peaks (40S and 60S).

In ribosome dissociation experiments, cycloheximide was omitted from all the buffers and the KCl concentration in the cytoplasmic extracts obtained after centrifugation was increased to 0.5 M (high salt). Ribosomal subunits were resolved by centrifugation at 270,000 x g for 3 h 30 min at 4°C on a 10 to 30% (w/v) sucrose gradient containing 15 mM Tris-HCl pH 7.4, 500 mM KCl and 5 mM MgCl<sub>2</sub>.

In the steady-state subunit joining analysis, the cells were lysed with buffer B (15 mM Tris-HCl pH 7.4, 80 mM KCl and 0, 1, 2.5 or 5 mM MgCl<sub>2</sub>), containing 1% (v/v) Triton X-100, 40 U/ml RNasin and protease inhibitors. The lysates were loaded onto a linear 10 to 30% (w/v) sucrose gradient in buffer B, centrifuged at 270,000 x g for 3 h 30 min at 4°C and fractionated. To quantify the association between ribosomal subunits from either control and P1/P2-depleted ribosomes, the peak areas from the sucrose gradient profiles were calculated:

$$\% \text{ subunit association} = 100 \times \text{area 80S peak} / (\text{area (40S+60S+80S) peaks})$$

### Statistical analysis

The results were analyzed using the Student's t test and p values of less than 0.05 were considered statistically significant.

## RESULTS

### Selection of stable doxycycline (Dox)-inducible P protein silencing cell lines

TTR4 cells transfected with pSUPERIOR.puro vector (OligoEngine) were selected with puromycin. Stably transfected SP14 (Supplementary online material) and SP23 cells containing the P1 and P2 constructs were assayed by exposing them to doxycycline (Dox, 10 µg/ml) for 2 days. Among the SP14 transfectants only one clone showed an ~60% reduction in P1 protein and a parallel reduction in P2 protein of ~40% (Figure 1S, Supplementary online material). In contrast, three SP23 transfectants showed a reduction of P2 protein levels after Dox exposure above 90% (clones SP23-5, SP23-8 and SP23-10, Figure 1A) and notably, the amount of P1 proteins was also reduced when P2 was silenced in these clones. The effect of different Dox concentrations on P2 accumulation was tested by western blotting of SP23-5 cells (Figure 1B) and accordingly, Dox concentrations as low as 0.05 µg/ml significantly reduced P2 accumulation in these cells. Thus, the SP23-5 clone was used in the following experiments as a Dox-inducible P2 silencing cell line to induce P1/P2 proteins depletion.

### In human cells, P1 Protein is unstable in the absence of P2

To quantify stalk protein depletion, SP23-5 cells were treated with 0 (control), 0.1 or 10 µg/ml Dox for four days and the accumulation of P0, P1 and P2 proteins was assessed. Since the amount of P0 protein remained unchanged, this protein was subsequently used to normalize the values of P1 and P2 protein. A mean decrease in P2 protein of 90% and 95% was observed in cells induced with either 0.1 or 10 µg/ml Dox, respectively (Figure 1C). Significantly, in these same cells, the amount of P1 protein also fell by 70% or 85%, respectively (Figure 1C; see also Figure 1A and 1B). Thus, both acidic stalk proteins were depleted in these cells following Dox-induction.

To confirm that P2 expression was indeed silenced, the individual mRNAs present were measured by real time RT-PCR (Figure 1D). Within 4 days of Dox induction, there was a dramatic decrease in P2 mRNA transcripts (Figure 1D), with a depletion of P2 mRNA above 70% in the presence of 0.1 µg/ml Dox and of around 90% with 10 µg/ml Dox. Therefore, the loss of the P2 protein was correlated with a decrease in the P2 mRNA available following exposure to Dox. By contrast, the amount of P1 mRNA expressed, which is notably higher than that of P2 mRNA in the controls (P1mRNA/P0mRNA  $10.4 \pm 5.8$ ; P2mRNA/P0mRNA  $0.7 \pm 0.3$ ), is even higher after Dox treatment, suggesting that cells try to compensate for P1 protein depletion by increasing *RPLP1* gene transcription.

### Depletion of P1/P2 proteins reduces the proliferative capacity of the induced cells

The number of cells was monitored in order to analyze the effects of suppression of P1/P2 on cell proliferation (Figure 2). When the SP23-5 cells were cultured with Dox at different concentrations (from 0.1 to 10 µg/ml), their capacity to proliferate was significantly diminished when compared with the control, uninduced-cells. Moreover, upon the removal of Dox the proliferation of the SP23-5 cells recovered to control levels (Figure 2A). In order to quantify these changes, the doubling times (DT) of the cells was calculated from the growth curves (Figure 2B). Thus, the DT of the SP23-5 cells increased from  $21.9 \pm 0.2$  h to  $44.7 \pm 2.9$  h following P1/P2 depletion on exposure to 0.1 µg/ml Dox, the concentration used in the following experiments. This low concentration of Dox (0.1 µg/ml) had no effect on the proliferation of TTR4 cells (data not shown). When Dox was removed from the media, the DT returned to



29.4±1.6 h after 6 days or to 22.4±1.9 h after 12 days, indicating that the effect of P1/P2 depletion on the rate of cell growth was reversible. To test whether the suppression of the acidic stalk proteins induced by Dox-could also be reverted, a time course study of P2 silencing was performed. The kinetics of P2 silencing was examined in cells by western blotting, following a similar time course to the cell growth data (Figure 2C). Therefore, P2 depletion and its effect on cell growth is inducible and reversible.

### **Depletion of P1/P2 proteins does not affect cell viability or the proportion of cells in the different phases of the cell cycle**

The reduced growth of SP23-5 cells following P1/P2 depletion prompted us to evaluate the viability of the cells following Dox induction. At all concentrations and time points evaluated, the viability of P1/P2-depleted cells remained above 90% and this did not differ significantly from that of the control cells (91 ± 4%, Figure 3A). Thus, there was no indication that P1 and P2 proteins depletion induced cell death.

The effect of P1/P2 depletion on the cell cycle was also evaluated after a 4 day induction of SP23-5 cells with 0.1 µg/ml Dox. There was clearly no difference between the number of cells in the different phases of the cell cycle in control and P1/P2-depleted cells (Figure 3B). Therefore, acidic stalk proteins depletion did not affect the distribution of the cells in the distinct phases of the cell cycle. Furthermore, the apoptotic cells identified (subG0 population) were ~6% in both groups, confirming that there was no increase in cell death following P1 and P2 proteins depletion.

### **Effect of P1/P2 depletion on translation efficiency**

The polysome profiles were examined to determine if the decreased growth rate of P1/P2-depleted cells was due to a defect in some aspect of ribosome synthesis, assembly or function. In these assays, the number of ribosomes found within the polysomal mRNA fraction (mRNA containing two or more ribosomes) is a reflection of *de novo* protein synthesis [28]. The control ribosomal profile shows the translating polysomes (the 80S monosome peak harbors translating single 80S ribosomes and inactive couples), and free 60S and 40S subunits, respectively (Figure 4A, left panel). In comparison to the control cells, the P1/P2-depleted cells show a reduction in the total amount of monosomes and polysomes but not in the number and the relative proportion of the polysome peaks (Figure 4A, right panel). Furthermore, the reduction in the 80S peak was accompanied by the appearance of halfmers [29, 30] and an increase in the amount of free 60S subunits. These data, especially the obvious decrease in the amount of polysomes, suggest that the decreased growth rate of P1/P2-depleted cells was compatible with the reduced rate of translation initiation [31].

The proteins extracted from gradient fractions were resolved by SDS-PAGE and analyzed in western blots (Figure 4B). The P0 protein clearly co-sedimented with free 60S, 80S and polysomes, and it was absent from cytosolic and free 40S fractions from control and Dox-treated cells. By contrast, free P1 and P2 proteins were detected in the cytosolic fractions as well as in the particles where P0 was detected. P1 and P2 proteins were depleted in all ribosome containing fractions (4-10) in Dox-induced cells when compared to control cells, indicating the presence of active ribosomes lacking P1 and/or P2 proteins in the polyribosomes of P1/P2-depleted cells. It is interesting to note that the amount of P1 protein found at the top of the gradient, which corresponds to the free cytoplasmic pool, is much less affected in the Dox-treated cells.

The overall translation efficiency is defined as the proportion of rRNA participating in polysomes [28]. Thus, to determine whether depletion of P1/P2 proteins altered translational efficiency, the amount of rRNA actively involved in translation (associated with polysomes) was measured. In control cells,  $54 \pm 3\%$  of RNA was in polysomes and this dropped to  $43 \pm 4\%$  following acidic proteins depletion (Figure 4C). Thus, there was a reduction in global translation of ~20%.

An analysis of the polysome profiles showed that the depletion of P1/P2 proteins did not change the distribution of the polysomal mRNA (Figure 4A). Thus, the average number of ribosomes per translated transcript (mRNAs containing two or more ribosomes) was not altered ( $5.0 \pm 0.1$  in both cases, Figure 4D). In contrast, the relative ribosomal content, estimated from the polysome profiles as the ratio of ribosomes (monosomes and polysomes) and subunits (40S and 60S), fell from  $5.2 \pm 0.3$  to  $3.4 \pm 0.4$  (Figure 4E), indicating a defect in the equilibrium between ribosomes and subunits.

### **The depletion of P1/P2 proteins makes ribosomal subunit joining less efficient**

The total amount of 40S and 60S ribosomal subunits was analyzed in control and P1/P2-depleted cells after treatment with 0.1  $\mu\text{g/ml}$  Dox for 4 days. The cell extracts were treated with high- $\text{K}^+$  (500 mM KCl) in the absence of cycloheximide in order to fully dissociate the 80S ribosomes and in this way, the total amount of the 40S and 60S ribosomal subunits could be estimated [32]. P1/P2 proteins depletion did not affect the steady-state levels of 40S and 60S ribosomal subunits (Figure 5A).

The decrease in the relative ribosome content (Figure 4E), the production of halfmers in P1/P2-depleted cells and the accumulation of free 60S subunits (Figure 4A) while the overall subunit stoichiometry remains unchanged (Figure 5A), strongly suggested that the low levels of P1 and P2 proteins made subunit joining less efficient. To confirm this suggestion, the steady-state association capacity of 40S and 60S ribosomal subunits in control and P1/P2-depleted cell extracts was tested by sucrose gradient centrifugation at different concentrations of  $\text{Mg}^{2+}$  (Figure 5B). As expected, 80S ribosomes totally dissociated in the absence of  $\text{Mg}^{2+}$  in both cell types tested. The 40S to 60S ratio showed average values of  $2.8 \pm 0.3$  and  $2.6 \pm 0.3$  for control and Dox-treated cells, respectively, confirming that the steady-state ratio of 40S and 60S subunits was not affected by P1/P2 depletion. Increasing the  $\text{Mg}^{2+}$  concentration in the gradients from 1 to 5 mM highlighted the different degree of subunit association, which was always lower in the Dox-treated sample than in the corresponding controls (Figure 5B). This effect was most evident when the proportion of associated subunits was plotted as a function of the magnesium concentration (Figure 5C). As expected, the degree of association increases in function of the  $\text{Mg}^{2+}$  concentration for both control and P1/P2-depleted cells. However, the association capacity of the ribosomes from P1/P2-depleted cells was notably lower (~30%) than that of the control cells. Hence, it seems clear that the lack of P1 and P2 proteins clearly reduced the capacity of the 60S subunit to form 80S ribosomes in the cell extracts.

## DISCUSSION

Through the Dox-induced expression of short-hairpin RNAs (shRNAs) targeted to mRNA encoding stalk ribosomal P2 protein, both P2 transcripts and protein have been reduced in stably transfected human cells to levels below 10% of those in control cells. This depletion of P2 was associated with a similar decrease of its partner ribosomal stalk component, the P1 protein, without a parallel reduction in P1 mRNA, which rather increased significantly. These results indicate that P1 was unstable in the absence of P2 in human cells, consistent with data obtained in *Saccharomyces cerevisiae*. In yeast, free P1 protein is highly unstable, with a half-life of a few minutes, while P2 has a half-life of several hours. The yeast P1 protein is degraded by a proteasome-independent mechanism and its association with P2 protein protects it from degradation [33]. However, while P1 protein is not detected in yeast strains that lack the P2 protein [34], in human cells a significant amount of P1 is found in the supernatant when P2 is silenced in cells, considerably more than the residual amount of P2 detected (Figure 4B). This difference could be attributed to a higher intrinsic resistance to degradation of the human protein, possibly due to the lack of a serine at the second position in the amino end of the human P1, an important residue in the yeast P1 degradation signal ([33], Nusspaumer, Briceño, Remacha and Ballesta, unpublished results). Alternatively, the large excess of P1 mRNA detected in the SP23 cell line (P1mRNA >> P0mRNA ~ P2mRNA), which is not seen in yeast [33], could result in an excessive accumulation of protein that might saturate the degradation system. The fact that P1 protein can be detected in a yeast strain lacking P2 protein when overexpressed but not when it is expressed only from the endogenous gene [17] supports this possibility. However, further experiments will be required to determine how this phenomenon can be explained. Indeed, in contrast to yeast, where P2 proteins were not affected by P1 deficiency [33], the amount of P2 protein in human cells was reduced when P1 was depleted (Figure 1S), indicating that P2 was less stable in absence of P1.

As in yeast [10], human ribosomal P proteins preferentially form P1-P2 heterodimers [35] that attach to P0 through the P1 protein [9], forming the pentameric P0-(P1-P2)<sub>2</sub> structure on the ribosome [20, 36]. The interaction between P1 and P2 to form the P1-P2 heterodimer seems to be a crucial step for efficient assembly of the functional GTPase-associated centre, and P1 or P2 alone fails to bind efficiently to P0 [37]. The results presented here are consistent with the assembly model whereby the soluble heterodimers P1-P2 bind to P0 on the ribosome. For this reason, P1 that is more resistant to degradation accumulates in the cytosol when the levels of P2 are too low to form heterodimers, even though the ribosomes are defective in P1/P2 proteins.

As found previously in *S. cerevisiae* [19], we can conclude that the ribosomal stalk P1 and P2 proteins are not a prerequisite for ribosome activity and protein synthesis in human cells. Indeed, particles lacking both proteins are incorporated into polysomes (Figure 4A and 4B, right panels). However, the absence of these ribosomal components does have consequences for the cell. Recent studies have shown that ribosome synthesis is intimately linked with cell growth and the regulation of the cell cycle [38]. Although the details of this association are not yet understood, it is clear that the quality and quantity of ribosomes directly determine the growth rate of cells and by extension, the timing of cell division. Indeed, a reduction in the cell growth rate was the most obvious consequence of the absence of acidic P proteins in cells, with doubling times increasing from 22 to 45 hours. This inhibition was not correlated with a loss of cell viability and there was no evidence of cell death when the subG0 phase in the cell cycle was evaluated, indicating that the decrease in cell growth was not due to cytotoxicity. Moreover, there was no arrest of P1/P2-depleted cells at any specific phase of the cell cycle but rather, they

seemed to progress through it at a slower rate. It is therefore conceivable that the overall reduction in mRNA translation observed in cells lacking P1/P2 proteins affects genes involved in the cell cycle. This would explain the reduced rate of cell cycle progression. However, these effects are reversible and upon re-establishing P2 expression the growth rate returns to normal.

After P1/P2 proteins depletion, the polysome profiles show an increase in the 60S peak as well as a reduction in 80S and polysome peaks, without any apparent effect on average number of ribosome per mRNA transcript. In addition, halfmers appear in polyribosomes due to a late-stage translational initiation defect in which 43S preinitiation complexes are attached to an mRNA that already contains at least one translating 80S ribosome but lacks the corresponding 60S subunit [29]. These structures were first described *in vitro* in the rabbit reticulocyte translation system after sodium fluoride treatment [39], and *in vivo* in Ehrlich ascites cells treated with anisomycin [40]. Halfmers may reflect a stoichiometric ribosomal subunit imbalance caused by a reduced supply of 60S subunits, resulting in an excess of 40S over 60S subunits [41-43]. Alternatively, halfmers may be generated without altering the subunit balance from impaired translation initiation, whereby inhibitors of translation initiation [29] or defective ribosomal components [30, 31, 44, 45] prevent subunit joining. Thus, halfmers are indicative of a defect in ribosomal subunit joining [46], and coupled to the decrease in the amount of polysomes, their presence is compatible with defects at the level of translation initiation caused by depletion in P1/P2 proteins. However, the reduction in the average number of ribosome per translated transcript when initiation is blocked [28, 47, 48] was not detected in our system and thus, it is possible that acidic proteins depletion also affects other translation steps. This is not unexpected, since bacterial stalk acidic proteins influence the activity of initiation, elongation and termination factors [3, 49].

The ratio between free 60S and 40S ribosomal subunits in polyribosome profiles revealed an increase in the amount of free 60S ribosomal subunits relative to free 40S subunits in P1/P2-depleted cells. However, when the 40S to 60S ratio was more closely examined after ribosome dissociation on sucrose gradients with either high- $K^+$  or in the absence of  $Mg^{2+}$ , no changes were detected. These results indicate that the biogenesis of the subunits was not affected by P1/P2 proteins depletion and thus, a subunit imbalance is unlikely to be the cause of halfmer formation. Furthermore, the polysome profile of P1/P2-depleted cells showed a significant reduction in the relative ribosome content (accumulation of subunits). Consequently, the absence of P1/P2 proteins could produce defective steady-state subunit joining, and indeed, a decrease in ribosomal subunit association in P1/P2-depleted cells was directly confirmed by sedimentation analysis at different  $Mg^{2+}$  concentrations. Thus, 60S subunits depleted of acidic proteins were ~30% less efficient in joining to 40S subunits and forming 80S ribosomes at all  $Mg^{2+}$  concentrations tested. This illustrates the importance of the contribution of P1/P2 proteins to the subunit joining activity. In addition, a global decrease in translation efficiency that would be expected to contribute to a depletion of many proteins could account for the reduced growth rate of the P1/P2-depleted cells. This presumably results from a reduction in the rate at which ribosomal subunits can be loaded onto mRNA, as a consequence of the impaired association of ribosomes depleted of P1/P2 proteins. Thus, the P1/P2 content of the stalk could modulate the joining of the 60S ribosomal subunit. Although it is widely accepted that translation initiation is the rate-limiting step for translation and our results show that depletion of P1/P2 proteins significantly affect the ribosomal subunit-joining, we can not rule out an additional effect of the P1/P2 depletion on the elongation step of translation.

Data obtained from *S. cerevisiae* strongly suggest that the ribosomal stalk could function as a translational regulator of specific mRNAs [19]. The results presented here indicate that this

regulation could be achieved by modulating ribosomal subunit association. In agreement with this idea, several studies have indicated that translation can be regulated at the subunit joining stage [50-52].

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## FIGURE LEGENDS

### Figure 1 RNAi-mediated silencing of P2 expression in SP23-5 cells

(A) Stable clones carrying inducible P2 shRNA before (-) or after (+) induction with Dox for 2 days. (B) SP23-5 cells were exposed to the Dox concentrations indicated for 3 days. The position of the three stalk P proteins, as well as that of the molecular mass markers (kDa), is indicated. (C) SP23-5 cells were incubated with 0 (control), 0.1 or 10  $\mu\text{g/ml}$  Dox for 4 days. Proteins P0, P1 and P2 were detected by immunoblotting and the bands were quantified by densitometry. (D) The amount of P0, P1 and P2 mRNAs in the extracts was estimated by real time RT-PCR. The amount of P1 and P2 proteins (C) or mRNAs (D) were normalized to the respective levels of P0, which was not affected by Dox, and they were then expressed as a percentage of the values in control cells. The relative expression of mRNA in control cells were: P1/P0  $10.4 \pm 5.8$ ; P2/P0  $0.7 \pm 0.3$ . The bar graph shows the means of four independent experiments, and the standard deviation is indicated as error bars ( $\pm$  SD). Statistical comparison was made using the Student's t test for paired data, \*  $P < 0.05$ .

### Figure 2 Dox-induced P2 silencing reversibly reduces the growth of SP23-5 cells

(A) The cells were grown in the absence ( $\bullet$ , control) or the presence of 0.1  $\mu\text{g/ml}$  ( $\blacksquare$ ), 1  $\mu\text{g/ml}$  ( $\blacktriangle$ ), or 10  $\mu\text{g/ml}$  Dox ( $\blacklozenge$ ) for 5 days, and the media was then replaced with normal media without Dox at various time points. Cells were counted in samples taken at the times indicated. (B) The doubling times (DT) were calculated from the induced cells ( $\bullet$ ) and from cells after removing Dox for 6 days ( $\blacksquare$ ). The results shown are the average of three determinations with error bars showing the standard errors of the means. (C) Time-course of stalk P proteins expression. The protein extracted from the cells used to derive the growth curves in Figure 2A at 0.1  $\mu\text{g/ml}$  (upper panel) or 10  $\mu\text{g/ml}$  Dox (lower panel) were analysed by western blotting. The total days indicates the time from the start of the experiment as shown in Figure 2A. Treatment days correspond to the time from the beginning of each specific treatment indicated by an arrow in Figure 2A.

### Figure 3 P2-silencing by Dox does not affect SP23-5 cell viability or their distribution throughout the cell cycle

(A) The viability of SP23-5 cells cultured in medium with different concentrations of Dox was evaluated. Cells were collected after each time point, stained with trypan blue and counted immediately. Samples were evaluated in triplicate with the standard errors shown. (B) The effect of P2 silencing on the distribution of cells throughout the cell cycle was evaluated. The cells were stained with propidium iodide and the DNA content was determined by flow cytometry to assess the proportion of cells in each phase of the cell cycle. Histograms and bars represent the mean and standard deviations of triplicate measurements.

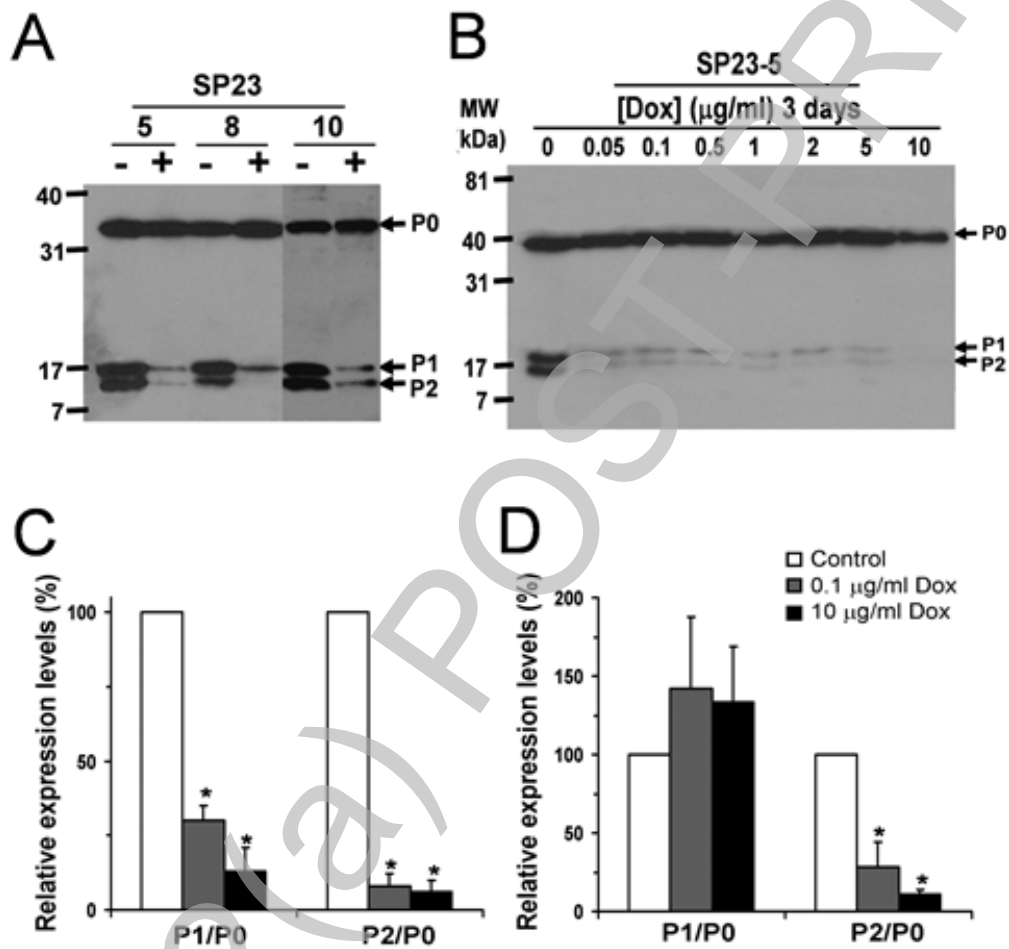
### Figure 4 Analysis of polysome profiles in P2-silenced cells

(A) Total extracts from SP23-5 cells before (control) and after exposure to 0.1  $\mu\text{g/ml}$  Dox for 4 days were resolved on a 10-50% sucrose gradient. The position of free small (40S) and large (60S) ribosomal subunits, monosomes (80S) and polysomes (2-8) are indicated. The arrows indicate the position of stalled preinitiation complexes (halfmers). (B) Fractions from the gradients shown in panel A were collected and the proteins were analyzed by immunoblotting. The position of proteins P0, P1 and P2 are indicated. (C) Translation efficiency in control and

P1/P2-depleted cells. The overall translation efficiency was estimated as the relative amount of rRNA participating in polysomes. **(D)** Average number of ribosomes per mRNA in polysomes. These values were calculated by differential integration of the profiles in A. **(E)** The relative ribosome content was expressed as the ratio of total ribosomes (monosomes +80S- plus polysomes) to subunits (40S plus 60S). Data are the means  $\pm$  SD and the results were analyzed using the Student's t test for paired data, \*  $P < 0.05$ .

### Figure 5 Analysis of ribosomal subunits in P2-silenced cells

**(A)** Total ribosomes were isolated from control and Dox-induced P2 silenced cells (0.1  $\mu\text{g/ml}$  Dox for 4 days), dissociated into 40S and 60S subunits in the presence of 500 mM KCl and 5 mM  $\text{MgCl}_2$ , and then sedimented on a 10 to 30% sucrose gradient. **(B)** Sucrose gradient analysis of  $\text{Mg}^{2+}$ -induced association of ribosomal subunits. Aliquots of ribosome preparation were fractionated on 10 to 30% sucrose gradient at the indicated  $\text{Mg}^{2+}$  concentrations and in the presence of 80 mM KCl. The position of the 40S, 60S and 80S peaks are shown. **(C)** Subunit association (see Methods) from three experiments such as that shown in Figure 5B was plotted as a function of the  $\text{Mg}^{2+}$  concentration in control (white bars) and P2-silenced (black bars) cells. Data are the means  $\pm$  SD and the results were analyzed using the Student's t test for paired data, \*  $P < 0.05$ .



**Figure 1**



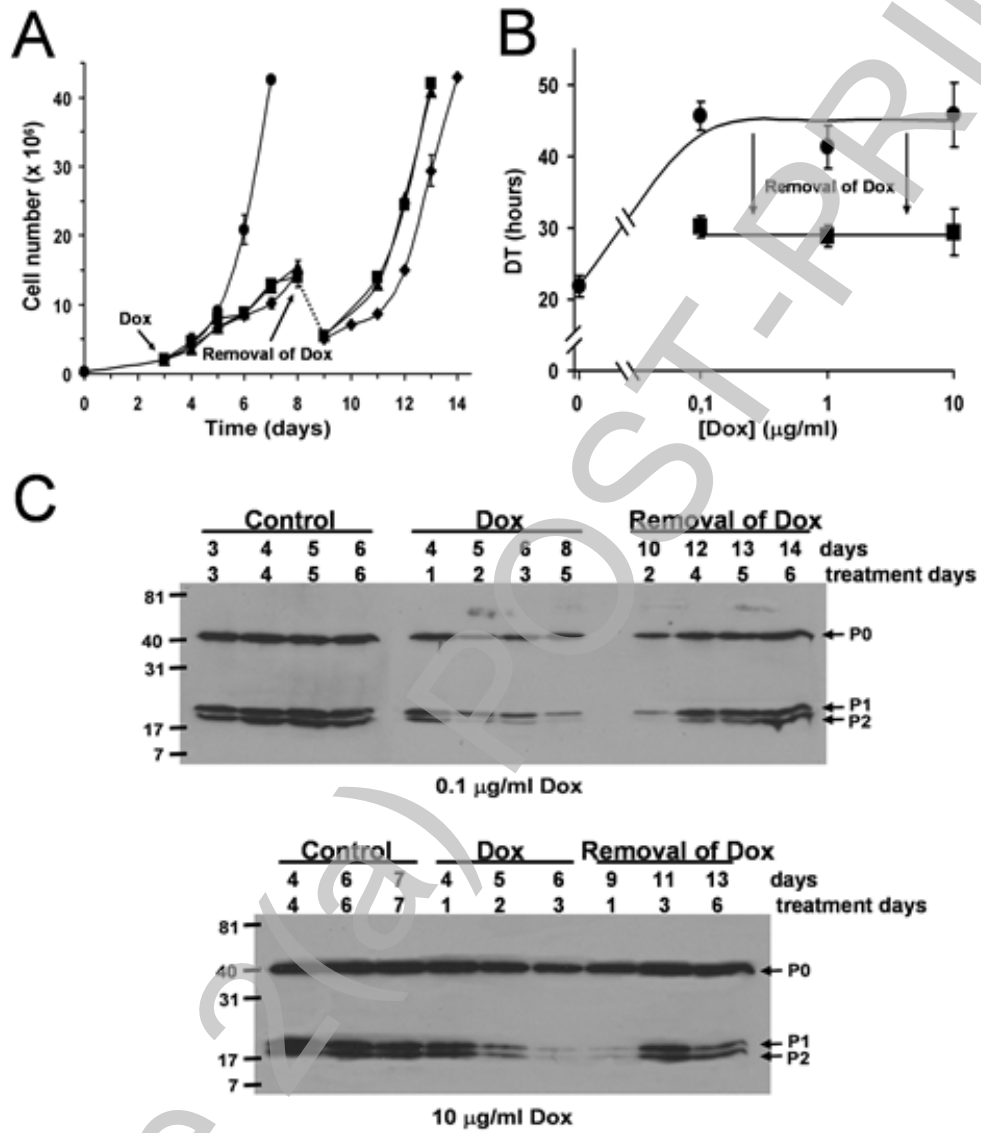
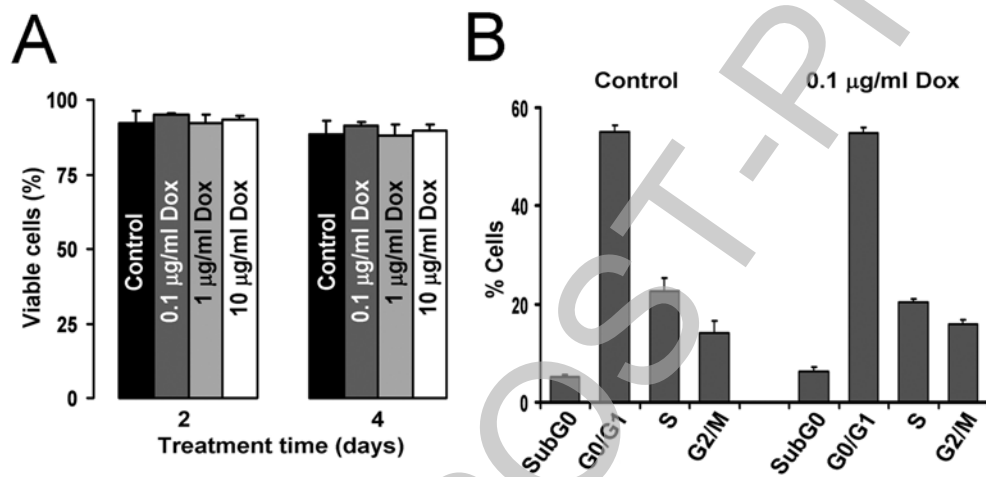
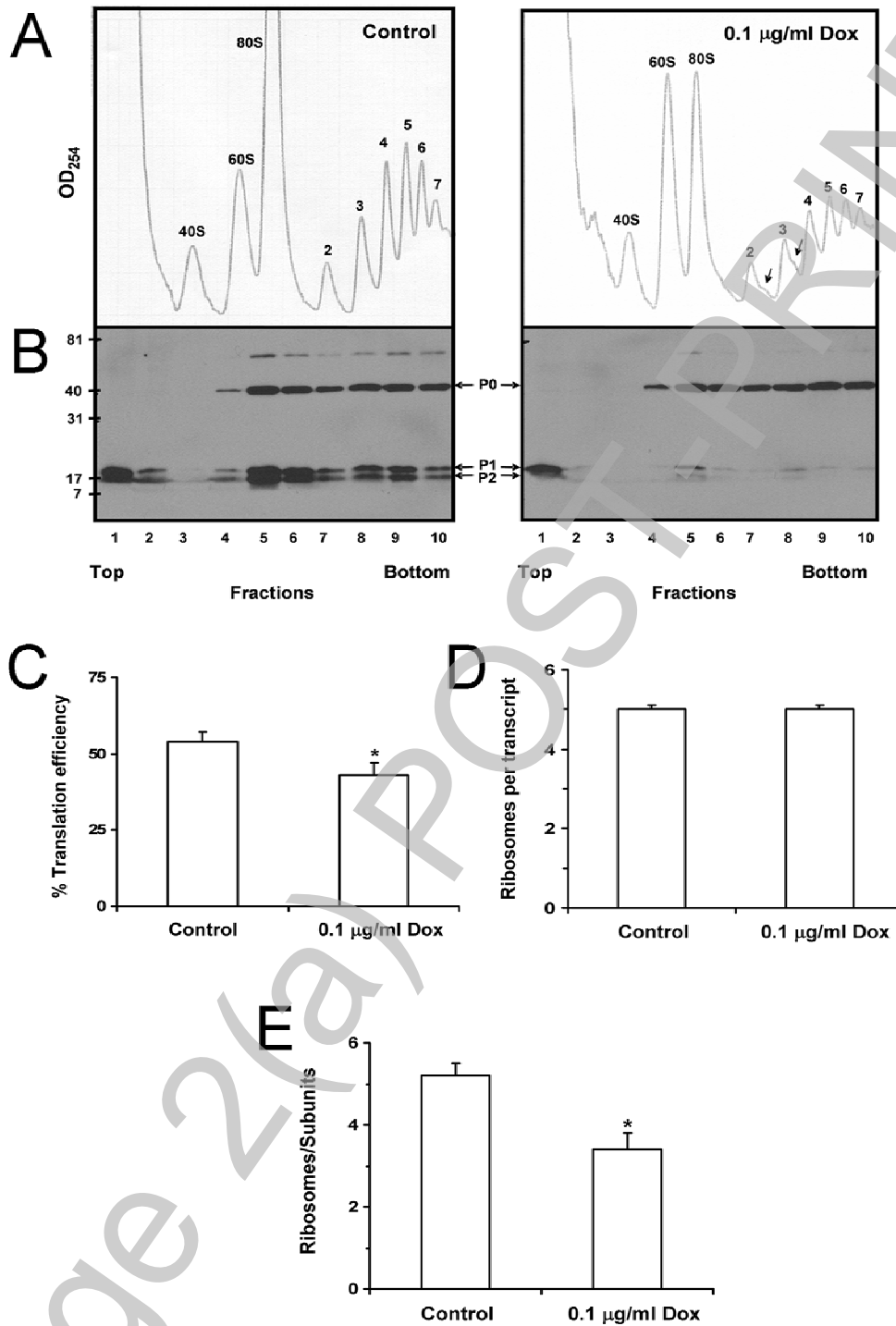


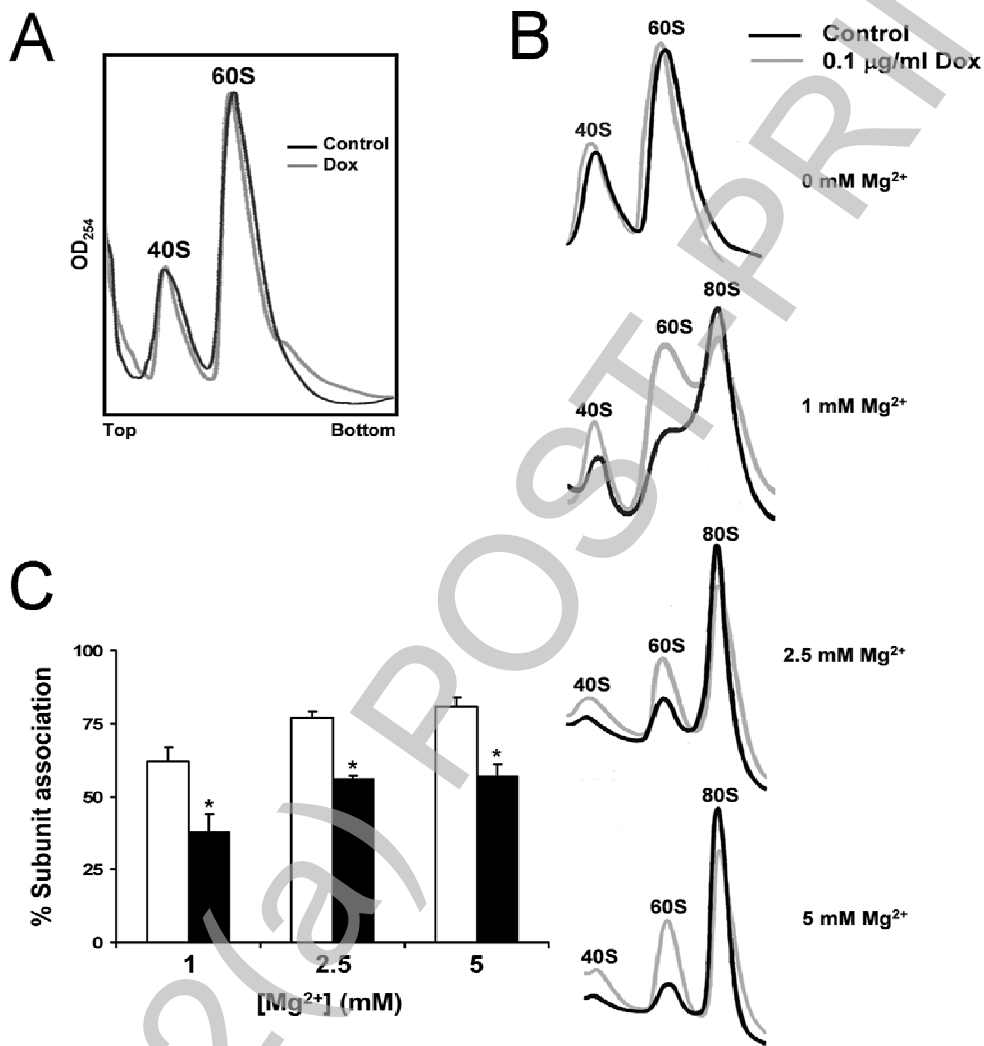
Figure 2



**Figure 3**



**Figure 4**



**Figure 5**