ARTICULO

Effect of the protein Stm1p on *Saccharomyces cerevisiae* initiation protein synthesis under normal growth conditions

Heriberto Correia, Alexandra Hernández, Ledia Triana, Elizabeth Ferrer and Flor Herrera

Instituto de Investigaciones Biomédicas de la Universidad de Carabobo (BIOMED-UC), Facultad de Ciencias de la Salud, Sede Aragua, Maracay, Aragua, Venezuela.

Correspondence: H. Correia

E-mail: heri_correia@yahoo.es

ABSTRACT

The possible function of Stm1p was evaluated in a cell-free system from *Saccharomyces cerevisiae*, grown in a rich medium. The cell-free system incubated with increasing concentration of anti-Stm1p monoclonal antibody showed an inhibition of the formation of the 48S initiation complex of up to 50 % with 10 µg/ml of the monoclonal antibody. On the contrary, non-reactive mouse hybridoma supernatant, used as control, caused a maximum inhibition of close to 20%. This data may indicate that Stm1p participates in this event of protein synthesis or may exist a competition for common or overlapping binding sites between Stm1p and some initiation factors. However, anti-Stm1p, also used on the translation of poly(U), did not interfere with the polyphenylalanine synthesis. In addition, Stm1p retained its association activity on α-sarcin-treated ribosomes which indicates that this protein, contrary to the elongation factors, does not bind to the α-sarcin domain of 28S ribosomal RNA in the process of protein synthesis. Finally, the ribosomal association activity was performed for another protein (although thermolabile) in the stm1Δ yeast mutant.

Key words: Ribosomal-association; Stm1p; α-sarcin; ribosomal function; protein synthesis

RESUMEN

Efecto de Stm1p en la iniciación de la síntesis de proteínas de *Saccharomyces cerevisiae* bajo condiciones normales de crecimiento.

En este trabajo se evaluó la posible función de Stm1p en un sistema libre de células de *Saccharomyces cerevisiae* crecidas en medio rico. El sistema libre de células se incubó con concentraciones crecientes del anticuerpo monoclonal anti-Stm1p y mostró hasta un 50% de inhibición de la formación del complejo de iniciación 48S con 10 µg/ml del anticuerpo. Por lo contrario, el sobrenadante no reactivo de hibridoma de ratón, usado como control, causó una inhibición máxima cercana al 20%. Estos datos podrían indicar que Stm1p participa en este evento de la síntesis proteica o podría existir una competencia o sobreposición de los sitios de unión entre Stm1p y algunos factores de iniciación. Sin embargo, al usar anti-Stm1p en la traducción del ARNm mensajero sintético poly(U), este no interfirió en la síntesis de polifenilalanina. Además, Stm1p retuvo su actividad asociante en ribosomas tratados con α-sarcina, lo cual indica que esta proteína, a diferencia de los factores de elongación, no se une al dominio α-sarcina

del ARNr 28S durante el proceso de síntesis de proteínas. Finalmente, la actividad de asociación ribosomal fue realizada por otras proteínas termolábiles presentes en la cepa mutante stm1Δ.

Palabras clave: Asociación ribosomal, Stm1p, α-sarcina; función ribosomal; síntesis de proteínas.

INTRODUCTION

Evidence has been presented showing that Stm1p participates in different biological mechanisms in the yeast cells. Earlier studies have established that it is a G4 quadruplex-binding protein (1) and later it was determined that Stm1p also binds a Pu motif triplex DNA (2). Studies have revealed the ability of STM1 to act as a multicopy suppressor of mutants involved in aspects of cell cycle control (3,4) and in the maintenance of telomere structure (5). Stm1p also participates in the process of apoptosis (6,7).

Likewise, the interaction between Stm1p and the ribosome has been documented which might point towards a specific function for Stm1p in protein synthesis (8,9,10). Recently, it has been reported (9) that Stm1p is required for optimal translation under nutrient stress conditions and is involved in the target of rapamycin (TOR) signaling pathway. In addition, over-expression of *Stm1p* can specifically suppress mutants related to the deadenylation process (11).

On the other hand, it has been shown that depletion of Stm1p activity due to deletion of the STM1 gene (*stm1*Δ cells) does not impair the growth of yeast cells (Hata et al., 1998, Ligr et al., 2001) (3,7). This indicates that Stm1p is not essential for translation; however, it may turn out that its function is necessary and it can be performed by another protein.

We found that Stm1p not only has sequence similarity with the association factor (AF) but also associates ribosomal subunits in normal growth conditions (Correia et al., 2004) (10). Besides, MAbs against each of the two proteins recognize both of them (being purified or present in cell fractions), which demonstrates that these proteins share the same epitopes (Correia et al., 2009, the accompanying manuscript) (12). However, only AF is glycosylated. Therefore, we wanted to investigate further the possible role of Stm1p in the protein synthesis mechanism. Here we report that it is needed for 48 initiation complex formation. Moreover, this protein does not share the same ribosomal binding domain of the translation elongation factors.

MATERIALS AND METHODS

Preparation of Yeast Extracts (S-100´). Strains of *S. cerevisiae* designated A-1455 (STM1 wild type) and A-1454 (stm1Δ) were grown to mid-log phase in rich medium as previously described (13). Yeast cells were stored frozen as a wet paste at -70ºC. Then, cells were thawed in 60 mmol/L Tris/acetate pH 7.2, 50 mmol/L NH_4Cl, 5 mmol/L $Mg(OAc)_2$, 5 mmol/L 2-mercaptoethanol, 0.1 mmol/L $PhMeSO_2F$, 1mmol/L iodoacetic acid and 20% glycerol (2:1, by vol.) and homogeneized by stirring in a blender using acid-washed glass beads. The extract was centrifuged at 100000 g for 30 min, and the resulting supernatant was aliquoted and stored frozen at -70ºC (14).

Preparation of ribosomes and high-salt ribosomal wash (HSRW). This was performed according to Herrera et al (14). Briefly: Polysomes, prepared following Gallis et al. (15), were suspended in buffer A [20 mmol/L Tris/acetate pH 7.5, 70 mmol/L KOAc, 3 mmol/L $Mg(OAc)_2$, 1 mmol/L dithiothreitol, 0.1 mmol/L $PhMeSO_2F$, 1mmol/L iodoacetic acid and 20% glycerol]. The resulting suspension was made up to 5 mmol/L Mg^{2+} and 500 mol/L KCl and centrifuged 3 h at 226000 xg. The ribosomal pellet was washed with buffer A, suspended in the same buffer and stored at -70ºC. HSRW was desalted through a Sephadex G-25 previously equilibrated with buffer B (20 mmol/L K_2HPO_4 pH 7.5, 100 mmol/L KCl, 10 mmol/l 2-mercaptoethanol, 0.5 mmol/L $PhMeSO_2F$, 1mmol/L iodoacetic acid and 25% glycerol), immediately heated at 90ºC for 15 min and then centrifuged at 30000 xg for 10 min.

Ribosomal association activity. The ribosomal subunits association activity was performed according to the methodology described by (14). In short: The ribosomes (0.5-0.7 A_{260} units), treated or not with α-Sarcin, were incubated for 5 min at 20ºC with 12 µg HSRW or with 0.1-0.5 µg purified STM1 in a final volume of 50 µL with buffer C (20 mmol/L Hepes/KOH buffer (pH 7.4), 210 mmol/L KOAc, 3 mmol/L $Mg(OAc)_2$, 3 mmol/L dithiothreitol and 10% glycerol).

The incubations were layered on top of 12 mL 15-40% (w/v) linear gradients of sucrose in buffer D (buffer A without glycerol). Centrifugation was performed at 2ºC in a Beckman rotor SW 40 at 39000 rpm for 4.5 h. The gradients were then analyzed automatically at 254 nm with a scanning recording spectrophotometer.

Purification of Stm1p. *E.coli* BL21DE was transformed with the plasmid pGEXStm1p that has inserted the gen for the yeast protein Stm1p fused to glutathione S-transferase. A bacterially expressed Stm1p was obtained after proteolytic removal of the glutathione S-transferase domain with factor Xa. These procedures were performed as described by Frantz and Gilbert (1).

Assays for 48S preinitiation complex formation. The formation of 48S preinitiation complex was followed similar to that described in Triana et al. (16). In brief: Yeast S-100´ extract (111 µg) was incubated for 15 min at 20ºC in a reaction volume of 50 µL in buffer C with the following components: 40 µmol/L each of 20 amino acids, 7 µg of ^{3}H-labeled poly(A)$^+$ mRNA and 12 units of human placental ribonuclease inhibitor (Amersham). In some experiments, S-100´ and poly(A)$^+$ mRNA were preincubated with different amounts of monoclonal anti-Stm1p antibody or nonreactive mouse hybridoma supernatant (NRMH), under conditions described above. Following incubation, samples were centrifuged as above on 15-40% (w/v) linear sucrose density gradients, fractionated, mixed with Aquasol scintillation fluid and counted in a ß counter.

Poly(U)-Dependent Polyphenylalanine Synthesis. Cell-free yeast extract (S-100´ fraction containing 111 µg of protein) was incubated with 4 µg of poly(U) in buffer C with 13 mmol/L $Mg(OAc)_2$, containing [^{3}H]phenylalanine (1,5 µmol/L final concentration, 49,7 Ci/mmol), 0,5 mmol/L ATP, 0,1 mmol/L GTP, 20 mmol/L creatine phosphate, and 10 µg of creatine phosphokinase in a total volume of 50 µL. The reactions were incubated for 40 min at 25ºC.

Polyphenylalanine was followed by measuring the incorporation of [^{3}H]phenylalanine in trichloroacetic acid-insoluble fractions. In some experiments, S-100´ and poly(U) were preincubated for 40 min at 25ºC with different amounts (0-50 µg/ml) of anti-Stm1p, anti-EF-1α or NRMH in a total volume of 28 µL in the same buffer as before. The S-100´ fractions pretreated with specific protein were assayed in the same manner as described above.

Treatment of the ribosomes with α-Sarcin. Ribosomes (6 A_{260} units), isolated as above, were incubated with α-Sarcin (0.2 µg) in 50 µL of 38 mmol/L Tris/HCl, pH 7.5, 38 mmol/L KCl and 5 mmol/L EDTA for 15 min at 37ºC. The reaction was stopped by the addition of 0.5 ml of 0.5% sodium dodecyl sulfate in 50 mmol/L Tris/HCl, pH 7.5 (17). Following incubations, the samples were centrifuged through 25 % glycerol cushion containing 20 mmol/L Tris/HCl (pH 7,5), 5 mmol/L $Mg(OAc)_2$ and 500 mmol/L KCl at 4ºC in a Beckman rotor 50 *Ti at 70000 x g for 12 h.* The precipitated washed ribosomes were resuspended in buffer A (with 70 mmol/L KCl instead of KOAc) to a final concentration of 50 OD A_{260}/mL, aliquoted and stored frozen at -70ºC.

Immunization protocol. Monoclonal antibodies (anti-Stm1p and anti-AF) production and Protein immunoblotting, were performed as described by Correia el al. (accompannyng manuscript) (12). In addition, MAbs against EF-1α were also prepared using a similar procedure.

RESULTS

We performed an analysis to see whether Stm1p participates in the formation of 48 initiation complex. For this, we cultured *Saccharomyces cerevisiae* in a rich medium and the cells were harvested during mid-log-phase of growth. Results presented in Figure 1 show that increasing concentration of monoclonal anti-Stm1p antibody inhibited the formation of the 48S initiation complex up to 50 % with 10 µg/mL of this antibody. On the contrary, the control NRMH caused a maximum inhibition close to 20%.

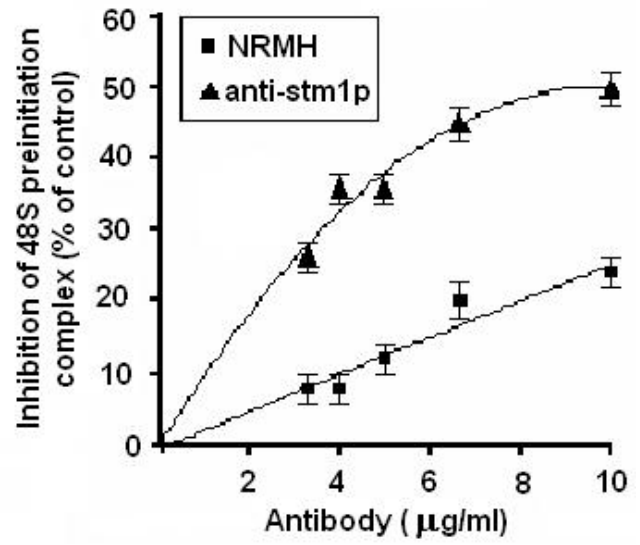

Figure.1: inhibition of 48S preinitiation complex by monoclonal anti-Stm1p antibody. Ribosomes from wild-type S-100' and [^{3}H]pmRNA were preincubated in the presence of (0-50 µg/ml) monoclonal anti-Stm1p (▲) or NRMH (■) as described in Material and Methods prior to the addition of the components for the formation of the 48S preinitiation complex. The complex was separated in a 15-40% linear sucrose gradients and was analyzed with a continuous monitoring system to determine the absorbance at 254 nm and fractionated to determine radioactivity. Incubation components centrifugation and analytical procedures are described in the text. 100% represents 1100 cpm. Vertical bars represent standard deviation.

To determine if Stm1p plays a role in elongation, anti-Stm1p was used on the translation of poly(U) (generally accepted which reflects the elongation event). Figure 2 shows that this antibody did not interfere with the polyphenylalanine synthesis. However (as expected) anti-EF-1α showed approximately a 60 % inhibition on the poly (U) translation.

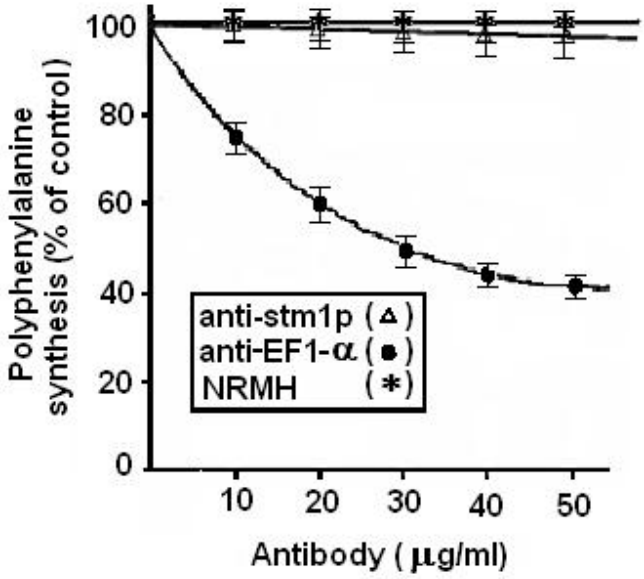

Figure 2: Effect of monoclonal antibody anti-Stm1p on polyphenylalanine synthesis. Ribosomes from wild-type S-100' and poly(U) were preincubated in the presence of (0-50 µg/ml) monoclonal anti-Stm1p (Δ), monoclonal anti-EF-1α (●) or NRMH (*) as described in Material and Methods prior to the addition of the components for the formation or the tritiated polyphenylalanine. The complex was separated in a 15-40% linear sucrose gradients and was analyzed with a continuous monitoring system to determine the absorbance at 254 nm and fractionated to determine radioactivity. Incubation components centrifugation and analytical procedures are described in the text. 100% represents 2400 cpm. Vertical bars represent standard deviation.

To further explore this point, we wanted to see whether the association activity of Stm1p depends on the binding site of the elongation factors. To this ends we used the protein α-sarcin. It is known that this protein cleaves a fragment from the 25S rRNA in the large subunit of yeast ribosomes; as a consequence, the binding of EF-1 and EF-2 to the ribosomes is prevented (18). *Saccharomyces cerevisiae* ribosomes were incubated at 3 mmol/L Mg^{2+} and analyzed by sucrose gradient centrifugation. The control ribosomes sedimented mainly as 40S and 60S subunits (Figure 3A). Figure 3B shows that Stm1p associated ribosomal subunits. Then ribosomes were preincubated with α-sarcin showing the same dissociated pattern (Figure 3C). These last ribosomes were associated by Stm1p demonstrating that the ribosomes were not affected by the α-sarcin treatment (Figure 3D). As usual, the polyphenyalanine synthesis was inhibited (~76%) when α-sarcin-treated ribosomes were used in the assay (data not shown).

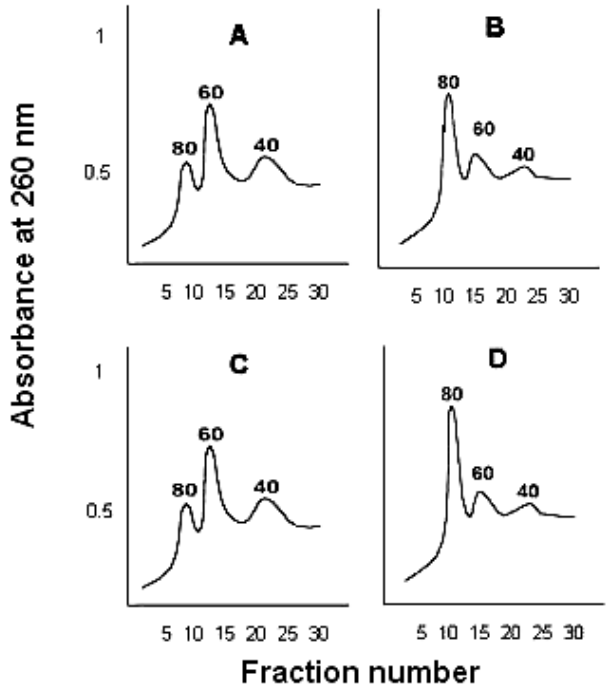

Figure 3: ribosomal association activity in the presence of α-sarcin treated ribosomes. Equal amounts of dissociated ribosomes (10 pmol) were treated or not with α-sarcin and incubated at 3 mmol/L Mg^{2+}, as described in Materials and Methods, under the following conditions: A) without treatment, B) without treatment plus Stm1p, C) pretreated with α-sarcin, D) pretreated with α-sarcin plus Stm1p. At the end of the incubation, the samples were layered over 15-40% linear sucrose gradients as described in Figure 1.

Since Stm1p is absence in a stm1Δ mutant, we investigated the association activity of this mutant, using the HSRW as a source of association factors. When stm1Δ ribosomes (Figure 4A) were incubated with its HSRW, the subunits associate at 3 mmol/L Mg^{2+} which demonstrates the presence of an association activity (Figure 4B). Then, to determine whether this activity was thermostable, the HSRW was heated at 90ºC for 15 min. As shown in Figure 4C, heated HSRW failed to form 80S monosomes. Control experiments reveal that the wild-type ribosomes were not affected by this treatment (Figure 4 D and E). The results of mixing the mutant and wild-type HSRW fractions with wild type ribosomes were also similar (data not shown).

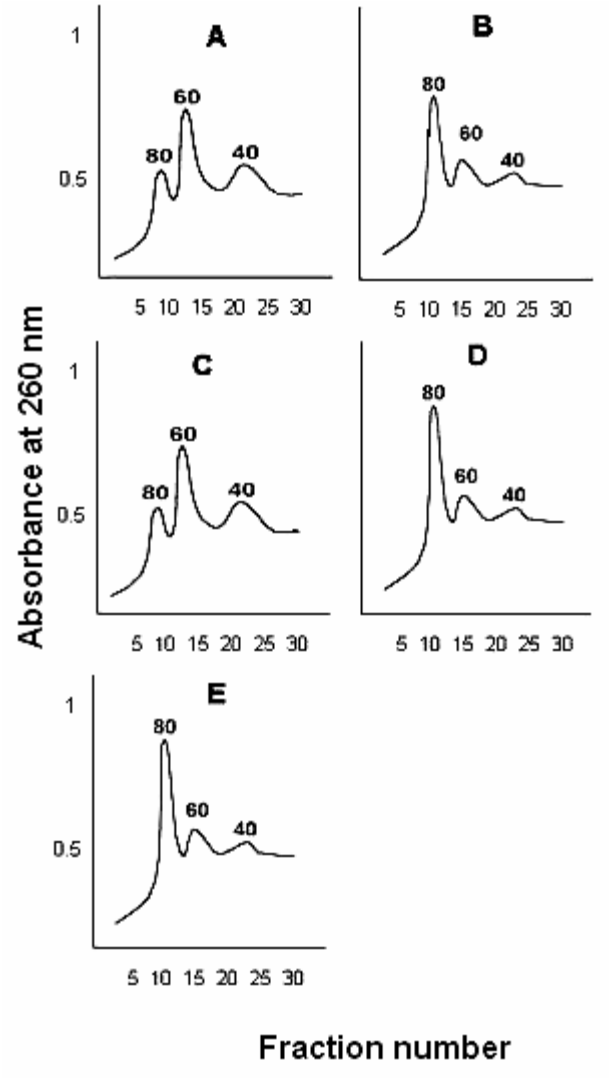

Figure 4: ribosomal association activity of the stm1pΔ. Mutant ribosomes (10 pmol) were incubated with HSRW, preheated at 90ºC for 10 min or not, as described in Materials and Methods, under the following conditions: A) ribosomes at 3 mmol/L Mg^{2+}, B) ribosomes plus HSRW (54 µg) from mutant, C) ribosomes plus preheated HSRW (37 µg) from mutant, D) ribosomes plus HSRW (54 µg) from wild-type, E) ribosomes plus preheated HSRW (37 µg) from wild-type. At the end of the incubation, the samples were layered over 15-40% linear sucrose gradients as described in Figure 1.

DISCUSSION

To investigate the possible role of Stm1p in translation under rich medium growth conditions, MAb against Stm1p was used in experiments with cell-free systems. Then, we analyzed the effect of anti-Stm1p in the initiation step. Experimental data presented in this communication show that anti-Stm1p inhibits 48S initiation complex formation about 50% which may indicate that Stm1p participates in this event of protein synthesis. Another possible explanation for the results obtained is the existence of a competition for common or overlapping binding sites between [anti-Stm1p.Stm1p] and some initiation factors. That is, If anti-Stm1p interacts first with its antigen, it may block the binding of one or more initiation factors to ribosome and, thus, the 48S preinitiation complex formation. On the other hand, the inhibition (<20%) observed with NRMH may be caused by nonspecific interactions between nonimmune serum proteins and initiation factors that could sequester and inactivate some of these proteins.

The observation that the anti-Stm1p has no effect on the polyphenylalanine synthesis could be explained because this assay has to be performed at high Mg^{2+} concentration in which the majority of the subunits remains associated. The 80S ribosomal formation, probably, resulted in hiding the protein Stm1p, thus suggesting the protein location at the subunit interface in the 80S ribosome. This latest point coincides with the previous work (9). These features are expected to impede the binding of anti-Stm1p to the protein. Therefore, Stm1p may bind proteins which are located in the subunit interface region of the 80S ribosome and as a result, associates ribosomal subunits. To strengthen this point, Stm1p was able to associate α-sarcin treated ribosomes. This indicates that this protein, contrary to the elongation factors, does not bind to the sarcin domain of 28S ribosomal RNA in the process of protein synthesis.

As mentioned before, Stm1p has been implicated in many different mechanisms. However, it is not an essential protein for the viability of yeast since deletion of its gene does not impede yeast growth. Therefore, this paradox could be explained if we consider that Stm1p does not act in a key process itself (like protein synthesis) but on its regulation. We could argue that Stm1p is the non-glycosylated form of AF (Correia et al., accompanying manuscript) (12), and that removal of glycosylation sites within AF induced changes in translation. Regulatory effects induced by protein glycosylation have been extensively investigated (19,20). To strengthen this point, we found that the inhibition of the 48S initiation complex formation was never higher than 50% indicating a system resistance to be totally inhibited. This may indicate a regulatory effect. Similarly, Van Dyke et al (9) reported that Stm1p is not required for all translation but for optimal translation under nutrient stress conditions. Besides, we show here the existence of a second ribosomal association protein (although thermolabile) in the stm1Δ yeast mutant. Taken together these data it seems that the couple Stm1p-AF affects translation at the level of initiation but the exact mechanism is still unsolved. We conclude that additional experiments are necessary to clarify these issues.

ACKNOWLEDGEMENTS *The authors are grateful to Drs. J. Daniel Frantz and Akira Sakai for their generous gifts of the plasmid pGEXG4p2 and the yeast strains A1454 and A1455 respectively; to Alexandra Infante and Gladys Jaspe for performing preliminary experiments and to Professor Henry Rupp for linguistic assistance in arranging the final manuscript. This work was supported by grant (Nº 99-011) from the Consejo de Desarrollo Científico y Humanístico of the Universidad de Carabobo.*

BIBLIOGRAPHY

1. Frantz J, Gilbert W. A novel yeast gene product, G4p2, with a specific affinity for quadruplex nucleic acids. *J Biol Chem* 1995; 270: 9413-9419.

2. Nelson, L.D., Musso, M., Van Dyke, M.W. The yeast STM1 gene encodes a purine motif triple helical DNA-binding protein. *J Biol Chem* 2000; 275: 5573-5581.

3. Hata H, Mitsui H, Liu H, Bai Y, Denis CL, Shimizu Y, Sakai A. Dhh1p, a putative RNA helicase, associates with the general transcription factors Pop2p and Ccr4p from *Saccharomyces cerevisiae*. *Genetics* 1998; 148:571-579.

4. Utsugi T, Toh-e A, Kikuchi Y. A high dose of the STM1 gene suppresses the temperature sensitivity of the tom1 and htr1 mutants in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1995; 1263: 285-288.

5. Hayashi N, Murakami S. STM1, a gene which encodes a guanine quadruplex binding protein, interacts with CDC13 in *Saccharomyces cerevisiae*. *Mol Genet Genomics* 2002; 267:806-813.

6. Morton CO, Dos Santos SC, Coote P. An amphibian-derived, cationic, alpha-helical antimicrobial peptide kills yeast by caspase-independent but AIF-dependent programmed cell death. *Mol Microbiol* 2007; 65:494-507

7. Ligr M, Velten I, Fröhlich E, Madeo F, Ledig M, Fröhlich K-U, Wolf D, Hilt W. The proteasomal substrate Stm1 participates in apoptosis-like cell death in yeast. *Mol. Biol Cell* 2001; 12: 2422-2432.

8. Van Dyke MW, Nelson LD, Weilbaecher RG, Mehta DV. Stm1p, a G4 quadruplex and purine motif triplex nucleic acid-binding protein, interacts with ribosomes and subtelomeric Y' DNA in *Saccharomyces cerevisiae*. *J Biol Chem* 2004; *279*:24323-24333.

9. Van Dyke N, Baby J, Van Dyke MW. Stm1p, a ribosome-associated protein, is important for protein synthesis in *Saccharomyces cerevisiae* under nutritional stress conditions. *J Mol Biol 2006;* 358:1023-1031.

10. Correia H, Medina R, Hernández A, Bustamante E, Chakraburtty K, Herrera F. Similarity between the association factor of ribosomal subunits and the protein Stm1p from *Saccharomyces cerevisiae. Mem Inst Oswaldo Cruz* 2004; 99:733-737.

11. Ohn T, Chiang Y-C, Lee DJ, Yao G, Zhang C, Denis CL. CAF1 plays an important role in mRNA deadenylation separate from its contact to CCR4. *Nucleic Acids Res* 2007; 35:3002-15.

12. Correia H, Hernández A, Ferrer E and Herrera F. SALUS. 2009; accompanying manuscript Production and characterization of monoclonal antibodies against association factor and Stm1p of *Saccharomyces cerevisiae.*

13. Hartwell, L. Macromolecule synthesis in temperature-sensitive mutants of yeast. *J Bacteriol* 1967; 93:1662-1670.

14. Herrera F, Correia H, Triana L, Fraile G. Association of ribosomal subunits. A new functional role for yeast EF-1 alpha in protein biosynthesis. *Eur J Biochem* 1991; 200; 321-327.

15. Gallis B, McDonnel J, Hooper J, Young E. Translation of poly(riboadenylic acid)-enriched messenger RNAs from the yeast *Saccharomyces cerevisiae* in heterologous cell-free systems. *Biochemistry* 1975; 14:1038-1046.

16. Triana L, Ferreras AC, Cayama E, Correia H, Fraile G, Chakraburtty K, Herrera F. Involvement of a 50-kDa mRNP protein from *Saccharomyces cerevisiae* in mRNA binding to ribosomes. *Arch Biochem Biophys* 1997; 344:1-10.

17. Endo, Y., Wool, I.G. The site of action of ∝-sarcin on eukaryotic ribosomes. *J Biol Chem* 1982; 257:9054-9060.

18 Schindler DG., Davies JE. Specific cleavage of ribosomal RNA caused by alpha sarcin. *Nucleic Acids Res* 1977; 4:1097-1110.

19. Kudlow, J. Post-Translational Modification by O-GlcNAc: Another Way to Change Protein Function. *J Cell Biochem* 2006; 98:1062-1075.

20. Chen L, Yang Y, Han J, Zhang B-Y, Zhao L, Nie K, Wang X-F, Li F, Gao Ch, Dong X-P, and Xu C-M. Removal of the Glycosylation of Prion Protein Provokes Apoptosis in SF126. *J Biochem Mo. Bio* 2007; 40:662-669.