Ribosome biogenesis factors working with a nuclear envelope SUN domain protein
New players in the solar system

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The nucleolus, the most prominent structure observed in the nucleus, is often called a “ribosome factory.” Cells spend an enormous fraction of their resources to achieve the mass-production of ribosomes required by rapid growth. On the other hand, ribosome biogenesis is also tightly controlled, and must be coordinated with other cellular processes. Ribosomal proteins and ribosome biogenesis factors are attractive candidates for this link. Recent results suggest that some of them have functions beyond ribosome biogenesis. Here we review recent progress on ribosome biogenesis factors, Ebp2 and Rrs1, in yeast Saccharomyces cerevisiae. In this organism, Ebp2 and Rrs1 are found in the nucleolus and at the nuclear periphery. At the nuclear envelope, these proteins interact with a membrane-spanning SUN domain protein, Mps3, and play roles in telomere clustering and silencing along with the silent information regulator Sir4. We propose that a protein complex consisting Ebp2, Rrs1 and Mps3 is involved in a wide range of activities at the nuclear envelope.

Ribosome Biogenesis and Surveillance

All life on earth depends on the ribosome for protein synthesis. The ribosome is a complicated ribonucleoprotein nanomachine and its biogenesis in eukaryotic cells is extraordinarily complex, requiring four different ribosomal RNAs (rRNAs; 25S/28S, 18S, 5.8S and 5S rRNA) and about 80 ribosomal proteins as the constituent materials. Another 200 non-ribosomal proteins and RNA molecules serve as processors, modifiers and assemblers (reviewed in refs. 1 and 2). The multistep maturation process occurs sequentially in the nucleolus, the nucleoplasm and the cytoplasm, where pre-ribosome particles are converted to functional 40S and 60S ribosomal subunits (reviewed in refs. 3 and 4). In rapidly proliferating yeast cells, rRNA accounts for approximately 80% of total cellular RNA, and 50% of RNA polymerase II transcription is devoted to ribosomal protein genes (reviewed in ref. 5). Thus, the process of ribosome biogenesis is both high-throughput and high-precision, requiring quality control and feedback mechanisms to adjust itself to demand.

It has been suggested that many ribosomal proteins have extra-ribosomal functions, which help them serve as sentinels for ribosome biogenesis (reviewed in ref. 6). One of the best known examples is the function that monitors ribosome imbalance in mammals. Ribosomal proteins like L11, L5, L23 and S7, interact with M/HDM2 (the mouse/human ortholog of E3 ligase responsible for the ubiquitination of p53). Under conditions that block transcription of rRNA or stall ribosome assembly, increased binding of these ribosomal proteins to M/HDM2 leads to p53 accumulation and cell-cycle arrest. L11 can also bind and inhibit c-Myc, which functions as an activator of all three RNA polymerases. In addition, several factors that are involved in ribosome maturation connect defects in ribosome biogenesis with...
Ribosome Biogenesis Factors

We have previously shown that Ebp2, yeast homolog of human Epstein-Barr virus nuclear antigen 1-binding protein 2, binds to Rrs1, and both are required for the maturation of 25S rRNA and the production of the 60S ribosomal subunit in yeast.\(^{1-3}\) These processes mainly occur in the nucleolus. It was demonstrated that Rrs1 and Rpf2 form a subcomplex with L5, L11 and 5S rRNA for the coordinated recruitment of these components to pre-60S ribosomal subunits.\(^{1-3}\) While Ebp2 and Rrs1 bind to each other, they may not always work together in ribosome biogenesis. The interaction between Rrs1 and Ebp2 is weaker than that between Rrp41 and Ebp2.\(^{4,5}\) Moreover, double staining of Ebp2 and Rrs1, suggesting that targeting of the perinuclear localization of both Ebp2 and Rrs1 positioning at the nuclear periphery.\(^{6}\)

P53 accumulation and subsequent cell-cycle arrest (reviewed in ref. 6). Finally, it was shown that bona fide ribosome biogenesis factors can have functions in various other cellular processes in yeast, such as RNA replication and cell polarity (reviewed in ref. 8). Nonetheless, how ribosome synthesis itself is coordinated with other cellular mechanisms remains unclear.

Ebp2 and Rrs1 are Bona Fide Ribosome Biogenesis Factors

In several temperature-sensitive ebp2 and rrs1 mutants, each of which had defects in 25S rRNA maturation and in 60S ribosomal subunit production,\(^{4,5}\) the nuclear envelope became distorted into a non-spherical shape at the restrictive temperature. This was observed more rapidly than the defect in biogenesis of the 60S ribosomal subunit after the temperature shift-up. Fluorescence microscopy has shown that Ebp2 and Rrs1 are localized along the inner nuclear envelope at non-pore sites, as well as in the crescent-shaped nucleolus throughout the cell cycle (Figs. 1A and 2, left). Moreover, the perinuclear signals are lost in the ebp2 and rrs1 mutants when the cells are placed at the restrictive temperature (Fig. 1B). While Ebp2 and Rrs1 do not always work together in ribosome biogenesis (our unpublished data). Consistently, an ebp2 mutation causes the accumulation of different species of rRNA precursors from those that accumulate upon Rrs1 depletion.\(^{13,14}\) Moreover, double staining of Ebp2 and Rrs1 showed that Rrs1 generally had a more peripheral distribution than Ebp2 in the nucleolus, reflecting their slightly different stages of function in ribosome biogenesis.\(^{15}\)

Perinuclear Localizations of Ebp2 and Rrs1 Mediated by a SUN Domain Protein Mps3

We showed that both Ebp2 and Rrs1 interact with the C-terminal domain of Sir4 (Fig. 1A), and conditional inactivation of either ebp2 or rrs1 interferes with both the clustering and silencing of yeast telomeres, while telomere tethering to the nuclear periphery remains intact (Fig. 1B). Importantly, expression of an Ebp2-Mps3 fusion protein which is spatially limited to the nuclear periphery suppresses the defect of the ebp2 mutant for telomere clustering,
but not its defects in growth or ribosome biogenesis. Thus, we conclude that the defects in telomere clustering can be dissociated from the defects in ribosome biogenesis for Ebp2 and Rrs1, even though both proteins are clearly implicated in both events. In addition to our findings, several recent papers addressed the clustering mechanism and the relation with tethering and silencing of telomeres. A screen using GFP-Rap1 to mark telomeric repeat sequences has identified various mutants defective in telomere clustering and/or the localization to the nuclear periphery.\textsuperscript{28} Several mutants led to a similar telomeric clustering phenotype as ebp2 and rrs1, i.e., loss of clustering but not tethering. Intriguingly, a recent paper showed that overexpressing Sir3 leads to the hyperclustering of telomeres and silencing factors in foci localized away from the nuclear periphery, and could separate Sir3’s role in telomere clustering from its role in silencing.\textsuperscript{29} These results reinforce our own which identify distinct pathways for the clustering and anchoring of telomeres in yeast.

Figure 1. Roles of Ebp2 and Rrs1 at the nuclear periphery and summary of phenotypes in each condition. (A) Ebp2 and Rrs1 localize to the perinuclear region through Mps3, and thereby serve a structural function in the nucleus. Ebp2, Rrs1 and Mps3 are associated with the silent information factor Sir4, and function in telomere clustering in S phase cells and silencing. (B) Mutant forms of Ebp2 and Rrs1 lose association with the nuclear periphery at the restrictive temperature, thereby leading to the loss of the integrity of the nuclear envelope. Telomeres remained localized to the nuclear envelope but lost the cluster formation in S phase cells and the silent domain organization. (C) The ectopic expression of Mps3-N’ leads Ebp2 and Rrs1 away from the nuclear periphery, thereby compromising their telomeric roles and the structural role at the nuclear periphery. Mps3-N’ also titrate Est1, a component of telomerase which is required for Ku-dependent telomere tethering in S phase, from the perinuclear binding sites.\textsuperscript{21} (D) The lack of Sir4 does not affect the interaction of Ebp2 and Rrs1 with Mps3 and the perinuclear localization of Ebp2. Telomeres are detached from the nuclear periphery due to the absence of Sir4. The perinuclear Ebp2 and Rrs1 is sufficient for providing the structural role independently of telomeres. ONM, outer nuclear membrane; INM, inner nuclear membrane; M-N’, Mps3-N’; E/R, Ebp2 and Rrs1; NE, nuclear envelope; Tel., telomere; gray ellipse, unknown proteins constituting the perinuclear protein network; white circle, telomeric complex of Yku70/80 and telomerase.

Warner and McIntosh have proposed three criteria for the ribosomal proteins
which are truly acting in an extraribosomal capacity. If we expand the application range to ribosome biogenesis factors, Ebp2 and Rrs1 meet these criteria, as follows: (1) both Ebp2 and Rrs1 bind specifically to a component of the cell, Mps3; which is not involved in ribosome biogenesis; (2) the interaction has a physiological effect on a cell; and (3) the function occurs independently of the ribosome biogenesis. The perinuclear Ebp2 and Rrs1 have specific functions which are separable from ribosome biogenesis roles of nucleolar Ebp2 and Rrs1. This finding raises the next question of whether the peripheral Ebp2 and Rrs1 function exclusively in telomere clustering and silencing.

Instead, we propose that Ebp2 and Rrs1 act as a scaffolding or coordinator for various chromatin reactions, much like their ligand, Mps3. Indeed, Ebp2 and Rrs1 bind to the nucleoplasmic region of Mps3 independently of Sir4, and in addition, that Ebp2 is localized at the nuclear periphery in sir4Δ cells (Fig. 1D). These results support the idea that perinuclear Ebp2 and Rrs1 can act with Mps3 independently of the presence of telomeres at the nuclear periphery.

The nuclear envelope is one of the key structures that regulates the positioning of various chromosomal loci in yeast. Recent studies showed that the damaged DNA is recruited to the nuclear envelope and that the relocation is important for the DNA double strand break (DSB) processing. Both nuclear pores and the SUN domain protein Mps3 are thought to provide the landing sites to the persistent DSBs, whereas the latter also provides the site to telomeres. It is expected that Mps3 works as a control hub for distinguishing and differently managing telomeres and DSBs, both of which contain chromosome ends and share many regulatory factors, such as the Ku complex, MRX (MRN) and Tel1 (ATM). Although many factors have been identified as targeting or regulating factors for repair or maintenance of telomeric ends, we know only a few proteins implicated on the side of the nuclear envelope. Intriguingly, ebp2 and rrs1 mutants show hypersensitivity to DNA damaging agents, such as hydroxyurea and methyl methane-sulfonate, in some cases more severely than mps3Δ145 (Horigome et al., unpublished data). The result raises the possibility that the perinuclear complement of Ebp2 and Rrs1 contribute the function of Mps3 not only in telomere maintenance but in DNA repair.

Ebp2 is Modified with the Small Ubiquitin-Related Modifier (SUMO)

Although the telomere organization in the nucleus is tightly regulated in cell-cycle dependent manner, the nuclear peripheral localization of Ebp2 and Rrs1 persists throughout the cell-cycle, suggesting that perinuclear Ebp2 and Rrs1 may be controlled by post-translational modification. Indeed, Ebp2 is sumoylated and interacts with the PIAS-like SUMO E3 ligase Siz2 as well as with two SUMO-related proteins, Uls1/Ris1 and Wss1. Although it was demonstrated that global perturbation of SUMO conjugation and deconjugation, impairs both the maturation and export of ribosomal subunits from the nucleus, the sumoylation of Ebp2 may have other functions. First of all, sumoylation-defective ebp2 mutants show the same growth rate as wild-type cells, and secondly, the sumoylation-defective forms of Ebp2 retained positive interactions with ribosome assembly factors such as Nop12 and Loc1. In contrast to this, sumoylation-defective ebp2 mutants show the same growth rate as wild-type cells, and secondly, the sumoylation-defective forms of Ebp2 retained positive interactions with ribosome assembly factors such as Nop12 and Loc1. In contrast to this, sumoylation-defective ebp2 mutants show the same growth rate as wild-type cells, and secondly, the sumoylation-defective forms of Ebp2 completely lost their binding to Siz2, Uls1/Ris1 and Wss1. Recent studies have demonstrated the importance of these SUMO-recognizing partners of Ebp2 in telomere maintenance and DNA damage repair at the nuclear periphery. Ferreira et al. reported that Sir2 sumoylates both Yku70/80 and Sir4 in vivo and is required for telomere anchoring—at least in part due to the sumoylation of the yeast Ku complex. Wss1 plays a role in removing SUMO and
ubiquitin from proteins undergoing pro-
teasomal degradation and genetically inter-
acts with the Sls5-Snb8 SUMO-targeted 
ubiquitin ligase complex,72,73 which is 
implicated in the DSB relocation to the 
periphery and the repair.21 Thus, the loss 
of interaction between Ebp2 and Siz2, 
Uth1 and Wss1, upon loss of sumoylation implicates Ebp2 in the pathway that 
positions telomeres in S phase and targets 
DSB to either nuclear pores or Mps3.

Ebp2, Rrs1 and SUN Proteins 
in Other Eukaryotes

Ebp2 and Rrs1 are highly conserved 
proteins and ribosome biogenesis is a 
conserved process within eukaryotic 
organisms. Human EBP2 and RRS1 are 
localized in the nucleus, suggesting that 
their roles in ribosome biogenesis are 
conserved.26,39 It has been reported the 
nucleolar localization of human EBP2 is 
dependent on nucleostemin, which is a 
nucleolar protein preferentially expressed 
dependent on nucleostemin, which is a 
nucleolar protein preferentially expressed 
dependent on nucleostemin, which is a 
nucleolar protein preferentially expressed 

human EBP2 might connect ribosome 
biogenesis with cell proliferation. 

While perinuclear localizations of EBP2 and 
RRS1 have not been reported to date 
except for budding yeast, we predict that 
the non-ribosomal functions of these 
proteins (which take place at the nuclear 
envelope in yeasts) are conserved in 
mammals and other species. As mentioned 
above, the yeast two-hybrid screen for 
S. pombe SUN protein Sad1 identified 
only two nucleolar proteins, the S. pombe 
Ebp2 and Rss1.17 Therefore, it seems that 
the interaction of perinuclear Ebp2 and 
Rss1 with the unique SUN domain 
protein is conserved in fission yeast, 
although the role of this interaction has 
not been studied in S. pombe. Human 
EBP2 and RRS1 are found associated with 
condensed chromosomes,28,39 suggesting that human 
EBP2, RRS1 and SUN proteins also 
colocalize at least transiently.

A study on human EBP2 offers valuable 
insight for the functional modality of the 
protein in ribosome biogenesis.33 Hirano 
et al. prepared a ribonucleo-protein-
containing nuclear matrix fraction of 
HeLa cells which is biochemically defined as 
an insoluble structure as to detergent-
and high salt-extraction followed by 
removal of chromatin (reviewed in ref. 46), and identified 83 proteins in the 
fraction by the peptide mass fingerprint 
(PMF). Coupled with many structural and 
RNA binding proteins (68 of 83 proteins), 
EBP2 and BXDC1 which is the human 
homolog of yeast Rpl2 were identified. 
Fluorescence microscopy and fluorescence 
recovery after photobleaching (FRAP) 
analyses showed that EBP2 and BXDC1 are 
more tightly associated with the 
nucleolus in an RNA-dependent manner, 
at least than nucleolar proteins B23, 
nucleolin and fibrillarin. Thus, it was 
proposed that EBP2 and BXDC1 are 
"dynamic scaffold" proteins which serve 
as a core structure for ribosome biogenesis.

While nucleoli appear to be detached 
from the nuclear envelope in higher 
eukaryotes, several studies revealed the 
existence of the structural links between 
the nuclear envelope and nucleoli. 
Transmission electron microscopy (TEM) 
analysis of the ribonucleo-protein-contain-
ning nuclear matrix fraction of HeLa cells, 
which includes EBP2 and BXDC1, revealed the existence of RNAse-sensitive 
fibers that extend throughout the nucleus, 
forming continuous association between 
nucleoli and the nuclear lamina.31 Given 
that the shape of the extracted nucleus 
was sensitive to RNAse, it was long 
proposed that RNA would be an important 
structural component of the nucleus. 
Alternative links may also exist, in the 
gerninal vesicle of Xenopus oocytes 
bundles of actin extend from nucleoli to 
the nuclear envelope.48,49 and lamin B1 
appears to form an external scaffold for 
nucleoli.50 In the case of budding yeast, 
the nucleolus forms a crescent-shaped 
structure that makes extensive contact 
with the nuclear envelope. It has been 
shown that a protein network including 
the inner nuclear membrane protein Sec1 
(also called Heh1) and Siz2 stabilizes the 
highly repetitive ribosomal DNA 
sequences.51 Sec1 also functions in 
subtelomeric gene expression and is 
embedded functionally in a network of 
factors, which participate in transcrip-
tion export (TREX) complex-dependent 
mRNA export through the nuclear pore 
complexes.52 We speculate that yeast 
perinuclear- and nucleolar-Ebp2/Rrs1 are 
conducive to a dynamic network between 
RNAs and proteins such as Mps3 con-
tributing to subnuclear structure.

Concluding Remarks

Ebp2 and Rrs1 are conserved nuclear 
proteins that act at the nuclear membrane 
with the SUN protein to maintain a 
spherical nuclear structure and probably 
to control chromatin repair and protec-
tion. Yet, we are only at the beginning of 
uncovering its function in processes such 
as telomere maintenance. Since the SUN 
proteins impact a wide range of activities 
at the nuclear envelope directly or 
indirectly, the understanding of the 
relationship between SUN proteins and 
Ebp2/Rrs1 is likely to shed light on the 
true nature of genome organization. 
Further studies will examine whether the 
nucleolar- and the perinuclear-Ebp2 and 
Rrs1 functions are related.

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