Under the Magnifying Glass: The Ups and Downs of *rDNA* Copy Number

by

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In this review, we will refer to the entire cluster of arrayed *35S* (also called the *45S*, depending on the organism) rRNA transcription units as the *bobbed* gene, and an individual copy of the repeated transcription unit and linked control sequences as a cistron. Likewise, *rDNA* array is synonymous with the *bobbed* gene locus and *rDNA* with a single cistron; *rDNA* copy number, then, means the number of cistrons in an organism or a cell.

We will not discuss the control of copy number of the ribosomal RNA (rRNA) cistrons within the rRNA gene cluster locus (*i.e.*, the *rDNA*) in *Saccharomyces cerevisiae*. This is perhaps an odd decision, given what all is known about *rDNA* copy number regulation in budding yeast. However, much of the biology of yeast *rDNA* seems driven by features unique to that organism. Yeast has a genetic system necessary to increase copy number within a "short" cluster (that is, a locus containing few copies of the rRNA cistron) (Kobayashi and Horiuchi 1996), and the rare form of closed-mitosis during daughter bud formation makes extrachromosomal *rDNA* accumulation a problem that is particularly pronounced in budding yeast (Boettcher and Barral 2013). Truly excellent reviews have been published on the yeast *rDNA* (Kobayashi and Sasaki 2017, Nelson et al. 2019), and those should suffice for readers curious about *rDNA* copy number regulation in that organism.

What follows here is mostly from *Drosophila*, in which research on *rDNA* copy number is the oldest and, outside of yeast, best researched. Our sincerest apologies to colleagues whose work on the *rDNA* of other organisms, or even *Drosophila*, was excluded here.

The *bobbed* locus of *Drosophila melanogaster*

Alfred Sturtevant discovered the seminal *X*-linked "*bobbed*" (*bb*) mutation in 1915, and subsequently identified multiple *bobbed* alleles with variable expressivity ranging from wild-type to lethal (Sivertzev-Dobzhansky and Dobzhansky 1933). Curt Stern later found that males or females bearing a normal *Y* chromosome were suppressed for an *X*-linked *bobbed* phenotype, and correctly inferred that the *Y* also contains a *bobbed* gene that could completely compensate for mutations of the *X*-linked *bb* gene, even if lethal (Stern 1927, Cline 2001). Further work found *Y*-linked *bobbed* alleles, and showed that the *X*-linked *bobbed* and *Y*-linked *bobbed* alleles were additive/redundant in their functions (Stern 1929, Muller 1932). Before identifying *bobbed* as the *rDNA* array, its unusual genetics – its redundancy, its correlation between copy number, expressivity, and penetrance, and its proclivity to revert – probably confused understanding of the nature of the gene that was coalescing at the time.

The *bobbed* loci map to the *X* pericentric heterochromatin and to the *Y* chromosome short arm, both near the cytological "secondary constrictions" of the Nucleolus Organizing Regions (NORs) (Cooper 1959, see also Palumbo et al. 1973). Later it was Ferruccio Ritossa that showed that the *bobbed* loci, the NORs, and the *rDNA* arrays were one-and-the-same by showing that the two largest ribosomal RNAs (the *28S* and *18S*) hybridized to DNA from flies harboring deficiencies of/within the NORs of both *X* and *Y* chromosomes (Ritossa and Spiegelman 1965, Ritossa et al. 1966). Whereas we refer to the *X*-linked *bobbed/rDNA* array as *X-rDNA* (and that of the *Y* as *Y-rDNA*), mutations of those arrays that confer a bobbed phenotype are *X-bb* and *Y-bb*. The complementarity further demonstrated how *rDNA* comprised 0.3 percent of the genome and consisted of hundreds of structurally-repetitive rRNA gene copies, and finally provided a clear explanation of the allelic series, additivity, and redundancy of the *X-rDNA* and *Y-rDNA* array loci.

We now know from molecular cloning that the *rDNA* array loci consist of hundreds of copies of the *35S* primary transcription unit which comprises the *18S*, *5.8S*, and *28S* subunits, preceded by an "External Transcribed Spacer" (ETS) and separated by intervening "Internal Transcribed Spacers" (ITSs) (Nelson et al. 2019). Individual transcription units are separated by Intergenic Spacer sequences (IGS; historically, these were called Non-Transcribed Spacers, NTSs, until they were found to indeed be transcribed (Miller et al. 1983)). In *Drosophila* as well as other insects, the *5.8S* is

cleaved into "*5.8S*" and "*2S*" components. These function analogously to the single *5.8S* of other organisms, as the *Drosophila 5.8S* and *2S* pair in much the same way that the *5.8S* self-anneals in other organisms (Pavlakis et al. 1979, Hori et al. 2000); it is not known if the *2S* or its cleavage product provides some unique functional role or whether the cleavage is essentially inconsequential.

The allelic series of *bobbed* and additive rescue demonstrated that the *rDNA* "polygene" has dose-requirements, with high copy numbers of the cistron necessary for full viability and cuticular/bristle protein production, fewer copies resulting in etched cuticles and truncated or insubstantial bristles (the "bobbed" gene was named for its resemblance to the then-vogue haircut), and fewer still was lethal (Ritossa et al. 1966). *bobbed* mutations also expressed delayed development in comparison to *wild-type* siblings. It is clear from decades of work that a certain copy number of *rDNA* is necessary for viability, though which tissues are most-sensitive remains unknown (Mohan and Ritossa 1970, Buszczak et al. 2014, Zhang et al. 2014). In *bobbed* alleles the cuticle, in particular the posterior dorsal abdominal tergites, show the phenotype most clearly. With the important caveat that not all *rDNA* copies are equal, it appears from ours and others' work that about 90 *rDNA* copies are necessary to confer viability, and about 120 are needed to avoid a bobbed phenotype altogether (Tartof 1973, Terracol and Prud'homme 1986, Paredes and Maggert 2009). One difficulty in this determination stems from common retrotransposon insertions that inactivate

expression of individual *rDNA* (see below). An additional (but understudied) source of variation comes from the expression control exerted by different IGS sequences (Miller et al. 1983), as individual *rDNA* expressed at a higher rate are expected to be more important in determining a phenotype than those that are expressed a little or not at all. The common view of the *rDNA* array is that epigenetic control is enforced to express some *rDNA*, and package others as unexpressed heterochromatin. This has not been subject to careful scrutiny in *Drosophila*, and in fact it remains possible that *rDNA* are expressed under direct control of the RNA Polymerase I enhancer sequences in the IGS (Hayward and Glover 1988) and polymorphisms within the transcription units, much like more-conventional genes.

We will return to the consideration of phenotypes that are affected by *rDNA* copy number. The case is unequivocal for *bobbed*, of course, and more-broadly for protein translation, but for other phenotypes this is an active area of research by many laboratories. It is extremely difficult work because of the pleiotropy expected for any locus that affects a biological process as fundamental as translation: experiments that probe "necessity" and "sufficiency" are largely beyond reach. It is also extremely difficult work because manipulating and determining *rDNA* copy number are laborious processes.

Ascertaining *rDNA* Copy Number

Understanding regulatory mechanisms controlling the maintenance of *rDNA* copy number rely on accurate quantification. The more sensitive, the better, since in general small changes could be meaningful, and it remains likely that different cells or tissues possess different *rDNA* copy numbers (Cohen et al. 2003, Paredes and Maggert 2009). One reliable method for quantification is hybridization with *rDNA* probes. In the case of fluorescence (*e.g.*, Warsinger-Pepe et al. 2020), great care must be taken to assure linearity and prevent bleaching artifacts, although cell-by-cell measurements are feasible with this approach. In the case of radiolabeled probes and slot- or dot-blot Southern hybridization, oftentimes whole animals are required, obscuring variation within organisms.

Recently sequencing technology has evolved to a point where investigators can sequence an *rDNA* array almost in its entirety (Kim et al. 2021, Sims et al. 2021, Nurk et al. BioRxiv). This allows direct counting of copy number, but relies on known genomic context and a relatively short *rDNA* array, which is not common and certainly not of broad experimental applicability. Other approaches come from shotgun or random sequencing, and assessing copy number by multiplicity of coverage (Gibbons et al. 2015, Morton et al. 2020, Sharma et al. BioRxiv). The general applicability of this approach remains unclear, however, as the apparent copy number can span more than 2-fold across an idealized contig of the *35S rDNA*. Such approaches have not yet been validated by comparison with some second approach, which may lead to artifacts

stemming from data acquisition or processing (Hall et al. 2021). For example, in quantifying *rDNA* copy number, CHEF gel estimation remains the gold standard foor validation (Morton et al. 2020). Alas, CHEF analysis is accurate to unequivocally determine *rDNA* copy number only if the flanks of the rDNA array are known and the length of each *rDNA* cistron is invariant; neither of these conditions is true outside of yeast, and so other PCR-based techniques have become standard.

Real-time ("quantitative") PCR (Paredes and Maggert 2009, Valori et al. 2020) and Digital Drop PCR (Xu et al. 2017) are two that are easily employed, though they do require specialized equipment. In the case of the former, linearity and correspondence with a *bobbed* allelic series have given some degree of confidence in the *rDNA* copy numbers obtained from PCR methods (Paredes and Maggert 2009).

Reversion of the bobbed Phenotype

Various researchers have commented on instability of the bobbed phenotype, and generally there are at least five mechanisms by which *bobbed* can be unstable. First, *Y* chromosomes are subject to hyperploidy through spontaneous (primary) nondisjunction of either sex chromosome in males or females (Bridges 1916, Koehler et al. 1996). Importantly, nondisjunction may be more likely in males with chromosomes bearing *bobbed* alleles (McKee and Karpen 1990) and the lack of genetic markers on

the Y makes its presence essentially invisible. Since the bobbed phenotype is dependent on the sum of *rDNA* copy number, even *Y-bb* chromosomes can complement a *bobbed* genotype in *X-bb/X-bb/Y-bb* females and *X-bb/Y-bb/Y-bb* males (compared to, respectively, *X-bb/X-bb* and *X-bb/Y-bb*). Such supernumerary *Y*s are used intentionally to maintain *bobbed* strains in the laboratory.

Second, in genotypes in which crossovers between two *X*s or an *X* and *Y* can occur, the *X-rDNA* locus can become the recombination point of an unequal homologue exchange. In female *Drosophila*, which are recombination competent, the products are an *X-bb* reverted to wild-type, and a more-extreme *X-bb* allele; the latter can be lost due to cell lethality, erasing any evidence of the reciprocity. In males, which do not undergo meiotic recombination, a mitotic exchange event can create paired *X-Y* and *Y-X* translocation chromosomes (Endow et al. 1984). Either product can be the recipient of the lion's share of the *X-rDNA* and *Y-rDNA*, and thus complement any *bobbed* alleles in the genotype. The smaller *X-Y* chromosome is essentially unmarked and can be considered like a *rDNA*-containing "B" chromosome that would be invisible without cytological examination or careful analysis of secondary nondisjunctional progeny (Bridges 1916, Maggert 2014).

Third, a purely-somatic form of phenotype reversion can occur in clones or individuals which bear mutations that genetically interact with the *rDNA* (Marcus et al. 1986), for example those that alter *rDNA* expression (Luo et al. 2020, Fefelova et al.

2021). In these cases, although *rDNA* copy number may remain unchanged, the expression of one or more cistrons can be increased. Debate still exists whether these are cases of derepression of previously-silent cistrons, are increased output of the already-expressed cistrons (as in yeast (Schneider et al. 2007)), or a mix of both possibilities. The appearance of recessive *suppressors-of-bobbed* in some laboratory strains may be common, and a general reason why the mechanism of true reversion had been debated for so long (Marcus et al. 1986, Hawley and Marcus 1989).

In a fourth (and fifth) type of instability, Ritossa described the bobbed phenotype reverting to wild-type in offspring of males with a moderate *X-bb* allele and a *Y-bb* allele that was either severe (*Y-bb-*) or entirely devoid of *rDNA* (*Y-bb*0). Ritossa monitored this "*rDNA* magnification" and found that nearly all of the offspring were reduced for *bobbed* expressivity, and the expressivity further lessened from generation to generation. It appeared that the *bobbed* locus was slowly healing itself over multiple generations, restoring the mutant allele to full function. Strikingly, within these slowly-magnifying populations were some offspring that fully-restored *bobbed* to *wild-type* in a single generation. Thus, magnification seemed to have two different but simultaneous manifestations.

Ritossa used DNA/RNA hybridization to search for clues to the mechanisms of *rDNA* magnification (Ritossa 1968). As expected, he found that reversion of the bobbed phenotype correlated with an increased *rDNA* copy number (Malza et al.

1972). However, in the “slow” magnification, he noted that the increase of *rDNA* copies was not inherited at the *bobbed* locus, but rather was recreated every generation *de novo*. Further, in this “slow” process the reversion usually back-reverted to a bobbed phenotype – in fact to the original bobbed phenotype – if taken out of magnifying conditions. Ritossa reasoned that the reason for reversion was the presence of extrachromosomal *rDNA*, either circular or linear mini-arrays, that were expressed but lost during gametogenesis (Ritossa et al. 1971, Ritossa 1973). Kenneth Tartof (Tartof 1971, Tartof 1973) studied the genetic requirements for this “slow” magnification, and showed that *rDNA* copy number could increase even at a normal *X-rDNA* array, and he named this form of magnification“disproportionate replication.” Disproportionate replication did not require a bobbed phenotype, but did require magnifying conditions, that is one *rDNA* array on the *X*, *bobbed* or not, and a homologous *Y*-linked *rDNA* array with a severe bobbed allele (i.e., *X*/0 males, *X*/*X-bb* females, or *X*/*Y-bb* males).

The “fast” type of magnification behaved differently. Here, revertants were sporadic (on the order of 15% of offspring showed reversion), complete (the reversion was to *wild-type*, not just to a milder bobbed phenotype), and permanent and heritable. Increases in *rDNA* copy number also attended this type of magnification, and restored *rDNA* were indeed found incorporated into the natural *X-bb* locus. This magnification required a *bobbed* allele on the *X* chromosome and magnifying

conditions including a *Y-bb-* or *Y-bb0* allele (Bonicinelli et al. 1972, Endow and Komma 1986), and possibly some still-unknown locus on the tip of the *Y* chromosome far from the *Y-rDNA* locus (Komma and Endow 1987). It was therefore evident that the "slow" and "fast" forms of magnification were different, although they coincided in magnifying males, suggesting that they may be different manifestations of the same mechanism, or at least two types of magnification induced by the same conditions.

Observations of Magnification and Likely Mechanism

To deduce the timing of magnification, Tartof and R. Scott Hawley (Hawley and Tartof 1985, Hawley et al. 1985) created a pair-matched set of chromosomes, a *Y-bb-* chromosome and a revertant, *Y-bb+*. This pair allowed them to discriminate between magnification and disproportionate replication by comparing offspring from *X-bb/Y-bb-* and *X-bb/Y-bb+* fathers. The former genotype produced clusters of identical magnification-bearing sperm chromosomes, indicating that magnification occurred in a stem cell or spermatogonium early in spermatogenesis. In contrast, the latter genotype produced only singular events, indicating that disproportionate replication occurred late in spermatogenesis (perhaps as late as meiosis). Both stages required some canonical DNA Damage Response genes, as mutations of the DNA repair gene *mus108* inhibited both forms of reversion, but other mutations could distinguish

between disproportionate replication and true magnification (Hawley et al. 1985). They homed in on the latter process.

True magnification occurred in *X*/0 males, which ruled out recombination between homologous *rDNA* arrays. Tartof put forth the hypothesis that magnification relied on unequal sister chromatid exchange (Tartof 1974). He predicted that such mitotic recombination events would produce sister chromatids with complementary losses and gains of *rDNA*, and promptly showed that magnifying *X-bb* chromosomes produced more severe *X-bb* chromosomes under magnifying conditions. He could not establish that these genetically-reciprocal events were both derived from the same event, and they did not appear in equal number. Nonetheless, it was a key piece of evidence that magnification and *rDNA* loss were linked.

Strong support for unequal sister chromatid exchange came from analysis of a magnifying *ring-X-bb* chromosome (a circular chromosome without telomeres and that bore a *bobbed* mutation, in a male genotype with a *Y-bb* chromosome to induce magnification). If magnification required unequal sister chromatid exchange, then exchange at the *rDNA* would produce unstable dicentric ring chromosomes as the two sisters formed a continuous figure-∞. This is contrasted to the alternative, if magnification did not involve sister-exchange, magnification should be able to revert individual sister *ring-X* chromatids without conjoining them. Tartof reported no magnification in 1500 offspring, which he interpreted as the obligate formation of

dicentric double-rings, lethal in subsequent mitoses, and support for unequal sister chromatid exchange (Tartof 1974). Sharyn Endow analyzed magnification products and directly observed the unstable dicentric ring chromosomes, cytologically proving unequal sister chromatid exchange in magnifying conditions and not in non-magnifying conditions (Endow et al. 1984).

The co-existence of “fast” and “slow” magnification fed debate over the nature and mechanism of magnification, injecting doubt about the role of unequal exchange, and it wasn’t until 1986 that the issue was satisfactorily resolved. Marcus and Hawley carefully analyzed the conditions and properties of “slow” and “fast,” and showed that “slow” magnification was not magnification at all, but rather a suppression of the bobbed phenotype by other mutations in the strain (Marcus et al. 1986); the aforementioned third and fourth types of reversion were the same. Finally, magnification fell neatly under a simple explanation. Unequal sister chromatid exchange appears to be the main, perhaps only, mechanism for the formation of the final *rDNA* magnification chromosomes (Endow and Komma 1986, Hawley and Marcus 1989). However the unequal sister exchange in the premeiotic divisions of the male germline requires a double-strand break to initiate exchange.

<u>Origin of the Magnification-Inducing DNA Break</u>

The *I-Cre*I meganuclease fortuitously recognizes the *Drosophila rDNA* sequence, and a heat-shock inducible transgenic *I-Cre*I is sufficient to create chromosome exchanges between the *X-rDNA* and *Y-rDNA* loci (Maggert and Golic 2005). Additionally, expression could be used to damage the *rDNA* and recover an allelic series of *bobbed* (Paredes and Maggert 2009). Upon a second exposure to *I-Cre*I, stable and heritable *rDNA* revertants could be recovered from *bobbed* mutants. We cannot assert that the mechanism is the same as true magnification, but we can conclude that a *rDNA* break in premeiotic divisions is sufficient to cause *rDNA* expansion.

Similar results have been obtained in mutations of the Bloom Helicase (*blm/mus309*), which is required to replicate "hard-to-replicate" DNAs (Ruchert et al. 2022). Defects in *blm* function cause replication fork collapse and double-strand breaks during S-phase. We have recently found that *blm* mutants produce *bobbed* offspring, enhance existing *bobbed* alleles, produce offspring with *X-Y* exchanges at the *rDNA*, and increase the frequency of *bobbed* reversion (manuscript in preparation). This strongly suggests that any *rDNA* damage is sufficient to alter *rDNA* copy number through homologue, sister, or intrachromatid repair.

If we accept that any break is sufficient, then the mystery becomes what about the "magnifying condition" makes the break? Whatever the answer, it must explain why unequal sister chromatid exchange happens only to *X-bb* alleles when the *Y* is even-

more severely *bobbed*. What about those two conditions conspire to elicit DNA damage in the *X-rDNA*? It may have to do with the mode of chromosome-specific *rDNA* regulation in male *Drosophila*.

In 2012, Frauke Greil and Kami Ahmad reported that the *X-rDNA* locus is silenced by a still-unknown process in *Drosophila* males, while the *Y-rDNA* array is active (Greil and Ahmad 2012, also Warsinger-Pepe et al. 2020). This observation is reminiscent of nucleolar dominance seen in interspecific hybrids of plants and animals (Pikaard 2000, McStay 2006). It also poses a conundrum: if only the *Y-rDNA* array is expressed, then the bobbed phenotype should reflect the *Y-bb* allele alone; the *X-rDNA* status (*bobbed* or not) should be recessive rather than additive in males. *Y-bb* alleles that are lethal when the sole source of rRNA should be dominant-lethal. *X*/0 males should die from lack of rRNA. And the magnifying conditions used by Ritossa, Tartof, and others should be impossible! So, it <u>must</u> be that a *Y-bb* allele that is incapable of sustaining sufficient *rDNA* expression triggers the derepression of the *X-rDNA* array in males. It is tempting to further propose that when the *X-rDNA* array is active in males, magnification occurs. In fact, perhaps *X-rDNA* expression is sufficient to make the necessary DNA breaks. How the *Y-rDNA* and *X-rDNA* arrays are regulated, specifically how a repressed *X-rDNA* array is derepressed in magnifying conditions, is an outstanding and persisting mystery of *rDNA* biology. Happily, Greil and Ahmad reported that *X-rDNA* loci are active in some strains, opening the possibility of

mapping this “derepression” phenotype. Experiments to deduce whether monoallelic expression is a property of the *X-rDNA*, the *Y-rDNA*, or of some unlinked mutation may well provide the insight needed to understand why magnifying conditions lead to magnification.

A tantalizing clue may come from other mutations that cause damage at the *rDNA* – heterozygous mutations in *Su*(*var*)*3-9* and *Su*(*var*)*205* (which encode a histone H3 Lysine 9 methyltransferase and the H3K9-methyl binding protein HP1, respectively) deregulate the *rDNA* and cause permanent *rDNA* loss (Peng and Karpen 2006, Paredes and Maggert 2009, Greil and Ahmad 2012, Aldrich and Maggert 2014). It is as hard to imagine a loss of *rDNA* without DNA breaks as it is to imagine magnification without DNA breaks. The loss of *rDNA* copy number by these mutations may indicate an *rDNA* instability that leads to primary loss, but also gains/magnification; to our knowledge, it has not been determined if these *Su*(*var*) mutations exhibit increased magnification rates, or could be sufficient to create magnifying conditions, as does mutation of *blm*.

One might assume that in a normal male, whose *X-rDNA* is silent and whose *Y-rDNA* is active, the *Y-rDNA* is subject to constant magnifying conditions. But we know this is not so. If it were no *Y-bb* alleles could be maintained as stable strains, yet multiple strains are, including our own. Specifically, the *Y-bb* allelic series mentioned above has been maintained in stock for over a decade with no sign of changes in the

bobbed expressivity or the *rDNA* copy number. Therefore something must discriminate *X-rDNA* from *Y-rDNA*, even though the ribosomal RNAs that come from them appear to be identical.

Heterogeneities

Within the *rDNA* arrays are multiple dimensions of heterogeneity. As alluded to before, the IGSs are variant in their length, reflecting differences in copy number and sequence of individual sub-repeats. These appear to be regulatory (Hayward and Glover 1988), suggesting that a shared selective pressures may act upon individual cistrons to assure sufficient rRNA output of an active array.

In arthropods, an additional dimension of variation exists as multiple classes of retrotransposable elements are found within the transcription unit; in *Drosophila* the two – R1 and R2 (historically called Type-I and Type-II inserts, respectively) – both are found within the *28S* subunit at specific sites (Glover et al. 1975, Glover and Hogness 1977, Jakubczak et al. 1990); R1s are slightly enriched in the *X*-linked *rDNA* array, and the R2 slightly enriched within the Y-linked *rDNA* array (Tartof and Dawid 1976, Wellauer et al. 1978). Common for retrotransposable elements, both classes are subject to abortive reverse-transcription during integration. Thus, based on retrotransposable elements alone, the *rDNA* cistrons can have a dramatic amount of variation: uninserted

rDNA cistrons, R1-inserted, R2-inserted, double-inserted, and 5' truncations of those latter three classes (Averbeck and Eickbush 2005). As Oscar Miller first noted, inserted *rDNA* cistrons are poorly expressed (Jamrich and Miller 1984, also see Long and Dawid 1979, Zhou and Eickbush 2009). What keeps inserted cistrons at this low level of expression is not fully elucidated, but most-likely includes a combination of sequence-specific (Wellauer et al. 1978, Guerrero and Maggert 2011) and regional factors such as proximity or "epigenetic" memory (Long et al. 1981, Eickbush and Eickbush 2003, Luo et al. 2020). Silencing R1- and R2-inserted cistrons is desirable because if expressed, they are expected to alter the strict stoichiometry that naturally arises from the co-expression of the *35S* primary rRNA transcript (Long et al. 1981). As before, the connection between expression and sequence remains to be thoroughly understood.

Retrotransposition is stereotyped, with the R1s and R2s inserting reliably at base pairs 6851 and 6777, respectively, of the primary rRNA transcript. The POL subunit's strong sequence-specificity is better understood for R2, where it generates a single-strand break and primes the reverse-transcriptase-mediated DNA polymerization. During the final step of maturation and ligation, R2 POL creates the second break to create an end-protected double-strand break that finally incorporates the R2 element (Xiong and Eickbush 1988, Jakubczak et al. 1990, Eickbush and Eickbush 2015). This event could potentially go awry and create the necessary double-stranded break that repairs from a sister chromatid in S- or G2-phase of the cell cycle.

Others have noted that R1 and R2 expression are weakly correlated with magnifying conditions, although causality has not been determined (Long and Dawid 1979, Long et al. 1981, Labella et al. 1983). On the one hand, it is possible that magnification sufficiently remodels the *rDNA* chromatin and derepresses R1- and/or R2-containing cistrons. Recent investigations into the expression of R1 and R2 have shown that both are induced by stress (He et al. 2015, Raje et al. 2018). However, the authors argue that this may be a consequence of increased generalized *rDNA* transcription in pre-magnifying *bobbed* males since the level of transcription of the insertions is still well-below the level of uninserted cistron transcription. de Cicco and Glover (1983) also reported an increase in R1- and R2- inserted *rDNA* during the first generation of *rDNA* magnified males, but the increase was the same as the level of magnification of uninserted *rDNA*, implying R1 and R2 activity were not an essential part of magnification.

An investigation of the *rDNA* units that were lost during *rDNA* magnification showed that only uninserted cistrons were lost, while cistrons containing R1 and R2 were retained, arguing for a nonrandom distribution of uninserted and R1-/R2-containing carrying *rDNA* (Salzano and Malva 1984). A different analysis showed that uninserted *rDNA* increased more so than did R1-containing *rDNA*, and R2-containing *rDNA* barely changed at all (Terracol and Prud'homme 1986). A similar conclusion was reached by a follow-up study (Terracol 1987). Overall, these conflicting results likely

indicate that R1 and R2 elements are not randomly distributed in the *rDNA* arrays (Zhuo and Eickbush 2009, Bianciardi et al. 2012) and suggest that R1 and R2 mobilization *per se* is not necessary for magnification.

However, it is possible that retrotransposable element expression is necessary for magnification. Recent yet-unreviewed work from Yukiko Yamashita's laboratory suggests this, as R2 expression was deemed necessary and sufficient for magnification (Nelson et al. BioRxiv). If this is confirmed, it would be an exciting demonstration of an adaptive use of the R2 retrotransposon to create and maintain *rDNA* variation. The "purpose" of *rDNA* magnification may really be to assist "selfish DNA" elements increase their numbers, and its role in offsetting potential losses of *rDNA* a secondary benefit.

We consider the formation of the magnification-initiating break to be the "regulated" step, but it need not be so. Magnification and loss require the sister chromatid exchange to be unequal, which it normally is not. It is therefore a possibility that breaks are common in the *rDNA*, but they are faithfully repaired from allelic *rDNA* cistrons except in magnifying conditions. Some plausible support come from mutations in *blm* which recruits cohesin as an effector of silencing (Busslinger et al. 2017, Pugacheva et al. 2020), and mutants of *Su*(*var*)*3-9* and *Su*(*var*)*205* which reduce the function of the naturally cohesive heterochromatin. The same may be considered for any condition that alleviates repression of the *X-rDNA* array. Magnification may be

blocked in normal conditions by the cohesin- and heterochromatin-induced pairing of the inactive *X-rDNA*. Loss of *Y-rDNA* and subsequent derepression of the *X-rDNA* may diminish pairing and allow out-of-register unequal sister chromatid exchange. The difference in magnification between active *X-bb* and *Y-bb* may indeed stem from nucleolar dominance, but because of pairing rather than DNA double strand break formation on only one array.

***rDNA* Magnification Offsets *rDNA* Loss – sometimes**

One might surmise that magnification has evolved to offset natural losses of the *rDNA*. Instability and loss in the soma has been known for some time (Cohen et al. 2003), and its contribution to penetrance and expressivity should be considered. However our work and others suggest that the average *rDNA* amount between individuals varies very little, and so the detection of somatic *rDNA* loss may be limited to some cells.

Recently, Yukiko Yamashita and colleagues reported a very high level of loss of the *rDNA* through normal male germ line stem cell divisions, progressive with age (Lu et al. 2018). Because no bobbed phenotypes were reported in the offspring of aged fathers, it seems unavoidable that a new form of reversion must occur every generation to offset the germ line loss. This reversion must also occur in two phases in cells of the

soma and the germ line. In the soma, reversion must be an early embryonic event to explain why offspring of old males were not *bobbed* or show decreases in *rDNA* copy number. Whatever the mechanism, the reversion was seen to offset *rDNA* loss in the previous germline and return the *rDNA* copy number to the same level it was in the prior generation's soma. This is reminiscent of Ritossa's "slow" magnification, as it was not inherited, and may rejuvenate his hypothesis of extrachromosomal *rDNA*, or it may also be the consequence of some other factor that was not considered in the experiments. In the germ line, the observations were different. The *rDNA* in the pole cells of the embryo stayed with less *rDNA* until after coalescing into the testis germ line stem cells in the adult, when they quickly returned to the *rDNA* level of the previous generation, and began the cycle of loss and gain over again. This observation, if repeated, would represent an unprecedented dynamism and memory of *rDNA* copy number.

Until now *rDNA* has been considered to be very stable from generation to generation. This is informally seen by the maintenance of multiple *bobbed* strains through the decades of work on *rDNA* magnification and molecular biology; surely if *bobbed* alleles were unstable in non-magnifying conditions – either by sporadic magnification to suppress the phenotype, or by progressive loss to enhance the phenotype – it should have been noted (Bianciardi et al. 2012). Some studies have directly tested for instability leading to *rDNA* loss and found none (Tartof 1973, Tartof

1974, Tartof 1974, Malva et al. 1980, Hawley et al. 1985, Hawley and Tartof 1985, Hawley and Marcus 1989).

In our work, we detect *rDNA* loss in only three conditions: the expression of enzymes that specifically damage the *rDNA*; in genetic mutants that affect chromatin structure, rRNA expression, or DNA repair at the *rDNA* array; and when subject to profound nutritional stress conditions that alter rRNA expression (Paredes and Maggert 2009a, Paredes and Maggert 2009b, Guerrero and Maggert 2011, Aldrich and Maggert 2014, Aldrich and Maggert 2015, Ji et al. 2019). Otherwise, we find that *bobbed* alleles are remarkably robust.

rDNA stability is true even for arrays that straddle the bobbed-lethal/extreme-bobbed threshold (Paredes and Maggert 2009). When chromosome $Y\text{-}bb^{484}$ is the sole array (by placing it over an *X* chromosome devoid of all *rDNA*) it expresses as bobbed-lethal with rare extremely-bobbed escapers. $Y\text{-}bb^{183}$ expresses only moderate sub-viability when culture conditions are not optimal, and the flies bearing $Y\text{-}bb^{183}$ as the sole *rDNA* array are extremely-bobbed with gross loss of abdominal cuticle. These strains are normally kept in the presence of a normal *X-rDNA* array, but every time males are crossed to generate offspring with these *rDNA* arrays as sole *rDNA* array, their phenotypes reappear exactly as they did before, and quantifications are indistinguishable from previous determinations over decades, regardless of the age or conditions of the culture.

We have performed experiments investigating gradual loss in mutants and in conditions in which *rDNA* is destabilized by dietary manipulation (Aldrich and Maggert 2015), and do not observe loss in subsequent broods of males. It remains unresolved whether the phenomenon described by Lu and colleagues affects only a subset of chromosomes, acts only under a subset of conditions, is a consequence of other mutations in the genotype, or some other explanation.

A Brief Mention of the *5S rDNA*

Relatively little work has been done on the biology of the *5S* array. The *5S* is also a repeat array, comprising about 50 copies per homologue in *Drosophila* (the locus is not sex-linked, instead being on the left arm of chromosome *2*), is transcribed by RNA Polymerase III, and might be expected to show some complex genetic interactions with alleles of the *35S* or ribosomal proteins. Changes in *5S* copy number are not known to cause a *bobbed*-like phenotype in *Drosophila*, however increases in *rDNA* copy number are linked to dramatic growth phenotypes in plants (Schneeberger et al. 1991).

It is possible that ribosomal RNA stoichiometry may be maintained by coordinating magnification and loss between the *35S* and *5S rDNA* arrays (Gibbons et al. 2015) however those reports have come under question (Hall et al. 2021). Still, this locus might yet reveal some fascinating biology.

Phenotypes

High intra- and inter-individual *rDNA* copy number variability makes it difficult to assertively assign phenotypic consequences of deficient *rDNA* arrays in most organisms, including *Drosophila*, and even yeast. *rDNA* transcription and ribosome biogenesis manifest in the formation of the nucleolus, the most transcriptionally active site in the genome. This alone may influence all cellular and metabolic processes through sheer transcriptional and translational demand (Warner 1999). Such effects may alone exert metabolic phenotypes (Holland et al. 2016, Bughio and Maggert 2019). Limited *rDNA* might also be expected to cause limited ribosome biogenesis, and thus defects in translation. The characteristic abnormalities of a delay in time of development and reduction in the rate of egg laying may be consequences of reduced protein synthesis (Mohan and Ritossa 1970), but specific phenotypes have yet to be ascribed to this.

Many groups have shown that deletions within the *Y-bb rDNA* array results in the destabilization of heterochromatin and suppression of heterochromatin-induced gene silencing (Position Effect Variegation, PEV) (Paredes and Maggert 2009, Larson et al. 2012, Zhuo et al. 2012, however see Greil and Ahmad 2012). This effect, discovered in induced *rDNA* copy number variants and reproduced in naturally occurring polymorphic *Y rDNA* arrays, may also lead to changes in gene expression across the genome (Paredes et al. 2011) Again, though rDNA has been linked to subtle

expression differences, whether these steady-state mRNA differences result in selectable phenotypes has yet to be demonstrated.

Research in other organisms has shown that perturbations in *rDNA* copy number may be a cause or consequence of many cancers and age associated diseases. The highly proliferative nature of malignant transformation and the cumulative stressors of cellular aging could be aggravated by *rDNA* limitation, metabolic perturbations, or otherwise. An inconsistent pattern of *rDNA* copy number changes along with hypo/hyper-methylation in promoters and coding regions of rRNA genes has been observed in cancerous and aged tissues when compared to healthy controls in mammals, plants and fish (Stults et al. 2009, Xu et al. 2017, Valori et al. 2020, Watada et al. 2020, Hall et al. 2021, Shao et al. 2021, Hall et al. 2021). The lack of a clear pattern of losses or gains across all cancer types could merely reflect tissue-specific differences in rDNA regulation. It could also be that cancer compromises *rDNA* stability rather than copy number, allowing both gains and losses, the latter of which may be more likely (Valori et al. 2020, Milanovskaya et al. 2018).

In some conditions, *rDNA* copy number may aggravate conditions through specific genetic interactions. Deficiencies and mutations in DNA repair factors or chromatin components that lead to fluctuations in *rDNA* copy number may make replication, repair, or heterochromatin formation more reliant on yet other gene products. It is plausible that *rDNA* copy number may genetically interact with these

genes and contribute to the etiological basis of diseases such as Bloom syndrome, Werner syndrome, ataxia-telangiectasia, and even growth rate (Schawalder et al. 2003, Killen et al. 2009, Kwan et al. 2013). Furthermore *rDNA* copy number changes that have been implicated in metabolic dysregulation (Aldrich and Maggert 2015, Holland et al. 2016, Bughio and Maggert 2019).

There is a recently emerging consideration that rDNA defects play a pivotal role in neuronal biology, and may underly some neurological disorders. Whether it might do so through alterations to transcription, cell cycle and development, heterochromatin and transposon control, or metabolism is an area of active inquiry (Chestkov et al. 2018, Porokhovnik 2019).

Where Are We At?

Magnification of the *rDNA* is well-accepted, and supported by decades of work by many laboratories, working in collaboration or independently. Unequal sister chromatid exchange appears fully explanatory as a mechanism. However, how widespread is this phenomenon – are all chromosomes susceptible, whether it happens in normal genotypes – seems still open for discussion (and disagreement). The balance of information suggests that DNA breaks are involved in initiating magnification, but it is unknown whether this is a specific and regulated process (as, *e.g.*, it is in

Saccharomyces cerevisiae), or whether it is a consequence of misregulation of the *rDNA* under what we now call "magnifying" conditions. Perhaps evolution would not discriminate: why would a specific system evolve under conditions that cause it to happen anyway?

A persistent question that arises when considering magnification is of the consequence. Broadly, there seem to be three "reasons" for magnification. One is to avoid lethality from the loss of translational capacity. This itself is important, but perhaps no different from any form of DNA repair that offsets mutation. Magnification may be a special case as, for example, repeat sequences are more susceptible to loss than more complex sequences. A second reason would be to "tune" other phenotypes that depend on *rDNA* copy number. This is perhaps the most exciting possibility, as the wide variation in *rDNA* copy number that exists within populations may modify yet-unknown phenotypes. It could, then, explain some degree of heritability "missing" from GWAS studies that struggle to consider epistatic interactions (Rabanal et al. 2017, Press et al. 2019). Our laboratory, and many others, are exploring this possibility since the correlation between *rDNA* copy number, aging, cancer, and neurological disorders are too compelling, and too important, to leave alone. Finally, it is possible that *rDNA* magnification is neither important nor adaptive, but merely the consequence of the unusual biology of repeat DNAs. Perhaps our understanding of *rDNA* copy number regulation is hindered by our expectation that it is "unique," and not simply following

the same rules of satellite DNA and other repetitive elements. Magnification may be a phenomenon without a reason, one of the many spandrels that arise during the evolution of other complex biological systems (Gould and Lewontin 1979). Whatever the case, many exciting studies are being done to work it out.

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