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Mechanisms of rDNA copy number maintenance

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Abstract

Ribosomal DNA (rDNA), the genes encoding the RNA components of ribosomes (rRNA), are highly repetitive in all eukaryotic genomes, containing 100s to 1000s of copies, to meet the demand for ribosome biogenesis. rDNA genes are arranged in large stretches of tandem repeats, forming loci that are highly susceptible to copy loss due to their repetitiveness and active transcription throughout the cell cycle. Despite this inherent instability, rDNA copy number is generally maintained within a particular range in each species, pointing to the presence of mechanism(s) that maintain rDNA copy number in a homeostatic range. In this review, we summarize the current understanding of these maintenance mechanisms and how they sustain rDNA copy number throughout populations.

Introduction: rDNA copy number variations

As the source of ribosomal RNA (rRNA, see Glossary), an essential component of ribosomes, preservation of ribosomal DNA (rDNA, see Glossary) loci consisting of the highly repetitive rRNA genes represents one of the most critical aspects of genome maintenance. The tandemly repeated nature of rDNA copies creates an inherit instability for rDNA loci due to **intra-chromatid homologous recombination** (see Glossary) between copies (Fig. 1, **Key figure**) [1]. This instability, combined with the importance of the rDNA loci for cell functionality, demands special attention to maintain these loci. Despite its critical importance, rDNA has escaped genomics-era analysis due to the difficulty in sequencing the loci, because of its repetitiveness and large unit size (8 – 13 kb for each repeat [2–6]), limiting the ability to obtain precise copy number information through genome sequencing. Still, gross assessments of rDNA copy number have led to a widely accepted notion that rDNA loci are dynamic and copy number varies considerably between individuals across a species, and even likely between cells within a single organism [7–11].

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The copy number variation probably reflects a combination of the effects of natural copy number loss and the mechanism(s) to recover copy numbers to maintain the functionality of the loci. In this review, we summarize the current understanding of the mechanisms governing rDNA copy number instability and maintenance, mostly focusing on *Saccharomyces cerevisiae* and *Drosophila melanogaster*, where these mechanisms have been studied most extensively.

Sources of rDNA copy number variation

Both genetic and environmental factors have been reported to influence rDNA copy number, providing insights into the underlying mechanisms that create rDNA copy number variation. Only a subset of rDNA copies are needed to be transcribed for normal cellular function, and the unnecessary copies are maintained in the repressed state by heterochromatinization [12]. Silencing of unnecessary rDNA copies likely minimizes transcription-replication conflicts, which can lead to DNA damage and copy number variation. Extensive studies in *Arabidopsis* established that rDNA silencing is mediated by multitude of epigenetic mechanisms involving DNA methylation and histone modification [13–17], and defects in silencing leads to rDNA copy number changes [18]. *Drosophila* HP1a mutants that are maintained over many generations have reduced rDNA copy number [19], possibly due to their inability to silence rDNA. It was also shown that a nutrient-rich diet, which likely increases rDNA transcription via target of rapamycin (TOR) [20], results in heritable rDNA copy number loss in *Drosophila* [21]. Interestingly, it was reported that both mouse tumor model with an activated TOR and human cancer cells with high TOR activity have reduced rDNA copies [22].

Another cause of copy number variation within species is accumulated copy number loss that occurs during aging. rDNA copy number loss during aging has been inferred in studies that measured bulk rDNA amount in tissues from young vs. old animals, e.g. canine brain [23] and human blood cells [24,25]. However, the results from bulk tissues, which likely contain a mixture of cells with different ploidies, allowed limited interpretation. It was reported that mouse hematopoietic stem cells accumulate ‘replicative stress’ in the nucleolus [26], possibly reflecting rDNA copy number loss during aging, although copy number was not measured.

Budding yeast is the best-studied model system to address rDNA copy number instability during aging. Mother cells age progressively with each division and eventually die after ~20 cell divisions due to rDNA instability caused by intra-chromatid recombination that reduces chromosomal rDNA copy number and generates extrachromosomal rDNA circles (ERCs, see Glossary) (Fig. 1) [27,28]. These ERCs are selectively retained within mother cells during division and proposed to contribute to mother cell aging [28,29], whereas the daughter (bud) cells can reset their age and divide another ~20 times [27]. rDNA copy loss counterbalanced by restoration results in a relatively constant size of the locus throughout the population over infinite generations (Fig. 1).

While copy number dynamics are well characterized in yeast, little is known about the source of individual-to-individual rDNA copy number variation in multicellular organisms.

The restriction of rDNA loci to the sex chromosomes and availability of genetic tools has made *Drosophila* a useful model to examine rDNA loci in multicellular organisms [30]. ERC formation also causes rDNA loss in *Drosophila* [31,32]. Observation that individual rDNA copies (uniquely marked by transposon insertions) are frequently lost in a span of several generations [33,34] confirms the notion that rDNA copies are lost during cell divisions/generations in *Drosophila* as well. It was recently shown that rDNA copy number is decreased in the *Drosophila* male germline during aging: although the semi-quantitative nature of copy number estimation does not provide precise copy numbers, the data indicate that rDNA copy number might decrease by half during 40 days of *Drosophila* aging [35]. This copy number decrease in the germline leads to inheritance of reduced rDNA copy number by the offspring [35]. Strikingly, rDNA copy number is recovered in the germline of the subsequent generation [35], indicating *Drosophila* germ cells also have mechanisms to restore rDNA copy number. These results collectively suggest that *Drosophila* rDNA is also normally maintained by cyclical copy number loss and recovery. Given the common feature of rDNA across species (e.g. tandemly repeated, unstable rDNA loci) [36], knowledge obtained from yeast and *Drosophila* likely applies to other eukaryotes, including humans.

The mechanisms that recover rDNA copy number

The age-related reduction in rDNA copy number raises an interesting question as to how organisms can maintain rDNA through generations. As described above, the reduced rDNA copy number is inherited to the offspring, and continued rDNA loss from generation to generation would soon create loci with too few rDNA copies to sustain cellular/organismal viability. Therefore, mechanisms to restore rDNA copies and counteract the loss during aging must be in place.

Budding yeast has been an instrumental model system to dissect the precise mechanism for rDNA copy number expansion, and detailed description of this mechanism is found in [36]. Briefly, rDNA copy number expansion in yeast requires binding of the replication fork barrier protein Fob1 to rDNA intergenic spacer sequence (**IGS**, see Glossary) 1 downstream of the **rRNA cistron** (see Glossary) (Fig. 2A) [37]. This binding blocks the progression of replication forks, leading to the formation of a double-strand break (**DSB**, see Glossary) in the leading strand [37–39] (Fig. 2A). In this model, DSB formation in the leading strand leads to strand invasion and repair using its sister chromatid (lagging strand) as a template [40–43]. Owing to the repetitiveness of rDNA loci, strand invasion can occur in a way that completion of repair will increase rDNA copies in a process called unequal sister chromatid recombination (**USCR**, see Glossary) (Fig. 2A) [42,43]. Interestingly, the ability to expand rDNA copies is dependent on the expression of non-coding RNA transcribed from the E-pro promoter within IGS1. E-pro transcription displaces cohesin from the nearby rDNA, allowing the newly replicated sister chromatids to misalign during recombination (Fig. 2B) [44]. On the contrary, when rDNA copy expansion is not required, Sir2, a NAD⁺-dependent histone deacetylase [45,46], represses E-pro transcription and maintains cohesin binding [42,44], thereby preventing USCR and rDNA copy expansion (Fig. 2B).

In addition to USCR, reintegration of ERCs has been proposed as a potential mechanism to increase chromosomal rDNA copy number [47]. ERCs can be produced by intra-chromatid

recombination events (described above), then amplified using an internal replication origin. Alternatively, it has been proposed that excision of rDNA copies from replication bubbles can generate ERCs without reducing chromosomal copy number, through DNA replication that fills the excised copies [47–49]. Irrespective of the mechanism to produce ERCs, their reintegration can expand rDNA copy number on the chromosomal rDNA loci. It has been reported that cells with fewer rDNA copies produce more ERCs in a replication dependent manner [47,50,51], implying that those amplified ERCs may be integrated to increase the chromosomal copy number. However, ERC insertion events are reported to be very infrequent [28,47], making it unclear how much this mechanism can contribute to rDNA copy number recovery.

In multicellular organisms, where rDNA copy number loss has not been extensively characterized, the mechanisms of rDNA recovery have not gained much attention. Given the conservation in the structure of rDNA loci from yeast to humans [36,52], it is plausible that similar mechanisms operate in multicellular organisms. The phenomenon of ‘**rDNA magnification**’ (see Glossary) that was discovered in the *Drosophila* male germline over 50 years ago [53] provided a paradigm for understanding rDNA expansion in multicellular organisms. *Drosophila* strains with large rDNA deletions have a characteristic developmental defect called the *bobbed* phenotype, exhibiting disrupted abdominal cuticle and thin bristles [30]. The offspring of *bobbed* males were frequently found to develop normally due to expansion - or ‘magnification’ - of the rDNA locus [53,54], demonstrating the ability of flies to expand rDNA copies in the male germline. Although the physiological relevance of this phenomenon remained unknown, some of the genes known to be required for rDNA magnification (*mus-101*, the TOPBP1 homolog, and *mei-41*, the ATM homolog) [55,56] are also found to be required for normal rDNA maintenance from generation to generation [35,55], suggesting that rDNA magnification may be a manifestation of the normal physiological rDNA maintenance mechanism.

Similar to rDNA expansion in yeast, it has been proposed that rDNA magnification is mediated by unequal recombination between sister chromatids [57,58] and/or integration of amplified ERCs [59]. ERC amplification/reintegration was initially an attractive model because it easily explained the surprisingly large number of rDNA copies that are gained during rDNA magnification [60]. However, the distinct sequence variations between X- and Y-chromosome rDNA loci [61,62] and mapping of uniquely sized rDNA transposon insertions revealed that newly-gained rDNA copies are tandem duplications of stretches of the existing copies within the locus [63], disfavoring the model of integration of amplified ERCs. Instead, the data suggested that new rDNA copies are synthesized from local copies, likely through the process of recombination. Furthermore, circular ring X-chromosomes fail to expand rDNA copies and instead form dicentric chromosomes in magnifying conditions [57,64], indicating that magnification requires DNA exchange between sister chromatids. The requirement for sister chromatid exchange prompted the model that rDNA magnification is achieved by a process called unequal sister chromatid exchange (Fig. 3A) (**USCE**, see Glossary) [57,58], where recombination between misaligned sister chromatids causes one sister chromatid to acquire rDNA copies from the other sister chromatid. USCE is somewhat similar to USCR in yeast described above, but distinct in that USCE results in

reciprocal gain vs. loss of copy numbers between sister chromatids, whereas USCR is similar to **gene conversion** (see Glossary) and one sister chromatid gains copy number without the other sister chromatid losing any copies.

The USCE model is supported by direct observation of sister chromatid exchange in meiotic germ cells under magnifying conditions [64]. Moreover, the emergence of a small fraction of offspring that had further reduced rDNA copy number during magnification supported that magnification involves reciprocal exchange of sister chromatids [57,65]. Despite the attraction of the USCE model, the observation that some offspring magnified rDNA >4-fold disfavored the USCE model, based on the rationale that a single USCE event would generate a 2-fold expansion at most (by receiving all rDNA copies from the sister chromatid) [60]. However, a more than 2-fold increase in copy number could be explained by multiple USCE events occurring over successive cell divisions (Fig 3B) [65]. Indeed, mapping of magnified chromosomes indicated multiple duplication steps occur during magnification [63]. Therefore, it is likely that multiple USCE events occur in mitotically amplifying germ cells (germline stem cells (GSCs) and spermatogonia (SGs)), leading to >2-fold increase in rDNA copy number. GSCs are the most attractive candidate, where successive USCE events may lead to effective increase in rDNA copy number, particularly if GSCs can consistently inherit magnified copies. (Fig 3B). Moreover, GSCs can produce many differentiating cells/gametes with improved copy number through their self-renewing ability. In contrast, SGs, which committed to differentiation, will undergo only 4 rounds of mitotic divisions prior to meiotic commitment, thereby limiting the opportunity for USCEs. This model would explain why large clusters of offspring with expanded rDNA copies are produced at once by individual fathers [57] and how multiple magnified offspring can inherit identical rDNA loci [66].

The mechanisms that monitor rDNA locus size

Whereas the above mechanisms may explain how reduced copy number can be recovered to sustain rDNA integrity through generations, they raise questions as to how cells know within what range to maintain the rDNA copy number. There is a wide distribution in the number of rDNA copies across wild populations, yet individuals with insufficient or excessive copy numbers are rarely observed [67–69]. The majority of individuals within a population/species cluster around a median number of rDNA copies [68,70,71], suggesting that there are mechanisms that function as a ‘rheostat’ for rDNA copy number.

Recent work reported an elegant rDNA ‘counting mechanism’ in yeast to maintain a set number of rDNA copies. The Pol I UAF complex binds to rDNA promoters to promote Pol I transcription [72–74], but when rDNA copies are low, promoter binding is saturated and free UAF complexes instead bind to the Sir2 promoter and terminator [75]. UAF binding to the Sir2 locus represses Sir2 transcription [75], preventing E-pro repression which in turn leads to copy number expansion as described above (Fig. 2B) [44]. UAF preferentially binds to the rDNA promoter over the Sir2 locus, and accordingly the Sir2 locus is depleted of UAF as rDNA number increases, forming an autonomous feedback loop to maintain rDNA copy number [75]. In this way, the amount of UAF complexes in the cell sets the rDNA locus size, and manipulating the amount of UAF will adjust the number of rDNA copies [75].

Since the molecular factors responsible for rDNA expansion in *Drosophila* are still unclear, it is unknown if Sir2 orthologues and UAF factors have a similar function serving as an rDNA rheostat in the *Drosophila* germline. However, the size of the *Drosophila* rDNA loci has been proposed to be defined by the balance of opposing forces that contract and expand rDNA copies [69,71,76]. In this model, the upper limit of rDNA size is constrained by the inherent instability of these loci, where larger rDNA loci have a higher chance for rDNA loss to occur, while the lower limit is set by selective pressure and the frequency of USCE events. rDNA magnification only occurs under specific context [54,57,58,77] and the frequency of magnification events is dependent on the severity of rDNA insufficiency [65], indicating the presence of a mechanism to sense rDNA copy number and actively induce USCE when copy number is critically low. The observation that Y-chromosomes containing a large rDNA deletion are required for magnification [78,79] and are sufficient to induce magnification of normal-sized loci [80] suggest that the sensor and / or USCE inducer is a Y-chromosome factor. It remains unclear what these factors may be or how they may detect insufficient rDNA copy number and promote USCE.

Global rDNA transcriptional state is an additional feature that might function as a sensor for rDNA copy number. As rDNA copy number decreases, a larger fraction of the remaining rDNA copies will be expressed to keep up with the demand of ribosome biogenesis. Transcription of a greater portion of rDNA increases the probability of transcription-replication collisions and DSB formation [49], which could initiate USCE-mediated magnification. Additionally, the regulation of the choice of active rDNA loci (the phenomenon called '**nucleolar dominance**') may function as a sensor. Nucleolar dominance is a phenomenon, where a subset of rDNA loci is actively transcribed, whereas the other loci are silenced. Although nucleolar dominance was originally observed in interspecies hybrids [81,82], it has been shown/inferred to occur within species (i.e. in the non-hybrid context) ranging from *Arabidopsis* [14,82], *Drosophila* [35,83] and mammals [84,85]. In male *Drosophila* cells, using transcription-dependent deposition of histone H3.3-GFP onto the rDNA loci as well as single nucleotide polymorphism (SNP) *in situ* RNA hybridization that differentially labels X vs. Y rDNA transcripts, it has been shown that the Y rDNA locus is predominantly active, while X rDNA is silent [35,83]. As Y rDNA copy number decreases in GSCs during aging, transcription of X rDNA gradually increases [35], which may signal a need for rDNA expansion to the cell. Cells may further utilize distinct rDNA gene structures between X and Y rDNA [61,62] to sense the expression of X rDNA to trigger magnification. It is tempting to speculate that nucleolar dominance serves as a mechanism to regulate rDNA magnification. In this model, certain rDNA loci may be marked as 'active' and others as 'back-ups' by the mechanism of nuclear dominance, and the activation of the 'back-up' loci may serve as an indication of the need to expand rDNA copies.

Despite the apparent robust mechanism to expand rDNA copies in the *Drosophila* male germline, rDNA copies are progressively lost from the germline during aging [35]. Given that rDNA appears to be maintained by a balance of rDNA copy loss and expansion, the net loss observed during aging is likely due to a shift in this balance. An increase in rDNA transcription, as has been observed in some aged cultured cells and human muscle tissue samples [86,87], may enhance copy number loss in older GSCs through frequent replication-

transcription collisions (more than they help increase the copy number). Alternatively, the accumulated rDNA loss during aging may be due to a reduction in the amount of expansion, or a reduced sensitivity to recognize rDNA copy loss and activate expansion. Homologous recombination repair activity is diminished in the GSCs of old males compared to young males [88], raising the possibility that USCE activity is reduced in old animals and thus copy number expansion is reduced. These models of rDNA dynamics during aging are not mutually exclusive. Further understanding the molecular factors that regulate and execute rDNA copy expansion will help to uncover how maintenance is disrupted during aging.

Concluding remarks

Like telomeres, rDNA is an essential but unstable genomic element that requires active maintenance. However, the mechanisms that maintain rDNA copy number have been underexplored except for budding yeast, despite the marked importance of maintenance through generations of multicellular organisms. Similar to telomeres, rDNA maintenance is likely a prerequisite of supporting ‘immortality’ of cell lineage, and accordingly all immortal cells, including germline and cancer cells, may have specialized mechanisms to actively maintain rDNA copy number. Reflecting its importance, rDNA and its transcription and the nucleolus as an organelle for rRNA biogenesis are subjected to a multitude of regulation. Here we described the current proposed models for how rDNA copy number is regulated and maintained from generation to generation. Understanding how these mechanisms of regulation may be interconnected to each other for the ultimate purpose of supporting cellular viability awaits future investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

rRNA

Ribosomal RNA, the RNA components of the ribosome

rDNA

Ribosomal DNA, loci comprised of hundreds of tandem repeats of a large (8 – 13kb) sequence motif containing the genes encoding the RNA components of the ribosome

Intra-chromatid homologous recombination

DNA recombination between homologous sequences within the same chromatid

ERC

Extra-chromosomal rDNA circle, a loop of one or more rDNA repeats that has been excised and circularized from the chromosomal rDNA due to intra-chromatid recombination between two rDNA copies in the same rDNA locus

IGS

Intergenic spacer sequence, the sequence in between repeated copies of the rDNA cistron that is not transcribed as part of the cistron

rRNA cistron

The single transcriptional unit that contains the 18S, 5.8S, and 28S rRNAs. These rRNAs are separated and flanked by transcribed spacer sequences that are spliced out after transcription. Each rDNA copy contains a cistron, its promoter, and intergenic spacer sequences

DSB

Double-strand DNA breaks, DNA damage that creates breaks in both strands of a DNA molecule

USCR

Unequal sister chromatid recombination (at the rDNA locus), the recombination between two sister chromatids of rDNA, where the leading strand with a DSB invades the lagging strand to increase the rDNA copy number, while maintaining the rDNA copy number of the lagging strand

rDNA magnification

The phenomenon of rapid expansion of many rDNA copies within a single generation

USCE

Unequal sister chromatid exchange (at the rDNA locus), the exchange of sister chromatids at misaligned rDNA copies, which results in one chromatid gaining rDNA copies while the sister has a reciprocal loss of rDNA copies

Gene conversion

The process of one sequence replacing another near-homologous sequence by recombination between the two sequences during homologous recombination-mediated repair of double-strand DNA breaks

Nucleolar dominance

The phenomenon of entire rDNA loci being transcriptionally active or silenced and rRNAs are transcribed exclusively from specific rDNA loci

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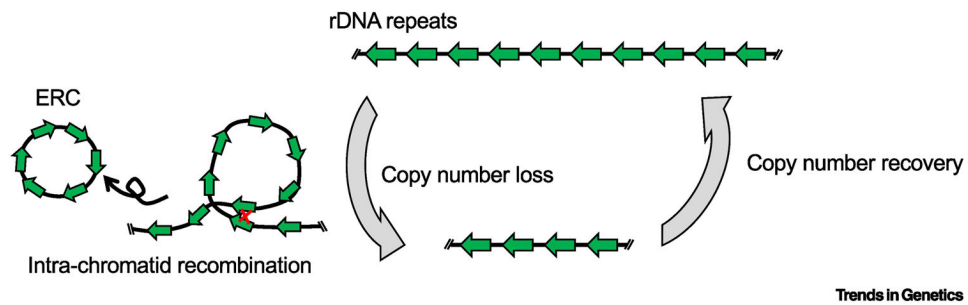
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Recent highlights

- Ribosomal DNA (rDNA) copy number is highly variable across individuals of a species and dynamically changes within a few generations
- Both genetic and environmental factors can alter rDNA copy number
- rDNA loci are inherently unstable and copy loss naturally occurs during aging
- rDNA copy number can be expanded during cell division via variations of homologous recombination mechanisms to offset rDNA loss
- The factors that initiate rDNA copy number expansion are not conserved across eukaryotes
- Age-related rDNA copy number loss in the germline is inherited to the next generation, but can be restored in their germline of *Drosophila*.
- Sensors may exist to monitor the rDNA copy number such that the mechanisms to increase copy number can be activated as necessary.

Outstanding questions

- The molecular factors and mechanisms to expand rDNA copies are well characterized in *S. cerevisiae*, yet homologues of the key components are not yet identified in multicellular organisms. Unequal sister chromatid exchange (USCE) is proposed to expand rDNA copies in *Drosophila*, but the molecular factors that initiate and execute this process for rDNA expansion remain largely unknown.
- An expected consequence of rDNA copy expansion by USCE is a reciprocal rDNA copy loss inherited by the sister cell, however there is a much smaller portion of offspring observed to inherit reduced rDNA copies than those with expanded copies. The underlying mechanism for this phenomenon is entirely unknown.
- rDNA copy numbers are maintained within a certain range in many organisms. The mechanisms that sense the ‘appropriate’ copy number such that the copy number restoration mechanism(s) can be turned on/off remains largely unknown, particularly in multicellular organisms.
- rDNA copy loss occurs in yeast and *Drosophila* germ cells during aging despite robust mechanisms to expand rDNA copies. How the factors that mediate rDNA copy loss and expansion are altered over time so that loss accumulates during aging, and why these factors cannot infinitely maintain rDNA within a single cell / individual, remains unknown.
- The specific mechanisms to expand rDNA copies are not conserved between yeast and *Drosophila* despite the importance of rDNA maintenance for all eukaryotes. It remains unknown whether either of these mechanisms is more widely utilized in other eukaryotes, or whether the pathways for rDNA copy expansion are divergent across species.



Trends in Genetics

Figure 1. rDNA copy number dynamics through cyclical copy number loss and recovery. The size of rDNA loci comprised of tandemly duplicated repeats of the rRNA cistron (green arrows) is dynamic due to loss and recovery of individual rDNA repeats. rDNA copies are most notably lost by intra-chromatid recombination between distant rDNA copies, leading to excision of the intervening copies in the form of an extra-chromosomal rDNA circle (ERC). rDNA loci are capable of expanding their copy number to recover lost copies. The multiple proposed mechanisms for rDNA copy number expansion are discussed throughout this review. Overall rDNA copy number is proposed to be maintained through balanced copy number loss and expansion activity.

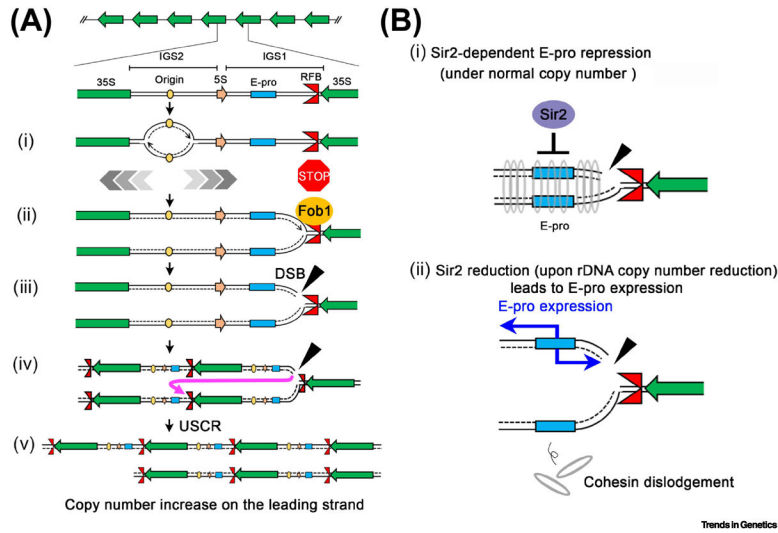


Figure 2. Model of rDNA copy number expansion in budding yeast

(A) The mechanism of rDNA copy number expansion in budding yeast through unequal sister chromatid recombination (USCR). Replication is initiated at the replication origin in IGS2 region (i). The replication fork that moves rightwards is arrested at replication fork barrier (RFB) in a Fob1-dependent manner (ii). The fork arrest creates double-strand break (DSB) in the leading strand (iii). The DSB repair by gene conversion between sister chromatids leads to copy number increase on the leading strand while maintaining the copy number on the lagging strand (iv–v). (B) The mechanism that regulates the decision of unequal vs. equal sister chromatid recombination. (i) In the absence of the need of copy number expansion, Sir2 represses the expression of IGS1-derived non-coding RNA (E-pro). (ii) When copy number is decreased, Sir2 protein is reduced, leading to E-pro expression, which displaces cohesin from the nearby rDNA, allowing the newly replicated sister chromatids to misalign and leading to USCR.

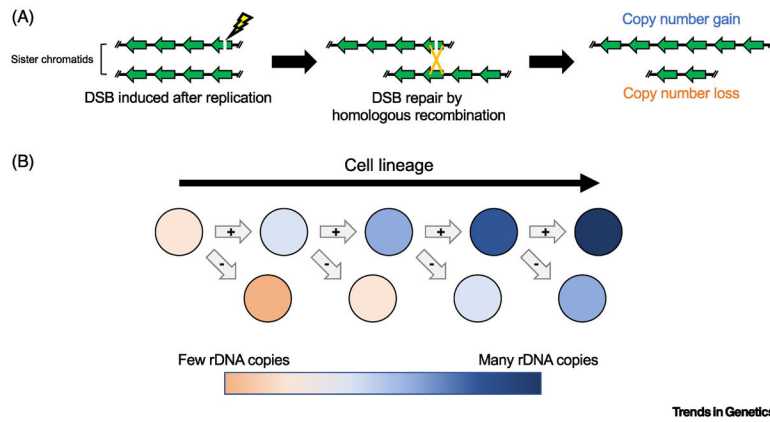


Figure 3. rDNA copy number expansion by unequal sister chromatid exchange in mitotic *Drosophila* germ cells.

(A) Unequal sister chromatid exchange (USCE) can create reciprocal rDNA copy number gain and loss between sister chromatids. When a double-stranded DNA break (DSB) occurs in an rDNA region post replication, the DSB can be repaired by homologous recombination with the sister chromatid. Homologous recombination between misaligned rDNA copies resolved by chromatid exchange increases rDNA copies on one sister while reducing the copies on the other sister. (B) USCE over successive cell divisions can rapidly expand rDNA copies (range from orange to blue). Repeated inheritance of the sister chromatid that gains copies during USCE will lead to rapid expansion of rDNA copies, beyond what could be gained by a single USCE event.