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The autosomal ribosomopathy SBDS with inherited bone marrow failure is regulated by essential 60S Ribosome Biogenesis Factor eIF6

ARUNIMA BISWAS*

Abstract

Recently, a number of congenital diseases, resulting from defects in ribosome biogenesis and maturation, have been discovered. Shwachman-Bodian-Diamond syndrome (SBDS) is one such ribosomopathy, caused by deficiency of the highly conserved SBDS protein involved in 60S ribosome biogenesis. Characteristic clinical features include exocrine pancreatic insufficiency, neuro-cognitive dysfunction, bone marrow dysfunction and failure, leukemia predisposition. Studies in yeast show evidence of genetic interaction between the yeast SBDS protein (called Sdo1p) and eukaryotic Initiation Factor 6 (eIF6). eIF6 is an essential, conserved 60S ribosome biogenesis factor, required for pre-rRNA processing in the nucleus. It also escorts pre-60S particles from nucleus to cytoplasm for final maturation. Sdo1p, with the cytoplasmic GTPase elongation factor-like 1 protein, facilitates the release of yeast eIF6, from the pre-60S ribosomes in the cytoplasm which is crucial for final maturation of 60S subunits and for recycling of eIF6 to the nucleus. Nucleo-cytoplasmic shuttling of eIF6 is also regulated by its phosphorylation and dephosphorylation, mediated by casein kinase 1 and Ca^{++} – dependent calcineurin phosphatase, respectively. Thus, eIF6 release and recycling seem to be due to concerted actions of SBDS protein and eIF6 phosphorylation-dephosphorylation events. Inhibition of this process leads to defects in ribosome biogenesis and maturation and associated pathophysiology.

Keywords:

Shwachman-Bodian-Diamond syndrome;
ribosomopathy;
eukaryotic Initiation Factor 6;
Nucleo-cytoplasmic shuttling;
phosphorylation-dephosphorylation.

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1. Introduction

Eukaryotic ribosome biogenesis and translation are linked processes that limit the rate of cell growth. Recently there have been many reports implicating ribosome dysfunction in certain genetic disorders which are characterized by bone marrow failure and cancer predisposition. Shwachman-Bodian-Diamond syndrome (SBDS) is one such autosomal recessive disorder, in which the central SBDS protein functions in 60S ribosomal subunit biogenesis. The disorder involves multiple organ systems and affects children and young adults. Characteristic clinical features include exocrine pancreatic insufficiency, neuro-cognitive dysfunction, and, as mentioned, bone marrow failure and leukemia predisposition. Additional organ

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systems, such as skeletal, hepatic, cardiac, immunologic, and dental systems, may also be affected [1], [2]. Studies in yeast show evidence of genetic interaction between the yeast SBDS protein and the essential ribosome biogenesis factor eIF6. Mutations in the yeast ortholog of this SBDS protein (called Sdo1p) lead to defective 60S ribosome synthesis, which is rescued by gain-of-function of yeast eIF6 mutants, indicating that yeast eIF6 (called Tif6p) genetically interacts with yeast Sdo1p. Sdo1p has also been shown to facilitate the release and recycling of yeast Tif6p, from the pre-60S ribosomes in the cytoplasm to form mature 60S ribosomes. This results in translational activation of 60S ribosomes. Additionally, genetic analysis in yeast has also shown that Sdo1p acts epistatically with the cytoplasmic GTPase Efl1p to promote yeast eIF6 release. This release is crucial for final maturation of 60S ribosomal subunits as well as for eIF6 recycling [3], [4].

Eukaryotic translation initiation factor 6 (eIF6), a highly conserved protein from yeast to mammals, is not a canonical translation initiation factor [5]; rather it is essential for 60S ribosome biogenesis and assembly. Both the mammalian and yeast eIF6 proteins are each 245 amino acid long and 72% identical in amino acid sequence. Most of our knowledge of the functional properties of eIF6 has been derived from molecular genetic studies examining the yeast eIF6 ortholog Tif6p in the yeast *Saccharomyces cerevisiae*. It has been shown that Tif6p is involved in 60S-specific pre rRNA processing, which is a nucleolar function [6], [7] (Figure 1). It has also been reported that Tif6p bound to the pre-60S ribosomal particles escort pre-60S complex through the nuclear pore to the cytoplasm for final maturation. Since eIF6 is a limiting component, it is imperative that following its release from the pre-60S ribosomal particles in the cytoplasm, it must recycle back to the nucleolus for continued 60S ribosome biogenesis.

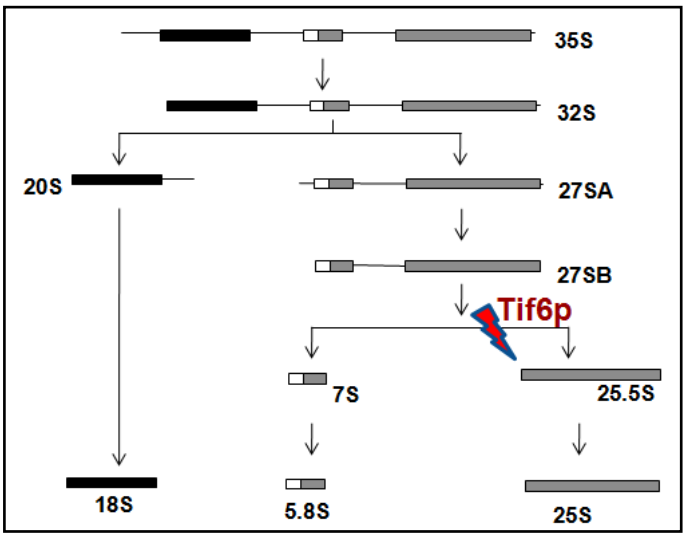

Figure 1. yeast eIF6 (Tif6p) is involved in pre-rRNA processing in nucleus [6]

Ribosome biogenesis is a complex process [7]. The nascent pre-60S particles are mostly assembled in the nucleolus and to a lesser extent in the nucleoplasm. Then they are exported out of the nucleus to the cytoplasm where final maturation of the pre-60S ribosomal particles occurs to form the mature, translationally competent 60S ribosomal subunits. Most of the trans-acting protein factors that associate with the pre-ribosomal particles during their nucleolar assembly are released in the nucleus prior to the export of the pre-60S particles in the cytoplasm. However, a small number of protein factors including eIF6 (Tif6p in yeast) remain bound as the pre-60S particles exit the nucleus and enter the cytoplasm. In fact, it seems that it is essential that eIF6 escorts the pre-60S particles in this way from the nucleus to the cytoplasm for its final maturation [7]. The cytoplasmic ribosomal maturation pathway involves sequential and ordered release of these bound protein factors by the action of specific energy-consuming cytoplasmic ATPases or GTPases, each of which associates with the pre-ribosomal particles to affect the release of a specific bound factor. The released factors are recycled back to the nucleus for another round of pre-60S ribosome assembly. In the ordered release of the bound factors, Tif6p and the nuclear export adapter Nmd3 are the last proteins to be released. Following their release, the pre-60S particles become mature 60S ribosomal subunits, competent to participate in translation [7]. Recent findings by Menne *et al*. (2007) and Finch *et al*. (2011) provide compelling evidence that the mechanism of eIF6 release from the pre-60S particles in the cytoplasm during the final maturation of 60S ribosomal subunits is highly conserved between yeast and mammals. As reported above, it involves cooperative interaction of SBDS (Sdo1p) and EFL1 (Efl1p) proteins in mediating the GTP hydrolysis-dependent release of eIF6 from the pre-60S particles [3], [4]. In yeast cells, deletion of either EFL1 or SDO1 confers a very slow growth phenotype. It was also observed that there was a large

accumulation of yeast eIF6 protein bound to the pre-60S particles in the cytoplasm of *efl1*Δ and *sdo1*Δ cells. Importantly, multiple gain of function eIF6 alleles that rescued the growth defect of either *sdo1*Δ or *efl1*Δ cells also restored both the nuclear export defect and nucleolar localization of yeast eIF6. Biochemical analysis showed that in contrast to wild-type protein, which binds to 60S ribosomal subunits with a relatively high affinity, mutant eIF6 in the suppressor strains has a much-reduced affinity for 60S subunits and can thus bypass the requirement of Sdo1 and Efl1p for eIF6 release [3], [4], [7].

In previous studies, it was observed that in both mammalian and yeast cells, eIF6 is phosphorylated at Ser-174 (major site) and Ser-175 (minor site) by the nuclear isoforms of casein kinase 1 or CK1 (CK1α or CK1δ in mammalian cells; Hrr25p in yeast). Ablation of yeast eIF6 phosphorylation, either by depletion of Hrr25p from yeast cells or by alanine replacement of Ser-174 and Ser-175 of yeast eIF6, abolished cell growth and viability, thus establishing the physiological importance of eIF6 phosphorylation at Ser-174 and Ser-175 [8], [9]. In addition to well-characterized conserved CK1 phosphorylation sites at Ser-174 and Ser-175 in nucleated species, eIF6 possesses a putative binding motif LxVP (x= any amino acid) for calcineurin (a Ca^{2+}/calmodulin-regulated protein phosphatase). This putative calcineurin binding motif LQVP is present at amino acid positions 177-180, which is immediately adjacent to the CK1 phosphorylation sites at Ser-174 and Ser-175 on eIF6 [7], [10] (Figure 2).

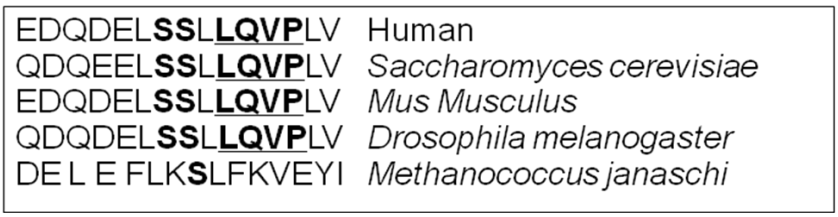

Figure 2. CK1 phosphorylation sites and putative calcineurin binding site on eIF6 [10]

It was shown that phosphorylation of eIF6 promotes its nuclear export [7], [10]. Nuclear import of eIF6 from the cytoplasm, on the other hand, seems to be promoted by activated calcineurin, which binds to eIF6 and presumably dephosphorylates it [7], [10]. However, it remains to be shown that sub-cellular localization of eIF6 is regulated solely and precisely by CK1-catalyzed phosphorylation and calcineurin-mediated dephosphorylation at positions Ser-174 and Ser-175.

Thus, eIF6 release in cytoplasm and recycling (back to nucleus) seem to be dependent on concerted actions of SBDS protein and eIF6 phosphorylation-dephosphorylation events. Inhibition of this process leads to defects in ribosome biogenesis and maturation and associated pathophysiology in SBDS patients. But the actual kinetics and sequence of these events are not yet known.

Hence, a detailed study of this pathway becomes essential in order to have a better understanding of this genetic disease and it may also help to understand, in general, the role of ribosome dysfunction in marrow failure and cancer predisposition. This can, then, be used as a platform for developing new therapeutics in future.

2. Research Method

Antibodies and Reagents — The different antibodies used in this study were purchased from Santa Cruz Biotechnology and Molecular Probes Inc. The other reagents used in this work like ionomycin (calcium ionophore), and other necessary chemicals and media were purchased from Calbiochem and/or Sigma Aldrich. Effectene transfection reagent was purchased from Qiagen.

Recombinant Plasmid Constructs — The various recombinant pcDNA3.1-Myc-His expression plasmids used in the study were kindly gifted by Prof. Umadas Maitra of Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, NY, USA.

Cell Culture and Expression of eIF6 —He La cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum , 2mM glutamine, 50units/ml of penicillin G - sodium and 50 µg/ml of streptomycin sulfate at 37°C in humidified incubators containing 5% CO_2. For expression of eIF6 from various recombinant pcDNA3.1-Myc-His expression plasmids, cells were seeded 18–24h before transfection and grown to 75–80% confluence. Transfections were carried out using Effectene transfection reagent (Qiagen) following the manufacturer's protocol. The transfection efficiency varied from 50–70% of the total cell population. Transfected cells were harvested 24–48h post-transfection as needed and further treated and analyzed as indicated.

Preparation of Nuclear and Cytoplasmic Fractions from Mammalian Cell Extracts and Western Blotting — This was done following standard protocol as described before [10]. Briefly, cells were harvested at 4° C and resuspended in buffer NE (10 mM HEPES pH 7.9, 100 mM KCl, 0.5 mM EDTA, 1 mM DTT, 1 mM

PMSF, 1.5 mM $MgCl_2$), containing a cocktail of protease inhibitors. The suspension was incubated on ice for 15 min, lysed by adding 1% NP-40 followed by vortexing for 10 sec. The lysate was immediately centrifuged at 1000 g for 5 min and the post-nuclear supernatant ("Cytosolic Fraction" or "C") was kept at 0-4° C. The nuclear pellet was resuspended in the same volume of NE buffer as that of the cytosolic fraction and the resuspended nuclei were vortexed, briefly sonicated, incubated on ice for 30 min and centrifuged at 1000 g for 5 min. The clarified supernatant was designated as the "Nuclear Fraction" or "N".

For Western blot analysis of eIF6, equal amount of each sample was subjected to SDS-polyacrylamide gel (10% gel) electrophoresis (SDS-PAGE) followed by transfer to Immobilon-P membranes (Millipore Corporation). Membranes were blocked in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and then treated with appropriate antibodies. After extensive washing, the immunoreactive bands were visualized by Enhanced Chemiluminescence (Amersham Biosciences). The Western bands were scanned, analyzed by densitometry using Image J software (National Institutes of Health, Bethesda, MD) and the results plotted as bar graphs in Microsoft Excel. Data are the average of three individual experiments. The error bars show the standard deviation from the average value, thus reflecting the range of the three independent values obtained in each case.

3. Results and Analysis

3.1. Intracellular localization of mammalian eIF6 is precisely regulated by CK1 and calcineurin — To explore whether sub-cellular localization of eIF6 is regulated solely and precisely by CK1-catalyzed phosphorylation and calcineurin-mediated dephosphorylation at positions Ser-174 and Ser-175, He La cells were transiently transfected with either the wild-type (WT) eIF6 construct or mutant constructs, as mentioned below.

To find out whether Asp replacement of the critical phosphorylatable Ser residues can mimic constitutive phosphorylation and reproduce the effects of phosphorylation, a phosphomimetic mutant was made with both Ser residues mutated to Asp. This mutant was named eIF6-SS174,175DD phosphomimetic double mutant. An attempt to make SS174,175EE double mutant (with Ser replaced by Glu residues) did not yield a functional protein. Next, to probe the importance of the LQVP site in calcineurin binding and subsequent nuclear import of eIF6, the putative calcineurin - binding motif LQVP in eIF6 was mutated to AQAA. This mutant was called eIF6-AQAA triple mutant. The objectives were: **1)** to find out whether nucleo-cytoplasmic shuttling of eIF6 is controlled precisely by phosphorylation-dephosphorylation events at the two serine residues or whether it can be mimicked and the effects reproduced merely by the presence of negative charges at these two amino acid positions (mimics constitutive phosphorylation); **2)** to probe the importance of the putative calcineurin binding site LQVP in promoting nuclear localization of eIF6. For these, He La cells were transiently transfected with the above mentioned eIF6 cDNA constructs that over-expressed wild type or mutant Myc-His-tagged eIF6. Cells were fractionated into nuclear (N) and cytosolic (C) fractions. The validity of the fractionation was verified by using antibodies for Nop1p and eIF5 as markers for nuclei and cytoplasm respectively (as shown in Figure 3 as an example). Western blot analysis using anti-His antibody showed that the wild-type Myc-His-tagged eIF6 was predominantly cytosolic in the transfected cell population, in agreement with previous findings [10]. Also, both the Myc-His-tagged eIF6-AQAA triple mutant and the Myc-His-tagged eIF6-SS174,175DD phosphomimetic double mutant showed predominantly cytosolic localization (Figures 3-5). The sub-cellular distribution of Myc-His-tagged wild type and mutant eIF6 was then tested in presence of calcineurin activator ionomycin (calcium ionophore) or calcium. While ionomycin treatment quickly promoted nuclear translocation of wild type eIF6, it had no effect on the sub-cellular distribution of the eIF6-AQAA mutant which continued to be localized primarily in the cytosol. Also, it failed to promote nuclear transport of eIF6-SS174,175DD mutant (Figure 6).

Co-immunoprecipitation analysis in transfected HeLa cells showed that activated calcineurin interacted with wild type eIF6 but not with eIF6-AQAA triple mutant in which the putative calcineurin binding site LQVP has been mutated. This shows the importance of this LQVP site for calcineurin binding to eIF6. Calcineurin also showed weak binding to the phosphomimetic eIF6 mutant with an intact LQVP site (data not shown).

Taken together, these results indicate that the LQVP motif on eIF6 is essential for binding activated calcineurin and also for subsequent nuclear import of eIF6. Mutation of this site to AQAA abolishes interaction between eIF6 and calcineurin. As a result, activated calcineurin can neither bind to nor dephosphorylate and promote nuclear localization of this mutant eIF6 and the protein exhibits nearly complete cytosolic localization. On the other hand, mutation of the critical serine residues to aspartate (phosphomimetic mutant), thus mimicking constitutive phosphorylation, leads to predominantly cytosolic localization of the mutant protein, in agreement with previous data that the phosphorylated form of the protein is cytoplasmic. But this mutant protein does not actually possess two phosphorylated serine residues at amino acid positions 174 and 175, but merely mimics phosphorylation. So although activated calcineurin

can weakly bind to the intact LQVP site on this mutant protein, it presumably cannot "dephosphorylate" this "constitutive phosphorylation-mimicking" mutant eIF6, and hence, cannot promote its nuclear import. Thus, the phosphomimetic mutant protein also remains primarily cytosolic in presence of activated calcineurin. Taken together, these data further emphasize the significance of eIF6 phosphorylation and dephosphorylation. It also emphasizes the role of calcineurin (and, in principle, that of intracellular Ca^{2+} level) in regulating nuclear import of eIF6, essential for continued pre-60S biogenesis.

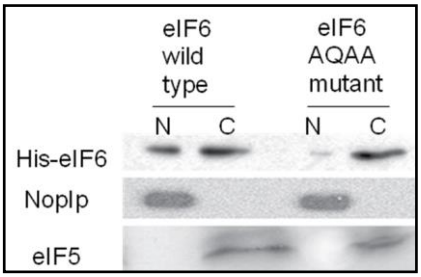

Figure 3. eIF6-AQAA mutant is cytosolic

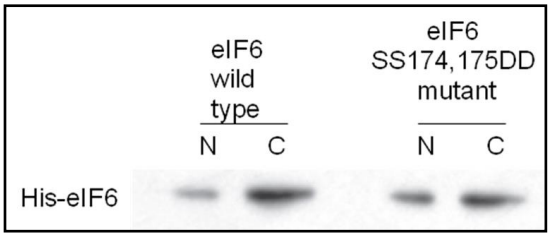

Figure 4. eIF6-SS174,175DD mutant is predominantly cytosolic

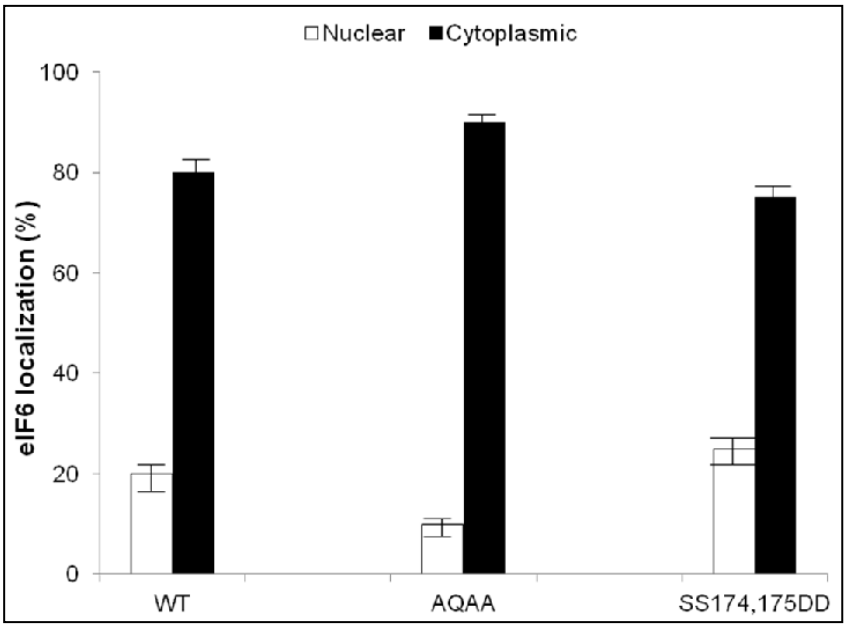

Figure 5. Sub-cellular distribution of wild type and mutant eIF6

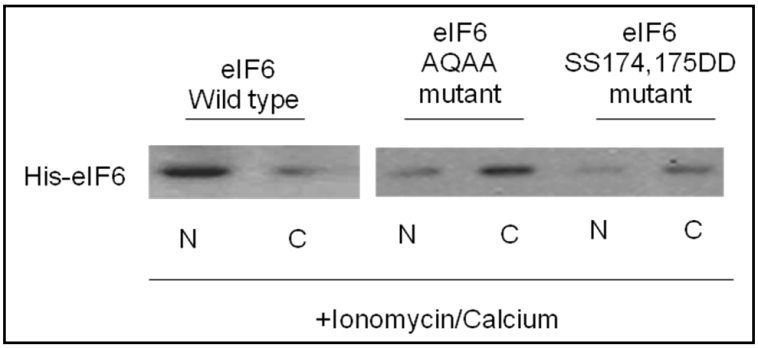

Figure 6. Activated calcineurin failed to promote nuclear transport of mutant eIF6

Thus, eIF6 release and recycling seem to be due to concerted actions of SBDS protein and eIF6 phosphorylation-dephosphorylation events. Hence, it becomes necessary to understand the proper sequence of events taking place in the cytosol, namely whether eIF6 release by SBDS and EFL1 is a pre-requisite for its dephosphorylation and subsequent nuclear translocation or whether dephosphorylation precedes eIF6 release from pre-60S particles. To investigate this problem, the following experiment was carried out.

3.2. Ribosome –unbound eIF6 recycles back to nucleus from cytoplasm — He La cells (treated or untreaed with Ca^{++} , as indicated) were fractionated into nuclear and cytosolic fractions. Each fraction was centrifuged at 2,00000g for 2 hrs in cold to crudely pellet down all ribosomes and ribosomal subunits. Equivalent amount of supernatant and resuspended ribosomal pellet from both fractions were subjected to SDS-PAGE and Western blot analysis using anti-eIF6 antibody. It was found that in the nucleus, endogenous eIF6 is almost completely bound to ribosomal particles/pellet. On the other hand, major amount of endogenous eIF6 in the cytoplasm is free of ribosomes and found in the post-ribosomal supernatant, although a low but significant amount of cytoplasmic eIF6 is reproducibly found to be bound to ribosomal pellet as well. Also, in agreement with previous data, total amount of eIF6 in the cytoplasm is more than that in the nucleus of mammalian cells. On Ca^{++} treatment, the nucleus gets enriched in eIF6 due to nuclear import of the protein, in agreement with previous data. Also, the eIF6 that is recycled from cytoplasm to nucleus on Ca^{++} activation, appears mostly to be the ribosome-unbound protein, while the ribosomal pellet in the cytoplasm also looks slightly depleted of eIF6 than in untreated cells. L3 is used as a marker for 60S particles in cytoplasm (Figure 7). What probably happens is that as ribosome-unbound eIF6 begins to enter the nucleus, more and more ribosome-bound eIF6 is released for continued recycling under calcium-activated condition. This result suggests that eIF6 is probably first released from pre-60S by SBDS and EFL1 and then the unbound eIF6 acts as a substrate for activated calcineurin which promotes its nuclear translocation. Clearly, further experiments are needed to confirm this hypothesis.

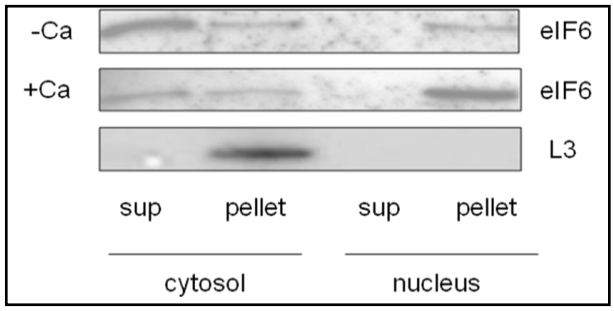

Figure 7. Ribosome –unbound eIF6 recycles back to nucleus from cytoplasm

4. Conclusion

From previous findings a model has been proposed depicting the functional pathway of eIF6 in 60S ribosome biogenesis and it is in agreement with the current results reported here [7] (Figure 8). In the nucleolus, eIF6 associates with the pre-60S particles and is essential for pre-60S ribosome assembly and pre-rRNA processing. eIF6 remains associated with the pre-60S particles during pre-60S maturation in the nucleoplasm as well as during the nuclear export of the pre- 60S particles. Nuclear export of eIF6 bound to the pre-60S particles requires phosphorylation of eIF6 at Ser-174 and Ser-175 by the nuclear isoform of CK1. In the cytoplasm, during the final maturation process, two cytoplasmic proteins SBDS/Sdo1 and EFL1/Efl1p interact with the pre-60S particles and catalyze the release of eIF6 coupled to GTP hydrolysis by EFL1. The released eIF6 that is presumably in the phosphorylated form then interacts with Ca^{2+}/calmodulin-regulated protein phosphatase calcineurin and the dephosphorylated form of eIF6 (either by itself or probably by interaction with another as yet unidentified protein factor X containing the NLS signal), is imported to the nucleolus to participate in another round of 60S ribosome biogenesis.

Inhibition of this pathway leads to defects in both new 60S ribosome biogenesis and existing pre-60S maturation resulting in associated pathophysiology in the Shwachman-Diamond syndrome patients. These findings identify SBDS, EFL1 and eIF6 proteins as critical, regulatory and inter-dependent components of a conserved pathway that is required for translational activation of ribosomes and link defective 60S subunit

biogenesis and maturation to an inherited bone marrow failure syndrome associated with leukemia predisposition. Identification of all the components of this Sdo1-Efl1-Tif6 pathway in yeast (and also in mammalian cells) will be of great interest for understanding the mechanisms underlying late cytoplasmic ribosome maturation. It may also focus the search for new genes implicated in bone marrow failure and leukemia. This pathway also provides a platform where eIF6 phosphorylation-dephosphorylation regulators (including calcium signaling) can be assayed for their therapeutic potential in SBDS patients.

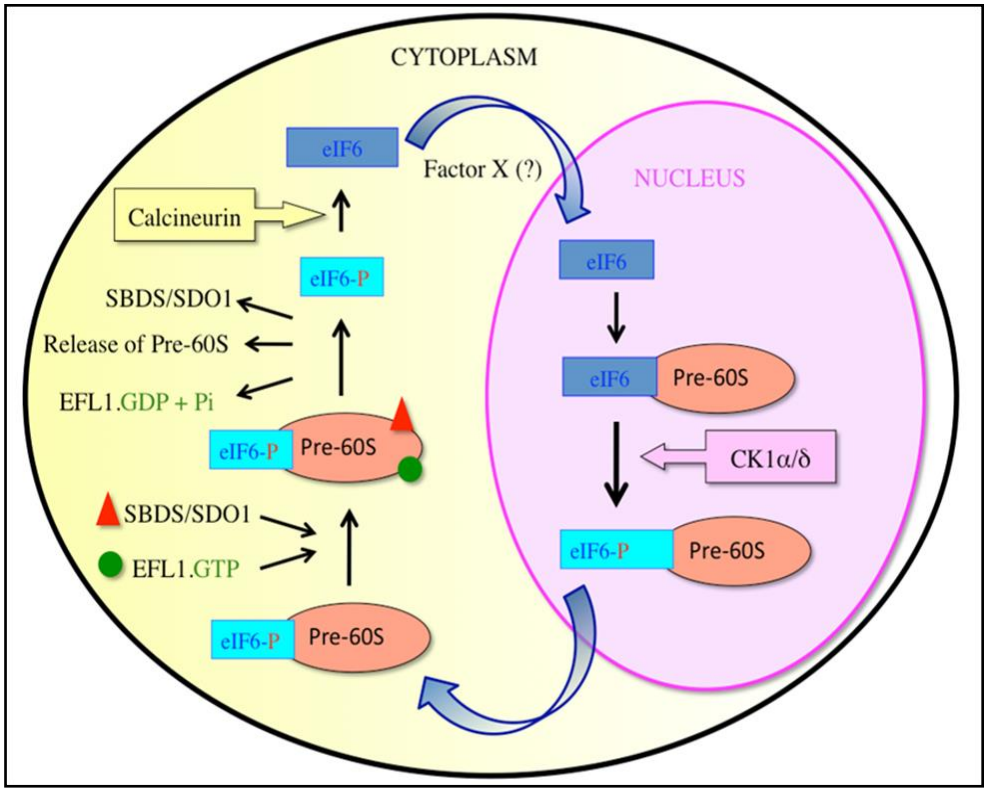

Figure 8. Schematic representation of the functional pathway of eIF6 in 60S ribosome biogenesis [7]

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