Seminars in Cell and Developmental Biology 159–160 (2024) 1–9

Contents lists available at ScienceDirect

Seminars in Cell and Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

rDNA transcription, replication and stability in *Saccharomyces cerevisiae*

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ARTICLE INFO

Keywords:
RDNA
Saccharomyces cerevisiae
Genome stability
Transcriptional silencing

ABSTRACT

The ribosomal DNA locus (rDNA) is central for the functioning of cells because it encodes ribosomal RNAs, key components of ribosomes, and also because of its links to fundamental metabolic processes, with significant impact on genome integrity and aging. The repetitive nature of the rDNA gene units forces the locus to maintain sequence homogeneity through recombination processes that are closely related to genomic stability. The co-presence of basic DNA transactions, such as replication, transcription by major RNA polymerases, and recombination, in a defined and restricted area of the genome is of particular relevance as it affects the stability of the rDNA locus by both direct and indirect mechanisms. This condition is well exemplified by the rDNA of *Saccharomyces cerevisiae*. In this review we summarize essential knowledge on how the complexity and overlap of different processes contribute to the control of rDNA and genomic stability in this model organism.

1. Introduction

The set of DNA sequences transcribed into major ribosomal RNAs and their intergenic spacers, collectively indicated as ribosomal DNA (rDNA), constitutes one of the most interesting chromosomal regions of the entire eukaryotic genome. In this review we will address major aspects of rDNA stability in *Saccharomyces cerevisiae*. This region displays a number of basic features that have been maintained throughout evolution, although there are also relevant differences in its organization as compared to other species [1].

As in other eukaryotes, also in budding yeast rDNA is confined in the nucleolus. Over the years, strong experimental evidence has accumulated on the association of the nucleolus with genomic instability and aging in both yeast and mammals [2]. The *S. cerevisiae* rDNA locus consists of a single cluster of repeated gene units. The cluster represents about 10% of the genome and is hosted on the right arm of chromosome XII [3] where it constitutes about 60% the total size of the chromosome [4]. Each rDNA unit spans about 9.2 kb and encodes the 37 S RNA, transcribed by RNA polymerase I and then processed into 18 S, 28 S and 5.8 S ribosomal RNAs. The main gene unit is tandemly repeated 150–200 times, depending on the yeast strain. In other species the repetitiveness of the rDNA units is higher, e.g., in humans there are about 300–600 rDNA gene repeats which are organized into more clusters, each residing on different chromosomes and giving rise to different nucleoli [5].

In *S. cerevisiae* the 37 S rDNA units are separated by an intergenic spacer (IGS), which in turn is divided into two parts by the presence of the 5 S rRNA gene, transcribed by RNA polymerase III [6]. Two IGSs are thus formed, IGS1 and IGS2 ([7]; see also Fig. 1). The latter contains, in addition to the RNA polymerase I promoter for the 37 S RNA, an Autonomous Replicating Sequence (rARS; circle in Fig. 1) where replication of an rDNA repeat unit can initiate [6]. Five nucleosomes occupy stable positions around the rARS region [8] and their acetylation level depends on Sir2 activity [9]. On average, one rARS out of three is engaged as active replication origin [10]. The IGS2 region hosts a cryptic promoter, transcribed at low efficiency by RNA polymerase II (thin arrows in Fig. 1), from which transcription of noncoding RNAs starts in the opposite direction of RNA polymerase I transcription [11,12]. In IGS1 there is a replication fork barrier (RFB), a 100 bp sequence located at the 3' end of the 37 S RNA gene, partially overlapping the 37 S RNA terminator and responsible for blocking the replication fork moving in the opposite direction to that of 37 S RNA synthesis [13]. The blocking mechanism relies on the binding of a specific factor, Fob1 [14,15] which also contributes to recombination. These processes occur locally at the RFB [14] due to the ability of Fob1 to form dimers able to connect different units [16]. In analogy to IGS2, also IGS1 contains a bidirectional cryptic promoter from which noncoding RNAs are transcribed by RNA polymerase II [11,12].

In addition to the transcriptional processes involving the three major RNA polymerases, and to the replicative events driven by the DNA

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https://doi.org/10.1016/j.semcdb.2024.01.004
Received 1 May 2023; Received in revised form 20 December 2023; Accepted 10 January 2024

polymerase starting from IGS2/rARS region, another important DNA transaction occurring at the rDNA locus is the recombination among homologous copies. This process keeps rDNA units homogeneous within a single cell [17]. Replication blocking and the presence of cohesins contribute to keep recombination at a low level [18], allowing to achieve the right balance between homogeneity of repeated sequences and genomic stability [19]. The fine regulation between transcription, replication, and recombination is ensured by the Sir2 protein through its ability to deacetylate histones (in particular, it is a class III histone deacetylase) and sense the cellular metabolic state (its activity is NAD^+ dependent) by a refined epigenetic mechanism that maintains transcription silencing [20,21], replication control [22] and locus stability [23].

In essence, the approximately 2.4-kb region encompassing IGS1 and IGS2 is a crowded molecular spot promoting fundamental processes such as replication, transcription and recombination via a complex and intricate series of interactions among DNA, RNA and proteins. The highly articulated dynamics of the processes occurring in this region implies that rDNA functionality affects major cellular aspects, such as overall genome stability and aging. In a study on the impact of about 4800 genes on genome stability in *S. cerevisiae*, Kobayashi and coworkers [4] reported that about 700 genes contribute to maintaining acceptable rDNA stability. This represents more than 10% of the total yeast genes, highlighting the demanding task the cell faces to ensure a correct and effective organization and function of the rDNA locus. Genomic instability processes are known to have a strong impact on cellular and organismal aging [24]. In yeast genomic instability and replicative aging have been well described [25,26]. Transcriptional defects in RNA Polymerase I have long been known to act on the integrity of the nucleolus [27], indicating how the global and three-dimensional organization of the rDNA locus is also genetically controlled to ensure its multifunctional activity. Several reviews approaching the topic of rDNA stability in yeast from different angles have been published previously [28-30].

Already twenty years ago, Moss and Stefanovsky [31] emphasized rDNA as being "at the center of life", stressing the importance of the locus and how its function could have far-reaching effects, well beyond ribosome production and protein synthesis. In more recent years several reviews have discussed many of the influences that the rDNA apparatus has on a number of cellular processes, attributing to ribosomal loci also the role of environmental sensors for mutagenic substances, calorie restriction, or replicative stresses [32–34]. In the following we provide an overview of key aspects and the major players (e. g. Sir2) that affect the overall stability of the rDNA locus in yeast in terms of interconnections among replication, transcription, recombination and genomic organization.

1.1. Control of yeast rDNA stability by major DNA transactions

It is well known that the dynamics of ribosomal DNA can be significantly affected by environmental changes [35,36]. Genome stability at the rDNA locus is crucial for cell survival and is ensured by strict interactions among replication, recombination, and transcription of rDNA units. Essential features of these interconnections are illustrated in the following sections.

1.1.1. Replication and Recombination

rARS elements are located within each rDNA unit [6] but only a subset of them is involved at the same time in a replication event [10]. In *S. cerevisiae* the timing of rARS firing is under strict control of histone deacetylases (HDACs). It is well known that the Sir2 HDAC is responsible for the specific timing of early firing rARS [22,36], whereas the Rpd3 HDAC controls late firing rARS in a Sir2 independent manner [37]. *SIR2* deletion increases the number of functional rARS [22]. When an ARS is fired, the replication bubble grows bidirectionally; then, while the rightward replication fork proceeds through the 37 S RNA gene and eventually merges with the adjacent replication bubble, the leftward fork reaches the 37 S RNA terminator and is halted at the RFB (middle section of panel A in Fig. 2). In addition, the efficiency of rDNA replication affects repeat expansion, and may also affect nucleolar fragmentation via ERC production [23] as discussed below in more detail.

The replication fork block at the RFB depends on the binding of a specific factor, a Fob1 dimer which interacts with the DNA via its zinc finger domain. In particular, it has been shown that a nucleosome-like arrangement forms, with the RFB DNA wrapping around the Fob1 dimer [38]. The Rrm3 helicase, which is part of the active replication machinery, is able to temporarily remove the RFB-bound Fob1, thus releasing the replication block. This ability is counteracted by the action of two factors: the Tof1 and Csm3 proteins, which protect Fob1 from being displaced, thus promoting the maintenance of the replication fork barrier. Two-dimensional gel electrophoresis assays indicate that yeast mutants *fob1*Δ, *tof1*Δ, or *csm3*Δ have the same phenotype, i.e. the unblocking of the replication fork at the RFB region ([39] and Fig. 2, panel C). Similarly to Tof1, also Tof2 is able to enhance Fob1 association with the RFB region, modulating recombination in the rDNA region [40].

Repeated sequences in the genome are known to be an effective recombination substrate and recombination events are crucial in DNA repair and in determining genetic variability. The stalling of the replication fork at the RFB, entailing frequent DNA breaks, is a major-

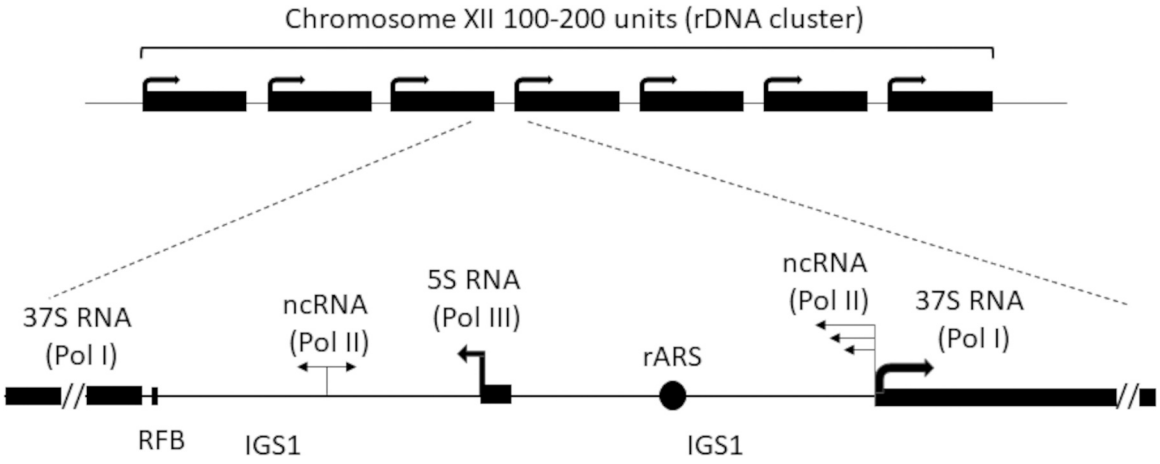

Fig. 1. *S. cerevisiae* rDNA organization along chromosome XII. Horizontal black arrows indicate products of RNA Polymerase I, II, or III transcription (thick arrows indicate 5 S and 37 S rRNAs; thin arrows represent ncRNAs potentially transcribed by RNA Polymerase II). IGS, Intergenic Spacer.

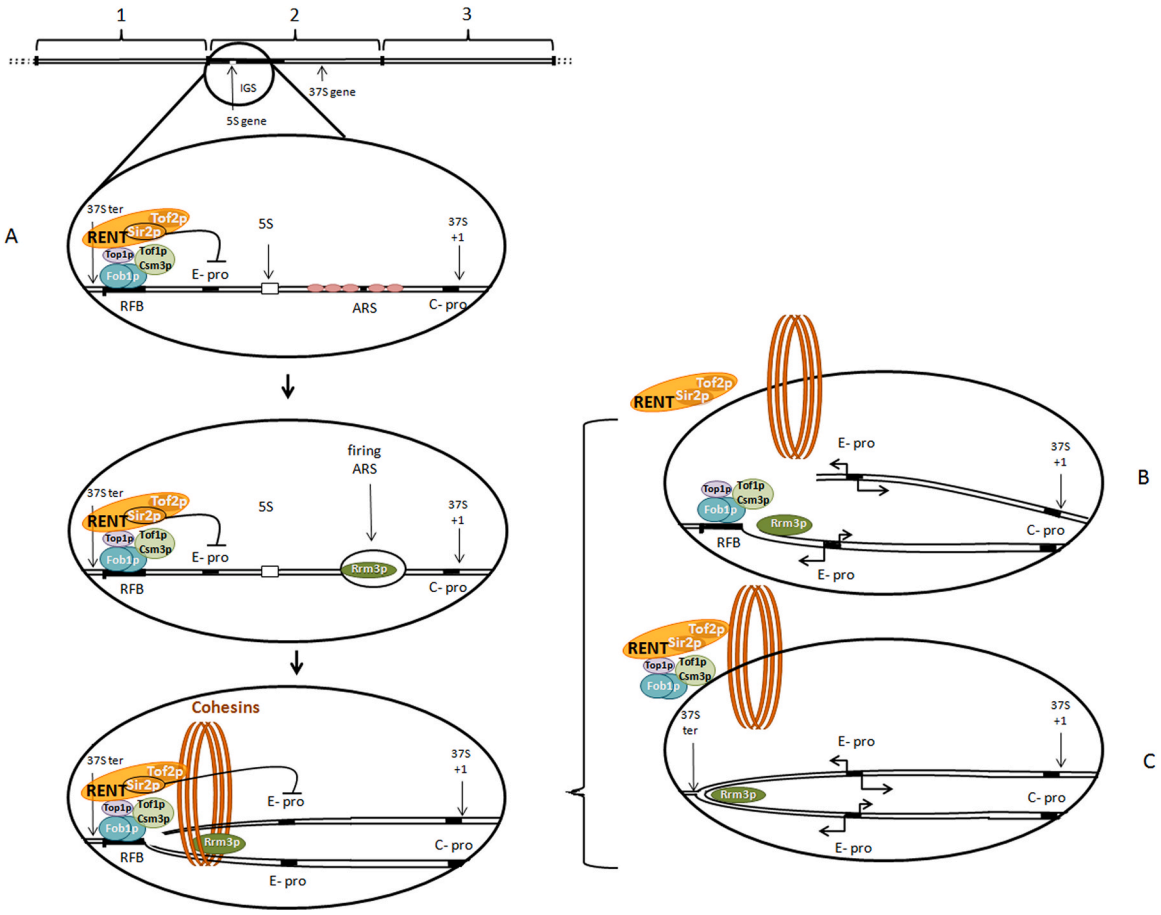

Fig. 2. Major DNA-Protein interactions in the IGS1 region of *S. cerevisiae* rDNA. A. DNA-protein interactions in a WT cell. Upper section: Sir2 maintains E- pro repression; pink ovals indicate nucleosomes. Middle section: Firing of the ARS. Lower section: The leftward replication fork stops when it arrives at RFB with consequent DNA breaks. Cohesins maintain the correct position of sister chromatids. B. If the RENT complex is absent, the leftward replication fork stops at the RFB, with consequent DNA breaks. E-pro de-repression causes cohesin displacement. Sister chromatids are not aligned. Recombination events will cause ERCs excision and/or modification of copy number, with consequent nucleolus fragmentation and locus instability. C. If Fob1 cannot bind RFB, Replication Fork Block does not occur and consequently the leftward replication fork proceeds to replicate the entire unit. Collision of replicative and transcriptional machinery occurs. Loss of Fob1 causes the absence of Top1, hampering the resolution of topological stress. Horizontal black arrows in B and C represent the ncRNAs transcribed from E- pro.

recombination-promoting event [41], according to a mechanism whereby the RFB/Fob1-induced replication fork block leads to single strand breaks (SSB) along the DNA. The DNA repair system then converts the SSB into double strand breaks (DSB) which in turn activate the homologous recombination machinery [42]. In the case of equal recombination, an exchange between two homologous rDNA units takes place and the DSB is repaired. If an unequal recombination event between non corresponding rDNA units occurs, some rDNA copies are replicated two times and, after the resolution of the recombination intermediates, the total number of units increases in a chromatid (amplification of unit number) and decreases in the sister chromatid (contraction). Hence, both increase and reduction of rDNA copy number could originate from unequal recombination between sister chromatids [43].

Especially in *S. cerevisiae* the stability of rDNA is a tightly controlled feature [44–46], in line with its relevance to aging [28], and in association with yeast evolution. After contraction or amplification, the system is able to return to the normal level [47]. This implies that the rate of recombination in rDNA is strictly regulated as compared to the whole genome [48]. In yeast a correlation between rDNA copy number and DNA replication stress have been demonstrated [49]: cells with a low number of rDNA repeats show less growth defects as compared to cells with more rDNA repeats.

When a recombination event occurs inside the same chromatid, single rDNA units can be excised from the chromosome as Extra chromosomal rDNA Circles (ERCs). Interestingly, ERCs accumulation is not limited to yeast but has been observed also in other organisms, e.g. amphibians, flies, and protozoa [50,51]. In yeasts there are generally only few episomal rDNA copies per cell and excision or reintegration of ERCs are crucial for modulating copy number [52] and are dramatically influenced by protein-protein and/or protein-DNA interactions at the ribosomal locus [53]. Cell division in *S. cerevisiae* takes place asymmetrically, with a mother cell producing a bud. This process does not occur indefinitely but rather takes place for a discrete number of times. This determines the replicative life span, which is essentially the number of (budding) divisions that a mother cell can undergo during its lifetime [54]. ERCs are stochastically produced during intramolecular homologous recombination at the rDNA locus and are asymmetrically inherited by daughter cells. The ERCs pool is preferentially maintained in mother cells and circles accumulation has been usually associated with replicative senescence because of nucleolus fragmentation [55].

Evidence suggests that aging may be influenced also by a mechanism not dependent on the accumulation of ERCs, directly connected to DNA damage [23,56]. Furthermore, it has been reported [57] that senescence often results from copy number shifting towards rDNA expansion rather than from accumulation of extrachromosomal units, allowing to hypothesize that in this case aging may be due to the replicative stress caused by the concentration of replication factors in the rDNA locus and their dilution in the rest of the genome [49,57]. It has been demonstrated that Fob1 is a positive regulator of the recombination event:

yeast cells with low rDNA copy number spontaneously return to the normal copy number after few divisions, while in a *fob1* null mutant, the restoration of the normal copy number is not possible [14]. Also in this case the alteration of copy number flexibility affects the replicative lifespan: it has been observed that overexpression of Fob1-repressors (RPS12, UBC4, CCR4) induces cell cycle arrest and shortens the lifespan [58]. Sir2 can act as regulator of recombination events, as implied by the fact that *sir2*Δ strains are characterized by high levels of ERCs accumulation, copy number unbalance, and reduced life span [59,60]. Overall, this indicates that the Sir2-mediated histone deacetylase activity is important to ensure equal homologous recombination and prevent rDNA instability.

It has been reported that DNA topoisomerases play a key role in controlling rDNA stability [19,48]. In *top1/top2* double mutants about 50% of the rDNA is found in episomal form [19]. In eukaryotic cells, DNA Topoisomerase I localizes mostly in the nucleolus [61]. In *S. cerevisiae* Top1 recognizes and cuts rDNA at specific sequences: once within the promoter region of the 37 S gene, twice at the terminator region in the RFB area [62]. The cleavage activity at the RFB has been demonstrated to strictly depend on the presence of Fob1 in the same region and it has been suggested that Fob1 is responsible to recruit Top1 at the RFB independently from replication fork block events [15]. Top1 also acts as a scaffold protein to recruit Sir2 in the nucleolus. In fact, in cells entirely lacking Top1, as well as in cells with Top1 lacking its protein-protein interaction domains, Sir2 levels at the rDNA are strongly decreased [63].

1.1.2. Transcription

About 50% of the 37 S RNA genes in yeast are actively transcribed and appear nucleosome free [64]. The ability to vary the transcription rate of the rDNA units by modulating the efficiency of RNA Pol I allows WT yeast cells to grow essentially independent of rDNA copy number [65]. Interference with cell growth is observed only when the copy number increases several fold or falls to very low levels [66,67]. Following entry into stationary phase rDNA transcription and the percentage of actively transcribed rDNA genes are reduced. In eukaryotic cells ribosome biogenesis is strongly dependent on environmental inputs, as growth conditions and nutrients availability, drastically affecting the rate of rRNA transcription and the translation rate [64, 68–70]. In S. *cerevisiae* there is no clear and continuous regulation of 37 S transcription by epigenetic signaling. Rather, the decrease in rDNA transcription during stationary phase is associated, through an as yet unclear mechanism, with the Rpd3 histone deacetylase activity [71]. After entry into stationary phase *rpd3* null mutants are unable to reduce the number of transcriptionally active rDNA units [71].

In terms of transcriptional dynamics, the possible relevance of Polymerase Switching on rDNA [72] is worth mentioning. Pol I is known to usually exclude Pol II from recognizing the 37 S promoter [73]. Only in case of a strong deficiency of the Pol I machinery (e.g., alteration of subunits), Pol II can recognize and bind the 37 S RNA promoter to initiate transcription from non-canonical transcriptional start sites [72]. In addition, Pol II is also able to recognize two specific cryptic promoters, E-pro and C-pro, located in the IGS1 and, respectively, IGS2 region, to produce several non-coding RNAs [11,12]. C-pro is unidirectional and partially overlaps the 37 S RNA promoter. The corresponding ncRNA is transcribed in the opposite direction with respect to the 37 S RNA transcription [11,12]. It has been suggested that the mechanisms which control the transcriptional silencing at C-pro may depend on the PolI-Net1-Sir2 interaction [74,75]. E-pro is a bidirectional promoter, localized between the RFB and the 5 S RNA gene. The so-called "transcriptional silencing of rDNA" consists in the repression of the two cryptic Pol II promoters, and the maintenance of a low-rate transcription of ncRNA is key to rDNA stability. The E-pro promoter maps 448 bp upstream the 5 S gene [76]. Its position, as well as its conservation, suggest that it could play an active role in rDNA regulation. RNAs transcribed from E-pro are produced by RNA Polymerase II [11], terminated by the Ndr1/Sen1 complex, and degraded via Ndr1/Sen1 pathway [12]. The major player in the silencing of the RNAs originating from the two rDNA cryptic promoters is Sir2 [9]. It operates by interacting with many other factors: Spt4 [77]; Nsi1 [78]; Smi1/Pnc1 [79]; Pol I/ Fob1 [80]; the RENT complex (REgulator of Nucleolar silencing and Telophase exit) [74,75,81] which includes Net1, driving the complex to the nucleolus; Cdc14, which catalyzes the escape from telophase [81]; three proteins, Tof2, Lrs4, Csm1, that recruit cohesins at the RFB [82]; and Fob1/Top1 [63,83].

Co- immune precipitation and Tap-tagging assays [84] have been used to identify macromolecular interactions among factors known to be involved in rDNA stability, revealing that Fob1 likely recruits Sir2 via RENT complex to the IGS region. Fob1 co-purifies with Top1 and it has been hypothesized that the two proteins could interact [84]. In fact, in *fob1* null mutants the two cleavages operated by Top1 inside the RFB region are lost [15]. Using two-hybrid systems Top1 was also found to be strongly associated with two nucleolar proteins, Tof1 and Tof2 [85]. The first is a direct Fob1 interactor and maintains the replication fork barrier operating with its partner, Csm3, to counteract the Rrm3 helicase which would displace Fob1 from the replication fork barrier [86]. Tof2 belongs to the RENT complex, physically interacts with cohesins to favor equal recombination [82] and contributes to accumulate Fob1 at the RFB [40]. In *fob1* yeast null mutants the activity of Top1 at rDNA sites is lost [15] and Sir2 localization at this locus is strongly decreased [63]. On the other hand, the lack of Sir2 or Top1 does not affect the replication fork barrier nor the presence of Fob1 at the RFB [63,83,84]. Taken together these evidences suggest that Top1 acts as a protein bridge between Fob1 and Sir2 within the rDNA locus [83]. Moreover, it has been well demonstrated that the dimeric nature of the Fob1 complex gives rise to a feature of rDNA, where the RFB regions of two rDNA units interact to facilitate the Fob1- mediated control of recombination and gene regulation [87].

1.1.3. Silencing and recombination

Recombination and transcriptional silencing in *S. cerevisiae* are tightly linked, and the stability of the rDNA locus is the result of a delicate regulatory balance among recombination, sister chromatid cohesion and transcription. Fob1 ensures the basal rate of recombination, giving the rDNA effective responsiveness to environmental changes and allowing this locus to maintain its balance, for example by re-equilibrating the number of rDNA units after a change. As shown in Fig. 2 panel A, Fob1 is also likely responsible for the RENT complex recruitment via Top1 [63,84]. Inside the RENT complex Sir2 maintains the rDNA cryptic promoters under negative control and Tof2 makes contacts with cohesins. This scenario easily favors equal inter-chromatid recombination. When cells lack Fob1 they lose the replication fork block. The absence of Fob1 leads to lack of Top1 at the rDNA locus (Fig. 2, panel C). Therefore, Sir2 accumulation at the RFB is severely affected, altering the physiological silencing of the cryptic promoters and possibly interfering with the Pol I/Pol II balance, known to be crucial for 37 S transcription [73].

The effects of the interplay between Fob1 and Sir2 are not limited to the silencing of rDNA cryptic promoters as it affects also the replicative lifespan: it has been observed that the replicative lifespan is increased in *fob1* mutants whereas it decreases in the absence of SIR2 [88]. This is in line with the observation that decreased Sir2 levels in the rDNA cause the accumulation of ERCs [88], which in turn negatively affects the replicative lifespan [29]. According to widely accepted models [89], while a yeast cell is aging, the activation of the rDNA cryptic promoters could lead to unequal or intramolecular recombination, resulting in strong locus instability (unit number expansion/contraction) and nucleolar fragmentation (ERCs excision). These events could eventually determine the death of the cell.

Overall, looking at the processes occurring at the rDNA during major DNA transactions makes the role of Sir2 stand out, as outlined in Fig. 3. An important target of the Sir2 deacetylase activity is lysine 16 of

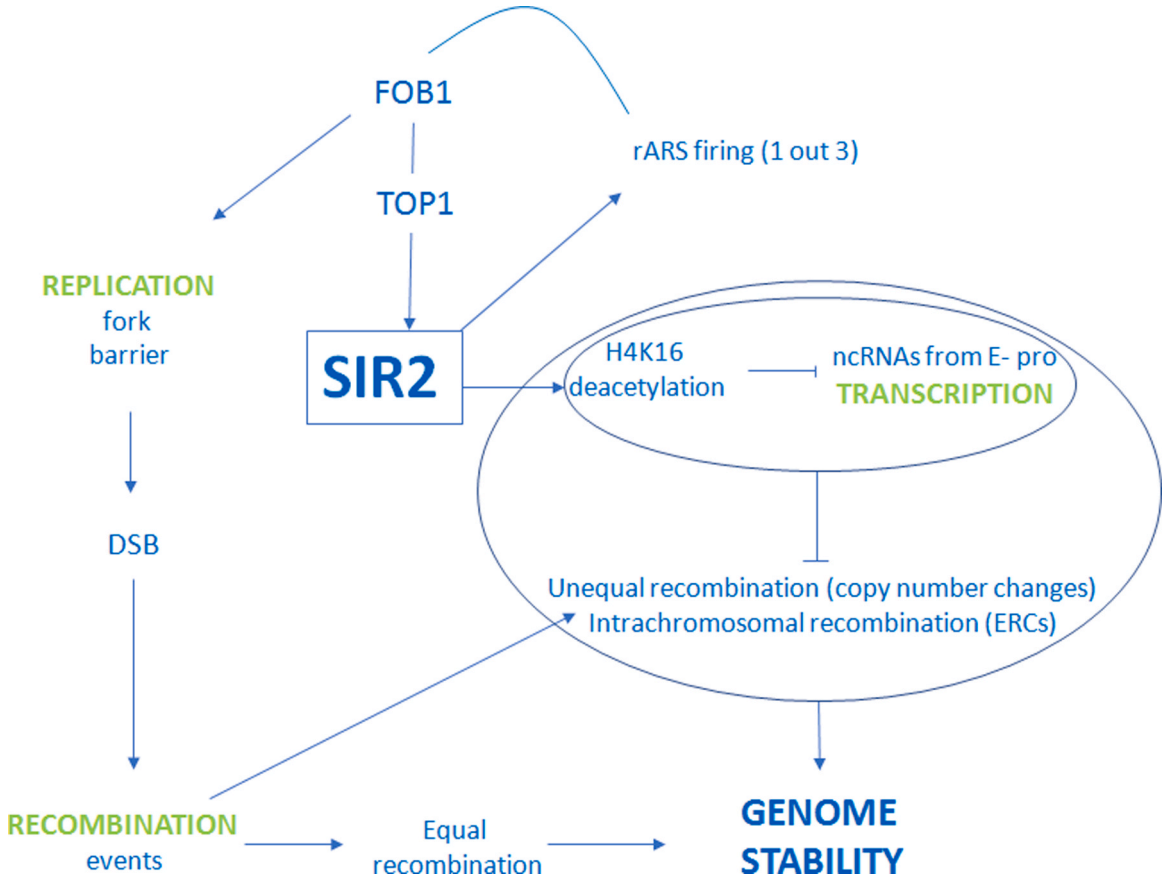

Fig. 3. Epigenetic control exerted by Sir2 on the main DNA transactions occurring in yeast rDNA. Sir2 action on replication (rARS firing), transcription (via H4K16 deacetylation and silencing of the ncRNAs originating from E-pro), and recombination is summarized. A circular mechanism is envisaged, through which transcription and replication in turn influence genome stability. Indeed, ARS activity is directly related to fork stalling and consequent double-strand breaks (DBS), and non-coding RNA transcription leads to cohesin dislocations, formation of extrachromosomal rDNA circles (ERCs), and changes in copy number. Thus, the epigenetic control operated by Sir2 is responsible for a significant portion of rDNA genomic stability.

histone H4 (H4K16). It has been observed that H4K16 is directly involved in ncRNAs silencing. In fact, *sir2*Δ strains show a significant increase of H4K16 acetylation throughout the IGS region as well as an increased transcription of rDNA ncRNAs [9]. H4K16 acetylation has been correlated also to ERCs accumulation: strains with high levels of rDNA circles show increased H4K16 acetylation at the rDNA IGS regions [9]. However, increased H4K16 acetylation due to SIR2 deletion can be uncoupled from decrease in lifespan and rDNA instability, as indicated by the observation that a repressible conditional promoter replacing E-Pro within the rDNA can rescue the lifespan and rDNA instability phenotypes of a sir2Δ strain [76,89]. H4K16 in rDNA is related also to meiosis progression in a Sir2-dependent manner [90]. An association between H4K16 and the silencing of a fraction of rDNA genes has been observed also in mammals, but in this case the acetylation of the residue is a signal for the recruitment of the silencing proteins [91]. Considering the relevance of H4K16 acetylation, it is not surprising that it has been reported to correlate with aging: H4K16 acetylation has been shown to increase in old cells, due to SIR2 repression [92]. Moreover, substitution of lysine 16 of H4 with glutamine, a condition which mimics H4K16 lysine acetylation, results in strong lifespan shortening [92]. In essence, epigenetic control is particularly important because it directly connects environmental conditions (such as glucose availability) to the NAD+ -dependent activity of Sir2. During yeast aging, the observation that the H4K16 residue is hyperacetylated suggests that increased ARS firing and transcription of rDNA cryptic promoters may lead to increased recombination, with overproduction of ERCs and strong genomic instability.

1.1.4. Structural organization feature of yeast rDNA

An articulated picture is emerging regarding the functional impact of the spatial distribution and organization of nucleolar chromatin in *S. cerevisiae* [93,94]. In this section we will summarize major aspects relevant to rDNA stability. A 3D model of the chromosomal arrangements in the nucleus, obtained by chromosome conformation capture approaches at kb resolution [95], shows the rDNA region as a single large aggregate along chromosome XII, protruding from the rest of the chromosome mass and abutting the nuclear periphery. This is in line with the presence, in this yeast species, of a single nucleolus, occupying up to one third of the nuclear volume, and with the observation that the rDNA region is tightly associated with the perinuclear membrane [96,97]. As opposed to other biological systems (e.g. the human NOR), where nucleolus-associated chromatin domains have been identified and their structural and functional relationships with the surrounding nuclear environment have been, at least in part, elucidated [98,99], the spatial arrangement of nucleolar chromatin in *S. cerevisiae* is as yet not fully characterized. A nuclear membrane-associated looped arrangement of 5 S and 37 S regions has been suggested [100]. In terms of overall 3D architecture, the relationship between recombination and structural organization of the genome in the nucleolus, possibly mediated by cohesins as elements known to act in recombination [18] and formation of chromatin sub-domains, e.g. via processes akin to loop extrusion [101], remains to be explored. In this respect it is worth noting that CTCF, a key element in loop extrusion models of many eukaryotic systems, has been reported to be absent in yeast [102,103].

Tethering of the rDNA cluster to the nuclear periphery is mediated by two major multimeric protein complexes, CLIP and Cohibin, involving

chromosome linkage inner nuclear membrane (INM) proteins (Heh1 and Nur1) and rDNA silencing associated proteins (Csm1 and Lrs4), and is required for the stability of the rDNA cluster [97]. Indeed, the depletion of either of these component complexes hampers the perinuclear positioning of the rDNA and promotes the formation of recombination foci destabilizing the cluster. Notably, it also disrupts the boundary between nucleoplasm and nucleolus [97]. In the absence of a specific boundary structure, the separation of the nucleolar space from the surrounding nucleoplasm is surmised to be controlled by physical properties [104] driving aggregation and phase separation, in particular liquid-liquid phase separation (LLPS) [105,106]. Essentially, in a LLPS model some protein components self-assemble into liquid-like droplets engulfing the chromatin fiber and allowing certain molecules to become more concentrated while excluding others [107]. LLPS-driven compartmentalization has been proposed also for other nuclear compartments and fractions of the eukaryotic genome, e.g. heterochromatin [108]. Besides LLPS, current views also stress the possible relevance of polymer-polymer phase separation (PPPS) as a mechanism sequestering yeast rDNA in the nucleolus, possibly as the result of transcriptional activity and R-loop formation [109].

While the exact mechanisms of phase separation processes are not yet fully elucidated, the current view is that the overall shape of the nucleolus may be determined by its possible "liquid-drop" nature [110], acting in parallel with other factors, e.g. rDNA size or metabolic events occurring at the nuclear envelope. In this context it is important to consider that the nucleolus, besides factors involved in ribo-biogenesis, also acts as a reservoir for the regulated sequestration of factors operating in different processes [105]. This occurs, for instance, for the Cdc14 phosphatase, confined in the nucleolus until the onset of mitosis [81]. On the other hand, exclusion from the nucleolar space is also possible, as found for some recombination factors [111]. Interestingly, also the nucleolar periphery can act as a sort of hub, as suggested by the transcription-dependent clustering at the nucleolar boundary of some yeast tRNA genes, transcribed by RNA Pol III and dispersed throughout the genome [112].

Considering the functional relevance of the nucleolar compartment, several aspects of its dynamics during the cell cycle are highly relevant for the cell. Nucleolar assembly is thought to be a self-organizing process, mediated by rRNA synthesis [113]. Not surprisingly, during the yeast cell cycle the nucleolar dimension and appearance vary with ribosome production [114]. Depending on the genetic or physiological context, the morphology of the yeast rDNA cluster undergoes significant changes in size and shape, e.g. the rDNA may fragment upon replicative aging when extra chromosomal rDNA circles originate and accumulate due to homologous recombination [82]. At mitosis the rDNA array is segregated equally between mother and daughter cells. In G1 rDNA units are scattered as discrete foci within the nucleolar space [115], with less condensed rDNA units possibly bridging the more condensed material. At the G2/M boundary the rDNA appears as a single focus or as an arc-shaped structure at the periphery of the nucleus and can eventually reorganize in the form of an rDNA loop structure [30,116]. Chromosome reshaping during cell division in yeast is subject to the action of multiple factors. In particular, both the cohesin and condensin protein complexes are essential for the transition towards the rDNA loop arrangement, while topoisomerase II, involved with SMC complexes in the condensation of eukaryotic chromosomes, seems not required [30,117]. Eventually, at anaphase, the rDNA loop structure is lost, with several cell cycle regulators playing critical roles in this reshaping step, e.g. Cdc5, and Cdc14 [118,119].

2. Conclusions

The capacity to sense the external environment and the ability to withstand harmful environmental changes are among the most powerful strategies for survival and evolution of living species. While this is quite evident at the macroscopic and organismal level, it is less obvious at the cellular and molecular levels. Nonetheless, as for the latter, clear evidence has accumulated stressing the control occurring in the nucleolus. Indeed, there is a variety of studies on nucleolar processes and mechanisms sensing environmental, metabolic, and chronological changes [120]. At the molecular level the rDNA locus is closely associated with the proliferation/quiescence state, the metabolic state of the cell (i.e. its level of nutrition), and the level of genome integrity. The interactions among different processes that ultimately affect the integrity of the yeast genome and are processed on rDNA have been well characterized [121]. In particular, the recruitment of Sir2 at the RFB region contributes to the stability of the locus: recently, it has been observed that Fob1 binding with RFB sequences elicits the recruitment of Top1, which in turn recruits Sir2, thus controlling the local stability [63,83].

The succession of events leading to genomic instability originates from the NAD^+-dependent histone deacetylase activity of Sir2. This significantly lowers the RNA polymerase II transcription efficiency from the IGS1/IGS2 cryptic promoters. A series of events is triggered when this silencing is lost, e.g. due to environmental causes, such as an excess of nutrients, or to a decrease in Sir2 functionality (physiological decline and loss of function during aging) [122]. RNA polymerase actively transcribes ribosomal ncRNAs, and this causes the displacement of cohesins that hold sister chromosomes together, altering the three-dimensional structure of the nucleolus. Thus, the alignment between homologs will no longer be sufficiently accurate, entailing the formation of extrachromosomal circles and/or alterations (increase and deletion) in the number of rDNA units. Furthermore, under conditions of low Sir2 functionality (aging or overnutrition), rDNA replication becomes deregulated. It goes from using about 30% of the rARS to more than 60%. Since rDNA replication from rARS is not exactly bi-directional (due to RFB), the additional stops lead to the production of breaks on the DNA which further induce the recombination system.

The interconnection between genetic and epigenetic components is also echoed by the overall organization of the nucleolus, where the action of cohesins, the CLIP and Cohibin silencing-binding complexes as well as the physicochemical features leading to phase separation, cooperate to maintain the proper integrity of the rDNA. In essence, taken together these processes/conditions tie transcription, replication and recombination events together under the environmentally mediated action of the Sir2 protein. This constitutes very clear evidence of how genotype and environment interact through the epigenetic space, allowing to control the integrity of genetic information.

Declaration of Competing Interest

none.

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